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Aquatic Microbial Ecology

Proceedings
of the
ASM Conference

7-10 February
1979
Clearwater Beach
Florida

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Aquatic Microbial Ecology

Proceedings of the Conference
Sponsored by the
American Society for Microbiology

7-10 February, 1979
Clearwater Beach, Florida

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Introduction

Aquatic microbial ecology has developed rapidly since the late 1960s when the science was recognized as a separate discipline. In only the last five years, new methods for collecting and evaluating samples and for measuring microbiological parameters have been devised. Particularly, ways to monitor environmental stress and the effects of stress on microbial parameters and processes have been implemented. And a variety of methods is now available for estimating biomass and the activities of microorganisms comprising the biomass. The conference brought together a group of scientists whose research is in the forefront of aquatic microbial ecology and provided a forum for discussing the significance of and interpreting the data gathered in laboratory and field work.

The talks and ensuing discussions on the effects of environmental stresses on microorganisms emphasized the heterogeneity of microbial populations in aquatic ecosystems, especially in those populations associated with inert surfaces

and with the surfaces of higher organisms, and the physiological interdependence of the microorganisms that make up those specific populations that develop in each ecological microniche. Each integrated population responds to environmental stress as a unit. As Dr. Mitchell pointed out, stress may disturb the delicate relationship between higher organisms and their specific adherent bacterial populations to the extent that normal commensal saprophytic bacteria become pathogenic for the animal or plant they have colonized. So the important consensus of this discussion was that to assess the degree of environmental stress on aquatic microorganisms, stable and integrated mixed populations should be used as test objects.

Microorganisms are normal components of both the internal and external surfaces of higher organisms. They function as decomposers and, through their increased biomass and/or extracellular secretions, also function as converters of poor quality dissolved organic matter to vitamins, amino acids, etc. In addition, microorganisms are direct food sources for many higher animals. Thus, the microorganisms play a ubiquitous role in energy transfer and nutrient flux at all trophic levels. Discussion of their effect on environmental processes pointed out that differential feeding of microorganisms by benthic meiofauna is related to their buccal size and questioned once again the conventional wisdom regarding carbon flux.

The role that microorganisms take in transforming toxic chemical substances is a critical process in an environment inundated with chemical discharges. Conferees examined the various known mechanisms by which microorganisms transform toxic substances in some detail. Discussants generally concluded that greater emphasis must be placed on studying the rates of degradation of xenobiotics at low concentrations in aquatic habitats.

A review of the role of microorganisms in biogeochemistry provided a broad perspective of elemental cycles and the discussion following the presentations brought out how little is known, even now, of the rates of most processes in natural environments and of the significance of microorganisms in mediating these processes. For example, although nitrous oxide is known to be important in ozone depletion in the atmosphere, the significance of microbes and the relative importance of nitrifiers versus denitrifiers in nitrous oxide formations are not yet well understood.

Presentations on microbiological methods surveyed current techniques for measuring biomass and activity and their deficiencies when used to describe natural microbial ecosystems, and the need by regulatory agencies for methodologies that accurately reflect microbial degradation of pollutants and the stress such pollutants create on microbial populations in nature. Discussions focused on problems in experimental design, data interpretation, and improvements in techniques.

Consensus pivoted upon four main points:

> Conversion factors employed in the various techniques, such as in ATP estimations, must be continually evaluated and their precision must be improved.
>
> Advanced statistical concepts such as factorial analysis should be employed in aquatic microbial ecology, especially when attempting to understand the microbial community structure and its interactions with the environment.
>
> Because of the diversity of aquatic ecosystems, no single method can describe biodegradation or microbial biomass and

activity for every aquatic system. Thus the goal of developing a universal standard method is meaningless.

Multiple methodological approaches are needed for research and regulation; specific questions are best answered if the test protocol is situation specific.

Addressing the contentious issue of the use of certain bacteria as indicators of the extent of fecal contamination and environmental alteration at the microbial level, speakers and participants questioned the application of the conventional indicators--coliforms and related microorganisms--and of the enteroviruses--the aeromonads--recently espoused as indicators. The suggestion was made that measuring a relatively refractory chemical component of feces would yield more accurate data on fecal pollution.

Many human pathogens, for example, *Vibrio parahaemolyticus*, *Vibrio cholerae*, and *Aeromonas* are components of aquatic microbial ecosystems. Ecological factors determine their persistence in and mobilization from these ecological reservoirs. If intestinal microorganisms become adapted and integrated into aquatic ecosystems, the resulting shifts in growth optima often make laboratory cultivation difficult. Thus, current methods significantly underestimate pathogenic bacteria and viruses when clinical laboratory methods are employed for their recovery and enumeration.

The need to integrate information regarding microbial communities and processes, in both space and time, was properly recognized as a priority by conference participants. And the importance of integrating with other disciplines was recognized. A multitude of approaches and robust statistical methods are now applied to problems, but clearly aquatic microbial ecologists must apply even

greater efforts to pursue the major goal of understanding the interactions of microorganisms. Scientists who are interested in discussing new concepts and recent developments should assemble for the purpose of integrating present knowledge and structuring it appropriately for meeting the needs of regulatory agencies. Dialogue must be established on general ecology theory that can be applied to microbial ecology.

Recommendations for research arising from the conference include joint collaborative research projects for comparative evaluation of methods, and studies to determine the function of the "nonculturable" organisms and to assiduously assess their activity. Metabolic evolution at the microscale level, determination of food web efficiencies, extension of the concept of diversity to microbial ecosystems, and relationships of microorganisms to other organisms--including microorganism-phytoplankton interactions, grazing of microorganisms by protozoa, and microorganisms as secondary stress factors--are other research problems requiring attention. Too frequently, values which are only rough estimates and based on preliminary data become embedded in the literature, without proper testing for accuracy. This problem requires continual, critical evaluation of "fudge factors."

Environmental monitoring, as presently carried out by regulatory agencies, often neglects the microbial components of ecosystems. However, until questions concerning indicator organisms, test selection, and reproducibility are defined and appropriate test systems are developed, microbiological aspects of monitoring will remain inadequate for the jobs to be done.

A consensus of the conference participants was that interactions between microorganisms and the environment can be fully understood only if

microbial communities and the aquatic environment are considered as a functioning unit. Stress effects must take into account the more robust nature of microorganisms in community.

In conclusion, research priorities for microbial ecology must include searching for answers to basic questions, meeting the needs of regulatory agencies for monitoring and regulation, and examining current dogma for defects in theory and application. Interaction and dialogue between microbiologists and scientists of other disciplines, the general public, and policy makers should be maintained and expanded.

Considering the issues raised and the highly technical level of the scientific discussions, this conference indicates that aquatic microbial ecology has important contributions to make to the fundamental sciences of microbiology and ecology and to the assessment and control of water pollution.

We gratefully acknowledge the assistance of Ray Sauber and Mildred Schwartznau of the American Society for Microbiology in arranging for the conference facilities.

The Conference Organizing Committee

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Carol D. Litchfield
Ralph Mitchell
James T. Staley

AQUATIC MICROBIAL ECOLOGY

Introductory Remarks

"To Cover the Subject Of Microbial Ecology..."

HERBERT C. CURL

We are in an interesting period in history. Recognition of the severity and nature of environmental problems has finally caught up with our ability to produce these problems. A recent Harris poll showed that more than 50% of Americans of all types favored correction of environmental problems, whatever the cost. More recently, pressures from industry have brought about a relaxation of air quality standards because of the apparent costs of cleaning up our acts. Indeed, there is an apparently unrecognized but implicit requirement that about 10% of the gross national product of the United States is required to prevent or mitigate environmental damage that results from industrial activities.

The war is clearly not over. But the battles have resulted in a lot of environmental assessment activity. Amidst the battles is the conflict of institutional needs for information in real time to address management decisions and the scientist's need for a complete data set and time for reflection on the meaning of the data. No attempt has

yet been made to resolve this conflict. Even so, it has resulted in much good science and has forced reexaminations of how to conduct many studies, particularly of how to relate the results of laboratory studies to observations in field studies.

This conference has been convened as a direct result of environmental assesssment activities, particularly those on the Alaskan Outer Continental Shelf. At the time when those studies were initiated in 1975, no one knew exactly how to conduct integrated field and laboratory studies in such a vast area in order to obtain information that could assist in making decisions. Midway through the Alaskan Outer Continental Shelf program, it became apparent that how microbiological studies fit into environmental assessment was not widely known. A series of working-group meetings was convened to consider what kinds of microbiological studies were required for the Outer Continental Shelf areas of the United States. These meetings resulted in a laundry list of techniques and their applicability, published as a National Oceanic and Atmospheric Administration Technical Memorandum in October 1977. Since the reasons for conducting a variety of microbiological studies were still unclear to many of us in federal agencies, it was decided that a gathering of scientists interested in the significance of microbiological processes to marine assessment was absolutely necessary, and this meeting today resulted.

At the risk of my personal safety, let me suggest that microbiologists have not persuaded general ecologists that microorganisms are important. Ecological textbooks depict the nitrogen cycle or the sulfur cycle with virtually no mention of the organisms involved, their trophodynamic roles, or the possible effects of environmental disturbances or contamination. This conference is a step in the right direction in that it deals with the two fundamental issues of ecological processes and environmental disturbance. The results of this

conference should raise the consciousness of environmental scientists and managers.

The charge to this conference as given in the *ASM News* (44:660, 1978) is "to cover the subject of microbial ecology, including basic principles, methods, and applications to problems of pollution, and to develop strategies and outline methods for microbial ecology that would be suitable for large-scale studies designed to evaluate environmental impact. . . ." As a result of ongoing large-scale studies, we have become aware of several scientific subjects that deserve special attention, and which I hope will receive that attention at this conference. These subjects include the use of microorganisms as indicators of environmental health, the effects of disturbance and contamination on community structure, and the effects of changes in microbial community structures on the health of the ecosystem. Because some pollutants are normally present in the environment and other compounds are altogether new and do not take part in normal biogeochemical cycles, the study of the biological formation of chemical pollutants is especially necessary. The subject of detrital food cycles, especially in the arctic, where organic matter tends to accumulate faster than it is decomposed, and the processes involved in the recycling of nutrients deserve attention. The roles of organisms such as yeasts, fungi, and protozoa need additional work.

Finally, let me say that I am personally very pleased to see this conference take place and that I think the organizing committee has done as excellent job of putting together a fine program.

Keynote Address

Factors That Affect Distribution Patterns of Aquatic Microorganisms

MOSHE SHILO

The vast majority of aquatic ecosystems can be considered homogeneous in composition and in physical properties; however, the margins bordering large water bodies are given to extreme fluctuations in conditions. Thus, in undertaking an analysis of the distribution patterns of aquatic microorganisms, we look for two completely different prototypes of microorganisms. Organisms living in the stable pelagic ocean environment should possess relatively uniform properties; they should be psychrophilic, moderately halophilic, and, at appropriate depths, barophilic. In contrast, organisms from marginal areas with fluctuating conditions should possess flexibility, i.e., physiological and metabolic versatility, rapid reactions to changing conditions, and a multimetabolic pattern with control mechanisms to open and shut off metabolic pathways as required.

In the majority of aquatic ecosystems, nutrient concentrations are extremely low, and flux of nutrients across the ecosystem is small, so the

effective turnover rate is low. Thus, ideal aquatic microorganisms should be oligotrophic, or, as Winogradski (1949) suggested, "autochthonous" nutritional types. These should have especially efficient uptake mechanisms, constant uptake capability, and the ability to accumulate reserves of a wide variety of nutrients. A common alternative life strategy for low-nutrient environments is escape to microhabitats where local nutrient adsorption creates higher nutrient concentrations (Marshall 1976). Indeed, most aquatic microorganisms are found adhering to interfaces, such as the air/water interface, where they are held in position either by physical forces or by means of specific attachment organelles or mucoid excretion bridges. They may also be bound to solid substrates such as suspended particles floating in the water, e.g., detritus, fecal pellets, flocculated bacteria, and phytoplankton. Any quantitative estimates of microorganisms in the aquatic milieu must take into account this heterogeneity of distribution in order to be meaningful.

Some examples of different types of aquatic microorganisms from both constant and fluctuating conditions will illustrate the problem outlined above and will demonstrate some of the major factors that play a role.

The first example deals with marine bdellovibrios, predatory bacteria which must carry out part of their life cycle--multiplication--within the periplasmic space of the prey bacteria they attack and penetrate. Marine bdellovibrios are found in oceans the world over. Their absolute need for high osmotic pressure for growth and their specific requirement for four major cations (Na, K, Mg, and Ca) during different stages of their morphogenesis (Marbach and Shilo 1978; Marbach, Varon and Shilo 1976) indicate that they are autochthonous to the marine environment. For a stable bdellovibrio population to be sustained, minimal prey-cell

populations must be constantly present. The prey-cell density required is, however, much higher than that of most marine environments in which bdellovibrios are found, as estimated from kinetics of extinction in predator-prey experiments using different densities of luminous bacteria (Varon and Shilo 1979; Varon and Ziegler 1978). An explanation of this paradox may be that growth and multiplication of bdellovibrios occur in interface microhabitats where host-cell densities may be sufficiently high.

Israel is located both on the Mediterranean Sea, connected with the Atlantic Ocean, and the Gulf of Elat, part of the Red Sea-Indian Ocean region. These bodies of water, which differ greatly in physical and chemical properties, are joined by the Suez Canal. Thus, we have a favorable situation for carrying out a simultaneous comparative study of distribution patterns of marine bacteria.

Detailed knowledge of the biochemical basis for bioluminescence and the taxonomic treatment of the group by Reichelt and Baumann (1973) favor the selection of luminous bacteria as indicator organisms for studying geographical and seasonal distribution. Four major clusters of luminous bacteria are now easily differentiated on the basis of nutritional requirements, flagellation, bdellovibrio sensitivity, and phage typing. These clusters are *Photobacterium phosphoreum*, *P. fischeri*, *P. leiognathi*, and *Beneckea harveyi*.

Three of the clusters are found as free-living organisms and also as symbionts in luminous organs of fish and cephalopods. Only one, *B. harveyi*, has not been found in symbiotic associations. Our results indicate that the distribution patterns in the eastern Mediterranean and in the Gulf of Elat are governed by four major factors: water temperature, lethal photooxidation, nutrient concentrations, and salinity. The adaptability of different types

to these factors determines their distribution (Shilo and Yetinson 1979; Yetinson and Shilo 1979).

The following examples concern factors governing selection of microorganisms in shallow, photic aquatic ecosystems in tropical and subtropical regions where light intensity is high and where diurnal fluctuations in the major physical and chemical parameters are particularly marked. Some organisms, such as benthic diatoms and some filamentous gliding cyanobacteria, can adapt by means of diurnal vertical migration. But most organisms, particularly other cyanobacteria which thrive in these conditions, have a capacity for shifting metabolic patterns as well as a capacity for overcoming the toxic effects of the highly reactive oxygen species.

The metabolic flexibility of cyanobacteria is particularly well illustrated by *Oscillatoria limnetica*, studied intensively both in the laboratory and in the field. It was isolated from the Solar Lake near Elat, a shallow monomictic hypersaline lake with steep gradients in temperature, oxygen, sulfide, light, pH, and other parameters. For ten months, the hypolimnion is anaerobic and rich in sulfide; for two, the lake is homogeneously oxygenated (Cohen et al. 1977). *O. limnetica* can shift from oxygenic photosynthesis to anoxygenic photosynthesis (Oren and Shilo 1979) (the first discovery of nonoxygenic photosynthesis in a cyanobacterium). Organisms with polyglucose-reserve granules can generate energy in conditions of darkness in one of three ways (regardless of the kind of photosynthesis): in the presence of oxygen, by aerobic respiration; in the absence of both oxygen and sulfur, by fermenting polyglucose to lactic acid; or, in the absence of oxygen but presence of sulfur globules, by anaerobic respiration, regenerating H_2S in the process (Oren and Shilo 1979). Metabolism in the dark may be of great importance for the maintenance mechanisms of

organisms which live in conditions of low light. Each shift among light, dark, aerobic, and anaerobic conditions leads to the specific induction activation, or repression, of different metabolic potentials of the organisms.

In addition, *O. limnetica* shows flexibility in its resistance to photooxidation, with anaerobically grown strains showing increased resistance and a concomitant increase in superoxide dismutase activity (D. Friedberg, unpublished data). When anaerobically grown organisms are exposed to oxygen, superoxide dismutase levels increase markedly, and there is a proportional increase in resistance to oxygen and light. Drastic changes in salt concentrations and fluctuations in temperature might also control the specific choice of metabolic potential.

We have become increasingly aware that oxygen and its reactive species are highly toxic to all living cells, anaerobic and aerobic ones alike. The problem is most severe in shallow aquatic ecosystems where heavy cyanobacteria blooms form scums, causing oxygen supersaturation in the upper layer, along with CO_2 depletion. Mechanisms for coping with oxygen and/or repair of damage are well developed in natural strains of cyanobacteria, whereas laboratory strains are sensitive. Resistance to photooxidation in pond strains is associated with high superoxide dismutase levels after exposure to photooxidative conditions; in exposed sensitive cells, superoxide dismutase levels drop (Eloff, Steinitz and Shilo 1976). The high resistance of some cyanobacterial species (*Microcystis*, *Anabaena*, and *Spirullina* types) to photooxidation seems to result from selective preferential synthesis of Mn-superoxide dismutase isozyme over the more prevalent Fe-superoxide dismutase isozyme. The former is greatly resistant to H_2O_2, whereas the latter is sensitive to H_2O_2. The sporadic mass mortality of even highly resistant cyanobacterial blooms to extreme photooxidative

stress seems to be linked to the inhibition of the physiological capacity of the cells to synthesize the superoxide dismutase at their regular high rate.

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Session I

EFFECTS OF ENVIRONMENTAL STRESSES ON MICROORGANISMS

Physiological and Genetic Implications of Mixed Microbial Populations and of Microbial Population and Microbial Community Growth

J. H. SLATER

The nature of the chemical and physical environment, whether under stable or stress conditions, influences the growth and physiology of microbial populations. Furthermore, the overall status and metabolic capability of a given population may be markedly influenced by the activities of adjacent populations within the same environment. As a result of these external parameters, microbial populations respond in a fashion which maximizes their chance of survival and rate of growth.

In this paper I shall concentrate on the features of various types of interaction of different microbial populations which serve to minimize the effects of self-generated environmental stresses on a given microbial population. Although the basic, nonbiological features of an environment influence the types of microbe located in that environment, self-induced environmental conditions and stresses are also extremely significant. For example, they result in such phenomena as successions of different species of microorganisms, each succeeding population developing in conditions generated by a preceding

population which are detrimental to the growth of the causative population. Interactions of different populations, which have been summarized extensively elsewhere (Meers 1973; Slater and Bull 1978), can serve to dampen potentially damaging environmental stresses and generally ameliorate the prevailing conditions.

Removal of Inhibitory Metabolic Products

Frequently, the products of the metabolism of a particular organism eventually accumulate to concentrations that restrict or inhibit the growth of that population (Slater 1978). Other populations capable of utilizing the undesirable end products of metabolism ensure that the environmental conditions do not change greatly, and permit the continued growth of the first population, while supporting still other dependent populations. For example, Wilkinson and his colleagues (1974) isolated a mixed microbial culture growing on methane as the sole carbon source (Figure 1). The stable association contained at least four different microbial species, only one of which, a pseudomonad, was able to oxidize and assimilate methane. Alone, cultures of the pseudomonad accumulated methanol, which rapidly caused inhibition of growth. The deleterious environmental change was prevented in the growing mixed culture since a secondary population, a species of *Hyphomicrobium* present at 4% of the total mixed culture, scavenged for and assimilated the excreted methanol. Moreover, the presence of the *Hyphomicrobium* species ensured that, under transient stress conditions, the optimal growth conditions were restored.

Under conditions where an exogenous pulse of methanol was added to the microbial community, the rapid increase in the methanol concentration resulted in a transient inhibition of the methane-oxidizing pseudomonad, but an increase (up to as high as

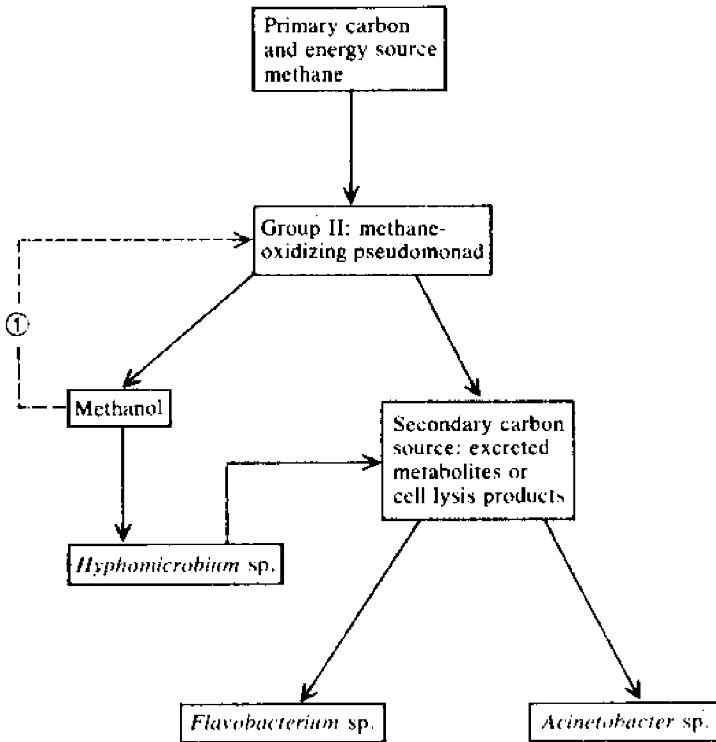

Figure 1. Isolation of a mixed microbial culture growing on methane.

25% of the total population) in the methanol utilizer. As the methanol concentration declined, the relative proportions of the two component populations returned to the prestress levels concomitantly with a restoration of the optimal growth environment.

Associations of microorganisms structured on this principle need to be fully assessed, particularly in terms of their type and frequency under natural conditions. It is well established as the basis of interaction of sulfur photosynthetic bacteria and sulfate-reducing bacteria (Gray et al. 1973; Wolfe and Pfenning 1973; Abstr. Int. Congr. Microbiol., 12th, Munich, 1978, p. 16). The association of heterotrophs with obligate chemolithotrophs may also be important in ensuring the optimal chemolithotroph growth rate by reducing the concentrations of excreted organic compounds (J. G. Kuenen and J. C. Gottschal, Abstr. Int. Congr. Microbiol, 12th Munich, 1978, p. 15).

Microbial Communities to Maximize the Rate of Growth

The rate at which a population grows ultimately determines how successful that population may be in exploiting a given set of environmental conditions. A limited amount of evidence suggests that associations of organisms may result in significantly improved overall basic growth parameters and hence growth rate. For example, a three-membered microbial community isolated with orcinol as the sole carbon and energy source was shown to contain a single primary organism, *Pseudomonas stutzeri*, able to utilize orcinol as a growth substrate in pure culture (A. Osman, A. T. Bull, and J. H. Slater, Abstr. Int. Symp. Fermentation, Berlin, 1976, p. 124). The other two organisms were unable to grow on orcinol and were therefore designated secondary organisms. However, the presence of the two secondary organisms significantly improved, by

approximately 30%, the affinity the community had for the aromatic compound compared with the affinity the primary organism had for orcinol in pure culture. In fact, orcinol was inhibitory to *P. stutzeri* and the influence of the two secondary organisms was a more complex relationship between the saturation constant and the inhibition constant (A. Osman, A. T. Bull and J. H. Slater, unpublished data).

Microbial Communities Based on Combined Metabolic Attack

In many cases the degradation of organic compounds, particularly complex substances, appears to be the result of the complementary metabolic activities of more than one population (Slater 1978; Slater and Somerville 1979). Some of the more notable examples concern the degradation of environmentally foreign compounds, particularly pesticides. Thus, although the number of such microbial associations that have been isolated and defined is limited, it seems probable that they are a common feature of natural assemblages of microorganisms. Their detection simply requires the use of appropriate enrichment and isolation techniques based on continuous-flow culture principles (Slater 1978).

Clearly, these microbial communities fulfill a crucial role when natural environments are stressed by the presence of xenobiotic compounds, which often have potent and environmentally hazardous properties. One example of a combined metabolic attack community comes from the work of Gunner and Zuckerman (1968), who showed that the insecticide Diazinon (*o*,*o*-diethyl-*o*-2-isopropyl-4-methyl-6-pyrimidyl thiophosphate) was appreciably degraded only by the joint activity of an *Arthrobacter* sp. and a *Streptomyces* sp. Neither organism alone could effect cleavage of the pyrimidine ring. Similar communities have been described for the degradation of surfactants (Baggi et al. 1978; V. Johanides and D. Hrsak, Abstr. Int. Symp.

Fermentation, 5th, Berlin, 1976, p. 426).

More recently, Daughton and Hsieh (1977) showed that the degradation of parathion (*o,o*-diethyl-*o*-p-nitrophenol phosphorothionate) depends on the interaction of *P. stutzeri*, which hydrolyzes the insecticide to two products, diethyl thiophosphate and *p*-nitrophenol (neither of which can act as a carbon and energy source for the hydrolyzing organism), and *P. aeruginosa*, which catabolizes one of the products, *p*-nitrophenol. In turn, the *P. aeruginosa* component of the community furnishes readily metabolizable compounds required to sustain the primary population upon which it is dependent.

Microbial Communities with More Than One Primary Utilizer

Stable microbial communities have been described in which two or more primary degraders coexist, although each one is capable of independent growth on the growth substrate. One such community, isolated by growth on the herbicide Dalapon (2,2'-dichloropropionic acid) (Senior, Bull and Slater 1976), initially contained three primary Dalapon utilzers, two bacteria and a fungus (Figure 2). Although the precise relationship between the placement members was not elucidated, the advantage to all the organisms appeared to be the stability of the system, particularly under environmental stress situations of variable pH and shock loadings of the substrate (Senior, Bull and Slater 1976).

Microbial Communities and Their Role in the Evolution of New Metabolic Capabilities in Response To New Environmental Conditions

A major environmental stress occurs when a novel, nonmetabolizable compound appears in the environment. Apart from the general importance of

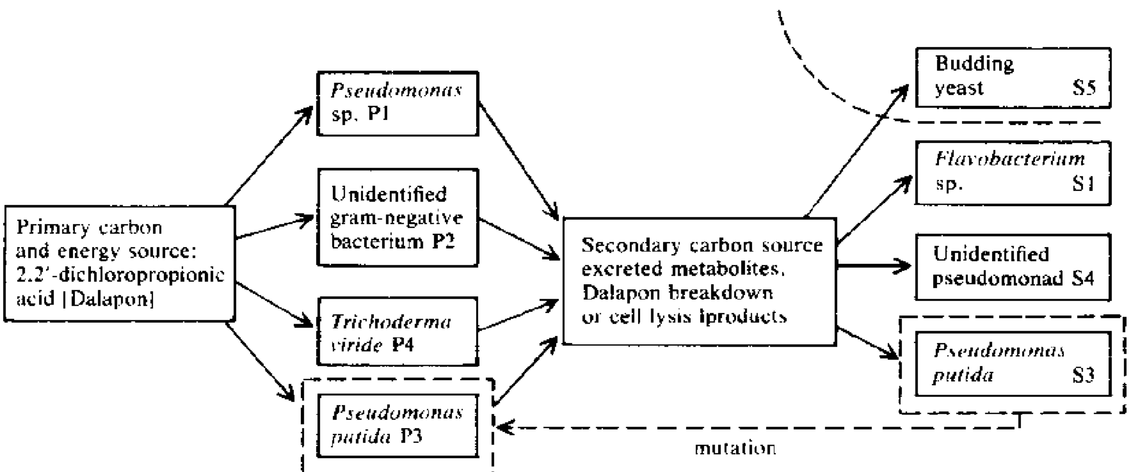

Figure 2. Microbial community containing three Dalapon utilizers, two bacteria and a fungus.

the economy of carbon and energy sources (that is, the exploitation of all available resources), the evolution of a degradative mechanism to mobilize such materials may be significant in maintaining the stability and character of a particular environment.

We have argued (Baggi et al. 1978) that microbial communities may have an important role to play in the development of new pathways. For example, the Dalapon community provided a permissive environment which enabled the selection of a fourth primary utilizer from one of the initial secondary organisms, a strain of *P. putida* (Figure 2).

Lovatt in our laboratory (Lovatt, Slater and Bull 1978) has shown that it is possible to adapt a multimembered community growing on picolinic acid to metabolizing partially the chlorinated analog 3,6-dichloropicolinic acid. It seems unlikely that such an adaptation would have occurred with pure cultures. More importantly, however, a particular compound may be degraded because a number of enzymes, originally evolved for different pathways and compounds, exist in two or more different microorganisms. The fortuitous activity of these enzymes on the compound in question could allow the compound to be degraded as a result of community activity.

This raises the question: Could these various enzymes, or rather their genes, be collected together in a single organism to construct a novel pathway, the individual enzymes of which could then be improved through well-established mechanisms (Clarke 1974)? It is now clear that the potential for genetic fluxes of this sort does exist, mediated by nonspecific phage, plasmids, or simply transformation. The intriguing question remains, however: Does such a mechanism operate in nature?

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Environmental Effects On Microbial Processes

RALPH MITCHELL

In recent years we have become increasingly aware of the pervasive effects of toxic pollutants on biological processes. The focus of our attention has been on direct toxicity. Minimal attention has been paid either to the role of pollutants as indirect agents of stress or to the destructive influence of sublethal levels of toxicants on essential microbial processes. Yet, it is likely that toxicants present in soil and water at low levels act either alone or synergistically to cause widespread ecological damage by perturbation of the indigenous microflora. The adverse effects are not limited to toxic chemicals. The eutrophic effects of sewage, chemicals, and thermal wastes on receiving waters also provide important sources of disturbance of the microbial community.

Microbial Diseases Caused By Pollutants

The accumulation of pollutants in an aquatic habitat often does not attract attention until a

massive fish kill occurs. It is rare to observe a sick or debilitated animal in its natural habitat. Ineffectual organisms are rapidly destroyed by predators, and the slow destruction of a population is often missed by human observers. The presence of pollutants at sublethal levels often provides the trigger for invasion by opportunistic pathogens, but the progress of these epizootics is rarely fast enough to result in massive mortalities. More frequently, the host population is slowly eradicated.

Industrial chemicals and pesticides present a major threat to the aquatic biota, even when present at sublethal concentrations (Mitchell and Chet 1978). Gill infections of Atlantic salmon by *Aerobacter liquefaciens* have been associated with zinc and copper pollution (Pippy and Hare 1969). There is increasing evidence that baculovirus infections of shrimp in the Gulf of Mexico are related to accumulation of polychlorinated biphenyls (PCBs) in the hepatopancreas (Nimmo et al. 1971). Fin erosion of English sole and starry flounder has also been shown to be correlated with PCBs in the fish tissue (Wellings et al. 1979), and chlorinated hydrocarbons have been implicated in widespread fin rot observed in the Dover sole populations of the New York bight. *Aeromonas* and *Pseudomonas* appear to be the pathogens (Mahoney, Midlige and Devel 1973) in the Dover sole epizootic.

The pesticide Mirex is used to control fire ants. Granulomas caused by acid-fast bacteria occur in goldfish when they are fed Mirex, and pink shrimp become infected by baculoviruses (Van Valin, Andrews and Eller 1968). DDT is known to predispose salmon to fungal infections (Cope 1965).

The wild fowl living in polluted habitats are equally susceptible to stress-induced disease. Although mollusks showed no ill effects when they were fed 100 mg of PCB per liter, ducks fed 50 mg of PCB per liter and exposed to duck hepatitis virus showed a mortality rate of 60%, compared to 15%

caused by the virus alone (Friend and Trainer 1970).

Destruction of the aquatic biota is common in waters that receive domestic sewage or nontoxic agricultural wastes. Usually, death is caused by the development of anaerobic conditions in the presence of excessive quantities of available organic matter. However, frequently conditions of partial oxygen deficiency exist in the water column, providing the stress necessary for the development of microbial opportunists. The myxobacterium *Chondrococcus columnaris*, common in eutrophic waters, causes columnaris disease in the fish population (Collins 1970). *Aeromonas* also predominates in nutrient-rich, oxygen-depleted waters and is often found in waters receiving agricultural runoff. In the waters of Florida, both fish and reptiles are often infected by populations of *Aeromonas* that utilize the citrate in the citrus-rich wastes as a carbon source (Shotts et al. 1972). *Aeromonas* is also prevalent in thermal effluents.

The genus *Aeromonas* provides an excellent example of opportunism. Although *A. hydrophila* is common and easily isolated in most fresh waters, fish disease is rare. Yet when the host population is under pressure and *Aeromonas* populations are high, fish infections become widespread.

Brevibacteria and corynebacteria are other opportunistic pathogens. They are stimulated by thermal elevation. The resulting disease has been described in detail in salmon (Smith 1964). The optimal temperature range for these pathogens in natural waters is 10 to 15°C. At temperatures above 15°C *Vibrio anguillarum* is often found in northern European coastal waters, causing "red disease" of eels (McCarthy 1976), and the fungus *Dermocystidium* is associated with oyster mortalities at water temperatures in excess of 25°C (Hewett and Andrews 1956).

The diseases described above involve an

intimate interaction of the host, the pathogen, and the environment. It is likely that sublethal levels of toxic pollutants suppress immune responses and allow opportunistic pathogens to develop. Nontoxic pollutants often are enriching to an indigenous microorganism capable of infecting a debilitated host.

We have used corals in my laboratory as model systems for studying the complex interactions involved in stress-induced disease (Mitchell and Chet 1975). Coral reefs are unusually sensitive ecosystems that are under increasing stress as a result of industrialization. The fragility of corals is probably caused by the complexity of the interaction of the coelenterate, its algal symbionts, and the associated bacterial population. In Hawaii and the Virgin Islands, sewage effluent discharged near reef areas has caused the slow death of corals. Sediment disturbance is destroying the reefs off the Florida keys, and oil pollution is slowly killing many species in the Red Sea.

In laboratory studies using isolated coral heads, we have found that sublethal concentrations of petroleum or heavy metals stimulate excessive mucus production by the coral polyps. In the presence of antibiotics the mucins do not adversely affect the coral. In the presence of the indigenous microflora, however, the contaminated coelenterates slowly die (Mitchell and Chet 1975). The coral mucins have been characterized as complex mixtures of polysaccharide, protein, and lipid, enriched in nitrogen and phosphorus (Ducklow and Mitchell 1979*a*. The structures do not appreciably change in the presence of pollutants.

The coral diseases appear to be stimulated by a change in the viscosity of the mucins that allows them to accumulate at the coral-water interface. The resultant bacterial population is dominated by *Vibrio alginolyticus*, an efficient mucus utilizer. This vibrio is also chemotactically attracted to the coral mucins. During the course of the disease,

oxygen becomes depleted at the coral surface. *Desulfovibrio* develops, and ultimately the dying polyps are coated with a film of *Beggiatoa* (Ducklow and Mitchell 1979*b*). It is probable that *Beggiatoa* behaves as an opportunistic pathogen on polyps damaged by the hydrogen sulfide. Filaments can be seen penetrating living coral tissue, probably hastening death of the coral.

Our observations on the role of mucins in coral disease suggest that excessive mucous secretions in aquatic organisms may provide an important key to pollution-induced disease. The relationship is unlikely to be confined to fish and mollusks. Bacteria also attack algae in polluted water. Both *Macrocystis* and *Ulva* develop lesions in the presence of pollutants, apparently caused by opportunistic pseudomonads. The role of mucins in pollution-induced disease of algae needs investigation in view of the excessive production of slime by the stressed organisms.

Chemoreceptor Blockage

Bacterial chemoreception plays an important role in aquatic habitats (Chet and Mitchell 1976). All motile bacteria have chemoreceptors. The capacity to detect concentration gradients of chemicals allows motile bacteria to locate food. Bacterial predators are attracted to their prey microorganisms. Chemoreception is also involved in symbiosis and in the surface ecology of microbial communities. It is probable that the presence of specific microbial chemoreceptors controls the composition of microbial communities in specific ecosystems by giving those microorganisms a selective advantage.

Some motile bacteria can avoid toxic chemicals by negative chemotaxis; i.e., they are repelled by the concentration gradient of the toxic chemical diffusing into the water. The vast majority of bacterial chemoreceptors are blocked by

concentrations of toxic chemicals small enough to have no effect on motility (Mitchell, Fogel and Chet 1972). This phenomenon has important implications for nutrient recycling in the oceans. Chemoreceptor blockage prevents motile bacteria from accumulating on substrates and causes severe inhibition of the decomposition of organic matter (Chet and Mitchell 1974).

Blockage of intermicrobial predation by pollutants may also retard self-purification processes in sewage treatment and in waters receiving human sewage. Sublethal levels of PCBs and the herbicide 2,4-D prevent predation on *Escherichia coli* by predacious marine bacteria (Walsh and Mitchell 1974). The evidence indicates that survival of the coliforms is linked to the inability of the predators to detect their microbial prey. In view of the chronic occurrence of low levels of toxic chemicals in sewage treatment plants and in coastal waters, these observations point to the urgent need for a better understanding of the subtle effects of pollutants on self-purification processes in natural waters.

Indicators of Chemoreception Blockage

One of the most subtle effects of water pollution is the development of aberrant behavioral patterns in the aquatic fauna. Low levels of toxic chemicals affect feeding and sexual responses. For example, petroleum hydrocarbons have been shown to affect the complex sexual behavior of lobsters (Atema and Stein 1974), and salmon feeding is retarded by sublethal levels of detergents (Sutterin, Sutterin and Rand 1971). These perturbations are caused by chemoreception blockage.

Motile microorganisms can be used as simple and reproducible indicators of chemoreceptor blockage by pollutants. Microbial chemotaxis might

provide an inexpensive tool for detecting either the presence of chemoreceptor blocking agents in the water or for studying synergistic effects of combinations of pollutants on chemoreception.

A serious drawback to the use of bacterial indicators of chemoreception blockage by pollutants is the low level of sensivity of most bacterial receptors to toxicants. All of the bacteria tested require at least 1 mg of toxicant per liter for blockage to occur. The green alga *Dunaliella* provides a more sensitive chemoreception indicator (Sjoblad, Chet and Mitchell 1978). It is sensitive to levels of toxicants below 0.1 μg/ml. In addition, the assay can be carried out rapidly and accurately by use of fluorometric techniques. Natural seawater samples taken from polluted locations totally block *Dunaliella* chemotaxis, suggesting synergistic effects of pollution on algal chemoreception. The presence of mixtures of either heavy metals or hydrocarbons in the water inhibits the chemotactic response of the alga.

It is apparent that motile eucaryotes can be used effectively to signal the presence of chemoreceptor blocking agents in polluted water. The data suggest the possibility of using these microbial indicators as alternatives to complex bioassays requiring the use of the aquatic fauna to study pollution-induced chemoreceptor blockage.

Disturbance of Host-Microorganism Balance

The populations of microorganisms on the surfaces of plants and animals living in aquatic habitats play an important role both in the provision of protection against pathogens and in nutrient recycling processes. Similarly, the intestinal microflora provides essential support for the physiological processes occuring in the digestive tract. Sublethal levels of pollutants disturb these

essential interactions.

In my laboratory we have observed these perturbations with crustaceans. The outer skeleton of the marine wood-boring isopod *Limnoria* is completely covered with bacteria (Boyle and Mitchell 1978). The digestive tract, however, is completely free from microorganisms. A similar situation exists with the terrestrial analog of *Limnoria*, *Oniscus*. In contrast, the termite *Reticulitermes* possesses a large microbial population in its digestive tract. *Limnoria* and *Oniscus* may utilize the microflora passing through their digestive tracts as a direct source of nitrogen. In contrast, the termite utilizes its intestinal microflora to fix atmospheric nitrogen symbiotically.

The delicate physiological balance permitting *Limnoria* and *Oniscus* to degrade microorganisms passing through their digestive tracts is easily upset. Levels of chemicals in the food well below the toxic level appear to block the bacterial lytic system. A common result is the observation of debilitated crustaceans containing a significant number of microorganisms in the digestive tract.

The need to develop a better understanding of the chronic and subtle effects of sublethal levels of pollutants cannot be overemphasized. Our observations of stress-induced disease, chemoreceptor blockage, and perturbation of essential physiological processes in crustacean digestive tracts is indicative of more widespread and subtle disturbances. A threefold approach to the problem is needed: (i) studies of the microbial ecology of the healthy biota in undisturbed ecosystems, (ii) a better understanding of the subtle detrimental effects of chronic sublethal levels of pollutants on essential microbial processes and on the relationships between microorganisms and higher organisms living in aquatic habitats, and (iii) an investigation of the synergistic effects of sublethal levels of pollutants on microbial processes in

aquatic habitats.

Acknowledgments

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Some Thoughts on and Examples of Microbial Interactions in the Natural Environment

PETER HIRSCH

It is a bad thing to discuss something you have learned during the lecture that went on while you were preparing your slides and talk at the same time. So I won't give a formal lecture, but just provide some ideas on Dr. Slater's talk and on symbiotic associations and the possible ways in which they may arise.

The first question I would like to pose concerns the slide of Dr. Slater's matrix: Could there be any communities present within the same environment which do not interact? I wondered about this many years ago when I read Dr. Brock's book on microbial ecology in which he suspected that no interaction between organisms adjacent to each other would not be possible. Since all living organisms require the same basic elements for growth of their cells, they should, for example, compete for these elements.

The second question concerns the discussion of community members and their joint efforts to

utilize a primary carbon source. Have there been any experiments where organisms that normally do not live together were brought together in a natural environment to see how they would cooperate or compete? In our laboratory systems we usually lack predators and we also lack diurnal changes of environmental factors. One should really study the interactions of organisms or communities under as natural conditions as possible.

Dr. Slater mentioned that a *Hyphomicrobium* participated in the methanol utilization system he had studied. We have done some work on hyphomicrobia during the past 15 years (Hirsch 1974), and I would like to report the strange observation that our *Hyphomicrobium* strains do not appear to lyse in pure cultures. So the organic input *Hyphomicrobium* add to his system may not be liberated by lysis but rather by excretion through living cells. We have some indications that there may be vitamins excreted by older cells of *Hyphomicrobium* spp.

I will now refer to close associations of organisms which thereby probably manage to better exploit the environment or withstand stress situations. I would like to offer some thoughts on the origin and purpose of such associations. We have to realize that in mixed populations there could be accidental interactions and associations from identical and simultaneous location; such interactions could be ephemeral. Some of these assocications could have a more stable character and eventually become permanent, with stress situations providing the selective evolutionary forces. My first slide demonstrates such an accidental association of microorganisms in a stress situation—it shows the microorganisms forming "bloody snow" (Figure 1). Here we find algal flagellates, diatoms and various bacteria brought together in small surface indentations of snow, where they are subjected to high light intensities, cold temperatures, and lack of nutrients.

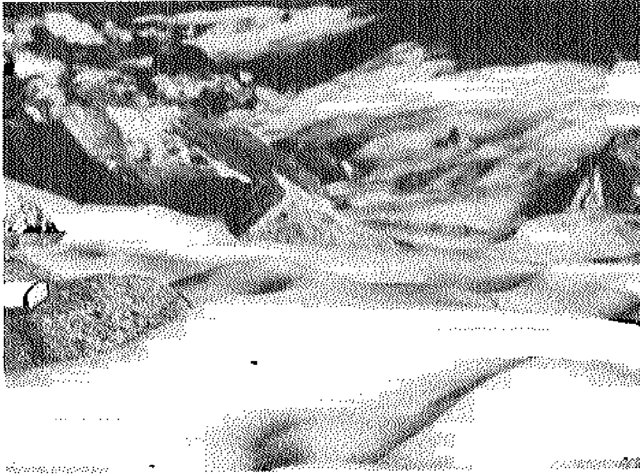

Figure 1. A case of "bloody snow" observed in the Jotunheim Mountains, Norway, by Dr. R. Rottger, Kiel. The dark portions, especially in indentations, contain a specially adapted microflora of algae and bacteria.

Many of the organisms found here adapt and manage to live quite well in this rather exotic "aquatic" environment. It is obvious that some of the members of this association develop an increasing interdependency, especially when this habitat remains undisturbed for a long time.

Hypolimnetic bacteria from Cassidy Lake, Michigan, demonstrate another interaction possibility (Figure 2). The anaerobic zone of such stratified lakes is rich in many diverse bacteria that normally live colsely together in the same location. Certainly many of them interact efficiently, but this is not always evident in microscopical samples. However, if one stresses this environment by shaking a sample with oxygen, many of these bacteria will attach to each other and clump, thus forming a precipitate. With the oxygen consumed again they will return to suspension. Hence organisms found together need not necessarily interact physiologically.

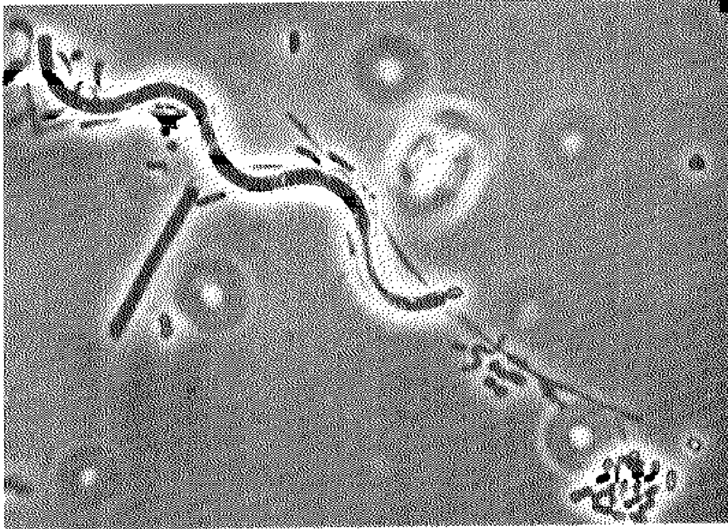

Figure 2. Bacteria from the hypolimnion (7 m) of Cassidy Lake, Michigan, July 1968. Magn. 1280 x.

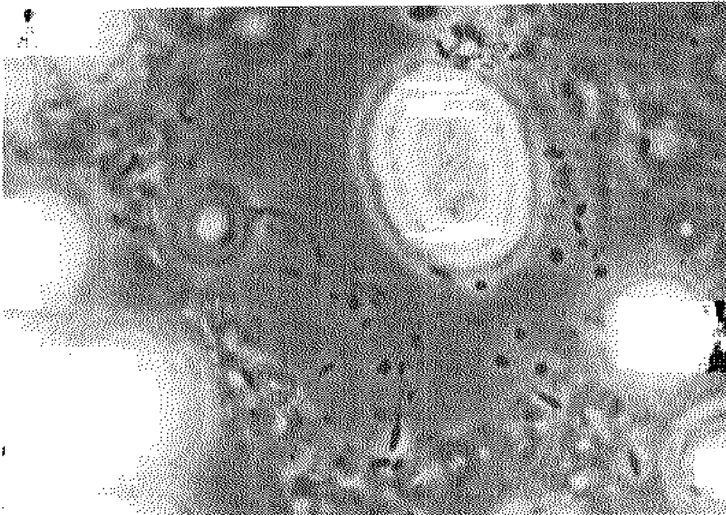

Figure 3. Oligotrophic bacteria found in *Gloeocapsa* capsules in a river water enrichment. Magn. 1280 x.

A more fortuitous association of bacteria with cyanobacteria is shown next (Figure 3). A *Gloeocapsa* sp., producing huge capsules, often harbors oligotrophic bacteria in the slime layers of such organisms as *Hyphomicrobium*, *Caulobacter*, *Stella*, etc. All of these morphologically distinct forms normally live quite well without the polymer, but in the natural environment, nutrient concentrations are higher in the polymer than in free water. Basically, the same holds true for the *Microcystis* capsules shown in Figure 4. However, here we often find large numbers of *Leptospira* spp. and other spirochetes, presumably living at the expense of the other bacteria enclosed by the polymer.

Some algae and protozoa can never be cultivated well in laboratory pure culture because they seem to require "epiphytic" bacteria. Figure 5 shows cells of an *Asterocytis* sp. clone, green-pigmented red algae isolated by Dr. Dedio in Kiel. The capsular surface is densely populated with rod-shaped or prosthecate bacteria, which can be readily cultivated without the alga. Growth of epi- and basiphytes is optimal only in the association of both.

An enrichment culture for hypomicrobia, inoculated with water from a polluted river on the Michigan State University campus, yielded a coccal cyanobacterium to which the *Hyphomicrobium* attached head on in large numbers. While both of the organisms were capable of growing alone in the respective media, growth in attached association in C- and N-free mineral salts medium was optimal and lasted for more than two years. Considerable amounts of organic matter were produced by this perhaps accidental and fairly efficient association. The forms associated have never been observed in natural water samples, and it is possible that the laboratory stress condition "caused" this association (Figure 6).

A still closer association is represented by

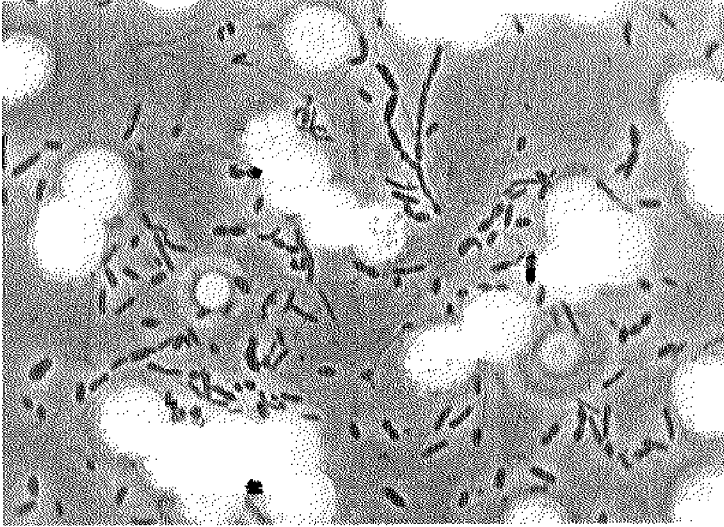

Figure 4. Bacteria inhabiting the capsular space between cells of *Microcystis* sp. during a bloom at Gull Lake, Michigan. Magn. 1280 x.

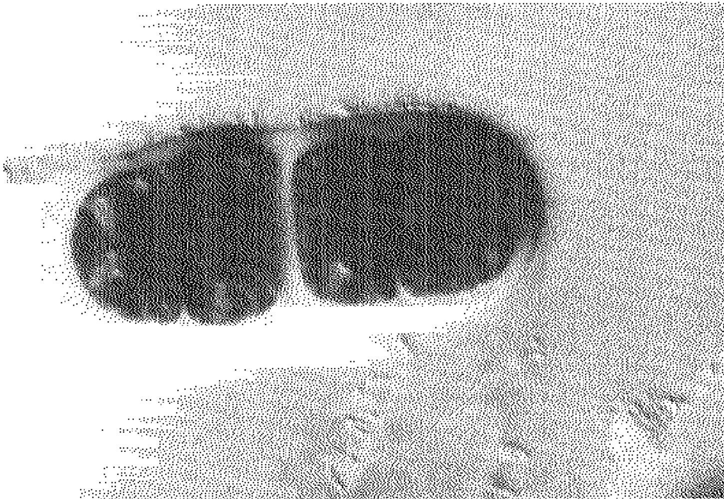

Figure 5. Four cells of the red alga *Asterocytis* sp. showing slime stalk and bacterial epiphytes. Laboratory culture Nomarski-interference micrograph by Dr. H. Volker, Kiel. Magn. 1280 x.

cyanobacterial chains of cells tightly covering the surface of a pondwater *Euglena*. Similar forms may be *Eugena spirogyra* or *E. splendens*, described in the literature. The association shown here was found in partially anaerobic water of a polluted iron spring, and it is possible that the interaction involves sharing of photosynthetically produced oxygen (Figure 7).

Consortia of anaerobic green sulfur bacteria (*Chlorobium* sp.) and central colorless bacteria can be found frequently in the anaerobic hypolimnion of freshwater lakes. Here some of the *Chlorbiaceae* have been obtained in pure culture (Mechsner 1957), but the colorless central organisms have not been cultured as yet. In fact, all natural samples containing these consortia also contain large numbers of free *Chlorobium* cells, but the central organisms do not appear to occur in the free state. These complete consortia have been grown successfully in Perfil'ev convection chamber gradients in the presence of sulfide and low concentrations of organic matter (Hirsch unpublished). Figure 8 shows a 6.25 m water sample from Cassidy Lake, Michigan, with possibly three different consortia: bluish-green *Chlorochromatium aggregatum* (A), olive green *Chlorochromatium glebulum* (G), and reddish *Pelochromatium roseum* (R). The *C. aggregatum* consortia in Figure 9 were slightly compressed to dislodge the green sulfur bacteria from the central organisms. The latter have pointed cell ends and occassionally carry dense storage granules (polyphosphate?) and lateral outgrowths. One can surmise that the interactions of these consortia members concern the exchange of reduced sulfur and organic compounds, perhaps acetate, as has been found for artificial associations of *Chlorobiaceae* and sulfate reducing bacteria (Biebl and Pfennig 1978).

An even tighter physical association can be observed with hypolimnetic bacteria termed "*Peloploca pulchra*." Up to now this slightly twisted

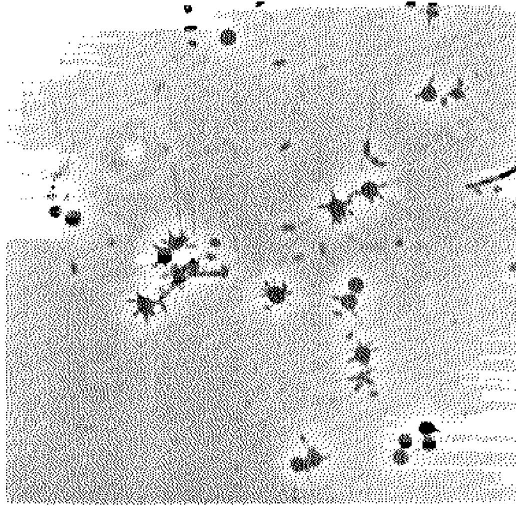

Figure 6. *Hyphomicrobium*-cyanobacterium association from a river water enrichment. Magn. 1280 x.

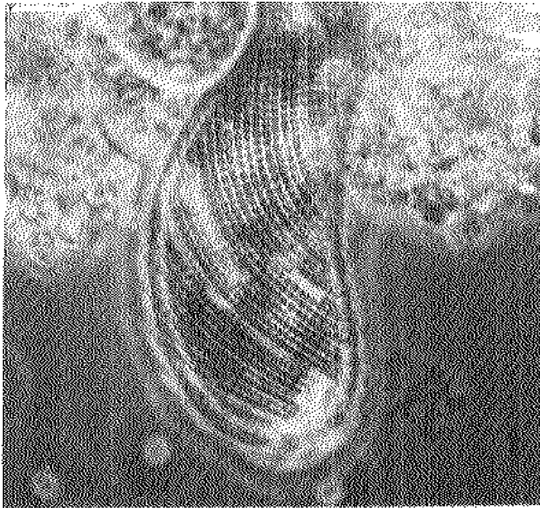

Figure 7. *Euglena* sp. covered with chains of cyanobacteria from a polluted iron spring near Itzehoe/Holstein. Magn. 1280 x.

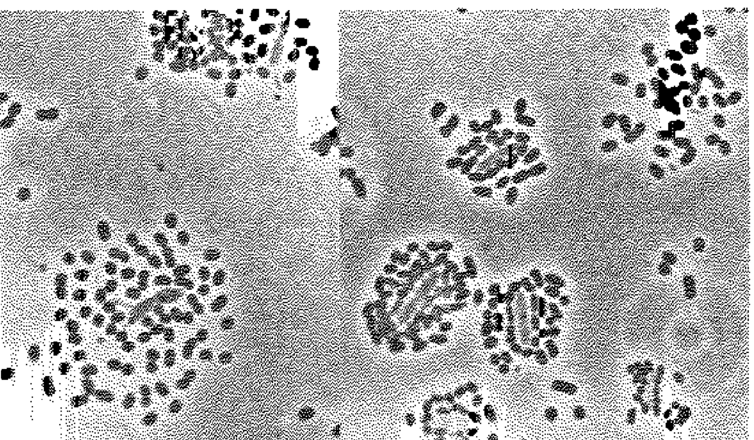

Figure 9. *Chlorochromatium aggregatum* consortia from Cassidy Lake, slightly compressed to show the colorless central bacterium. Magn. 1280 x.

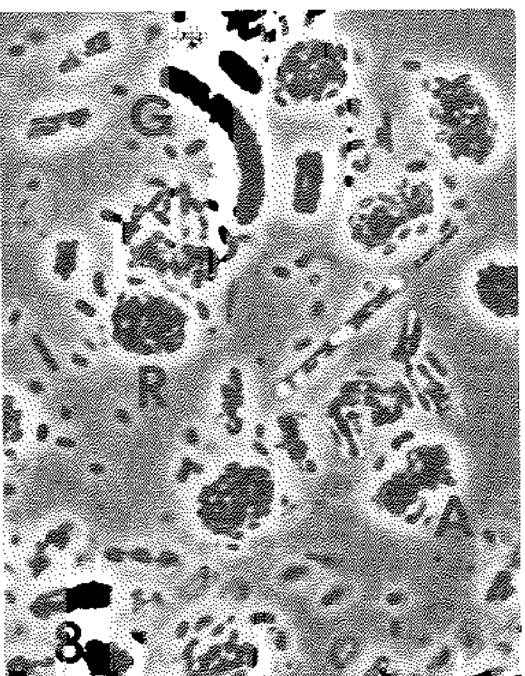

Figure 8. Hypolimnetic bacteria from a 6.25 m water sample from Cassidy Lake, Michigan, showing at least three different consortia: *Chlorochromatium aggregatum* (A), *C. glebulum* (G), and *Pelochromatium roseum* (R). Magn. 1280 x.

bundle of parallel cell chains was considered to be composed of only one organism. The consortium (Figure 10) photographed from Cassidy Lake by Dr. J. T. Staley clearly consists of alternating chains of cells with and without gas vesicles (silvery versus dark appearance). Exact measurements have shown that these two cell types differ in diameter (0.89 to 0.93 um as compared to 1.02 to 1.25 μm; Hirsch unpublished). Neither one of these two cell types has been found alone and all attempts to cultivate the bundles remained unsuccessful. The nature of the interaction is therefore still unknown.

Similar conditions may be assumed to prevail with the two-dimensional aggregates shown in Figure 11. Green bacterial rods without gas vesicles alternate with colorless rods that are packed with gas vesicles. This consortium may actually be identified as the *Pelodictyon parallelum* (Szafer) that Perfil'ev described in the literature.

Other tight associations of recent interest are the symbioses of larger foraminiferans (protozoa) with their algae. Figure 12 shows an individual of *Sprioline* sp. from Hawaii which contains unicellular red algae as endosymbionts (Figure 13). In the case of *Heterostegina depressa* and *Amphistegina lessonii*, close physiological interactions have been demonstrated (Rottger, Schmaljohann and Irwan, Kiel, personal communication 1980). Neither the *Spirolina* nor the red alga have as yet been cultivated alone, but symbiotic algae from other foraminiferans have been grown without the protozoan host. In one case the symbionts were shell-less diatoms which developed normal frustrules when grown without host. It can be expected that these occur freely in the environment. Since the natural habitats of these organisms--tide pools, tropical shore waters--are extremely inconstant with respect to some environmental parameters, it is thought

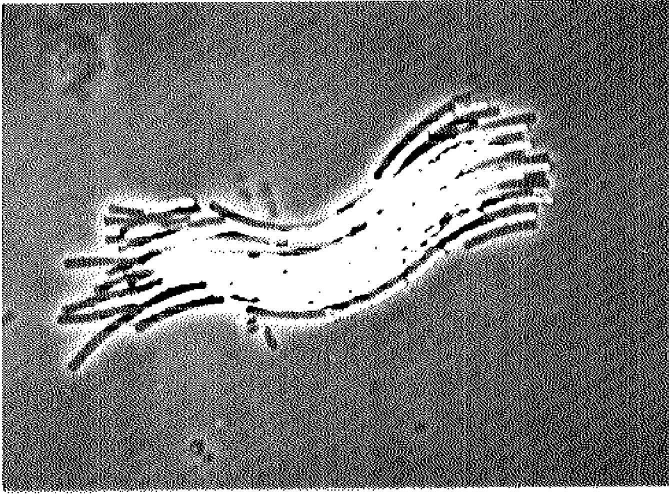

Figure 10. *Peloploca pulchra* from Cassidy Lake demonstrating filaments with and without gas vesicles. Micrograph by Dr. J. T. Staley, Seattle. Magn. 1280 x.

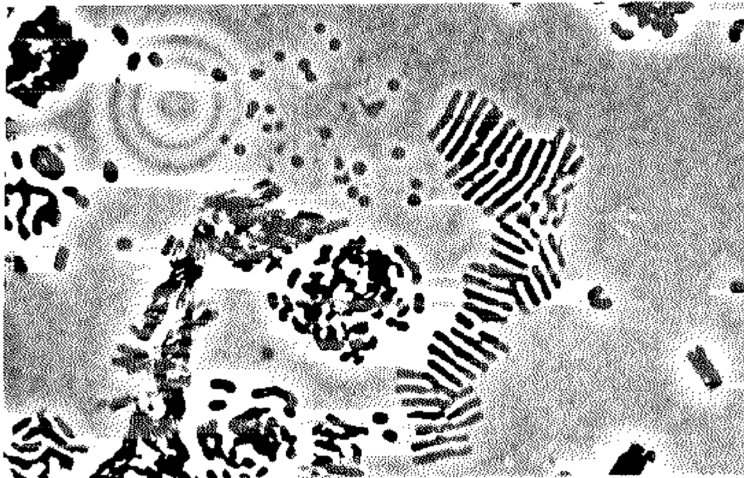

Figure 11. Hypolimnetic bacteria from Cassidy Lake, 6.5 m, Sept. 25 1969, showing an association believed to be *Pelodictyon parallelum*. Magn. 1280 x.

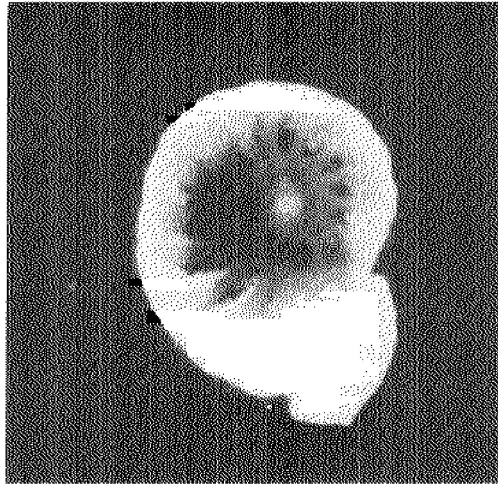

Figure 12. *Spirolina* sp., a larger foraminiferan from Hawaii that carries unicellular red algae as intracellular symbionts. Magn. 30 x.

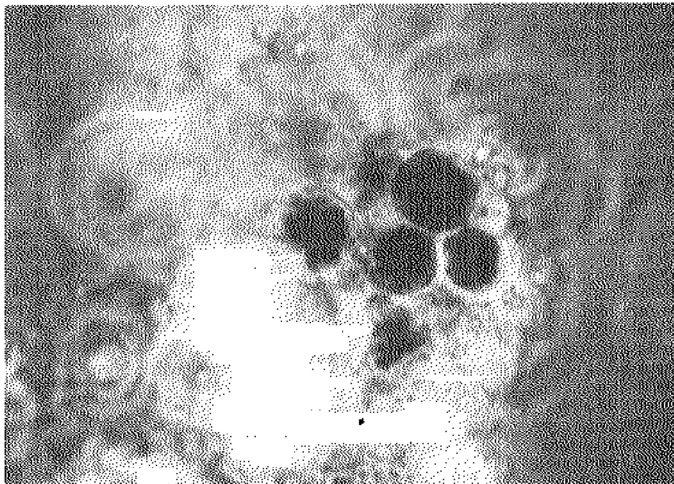

Figure 13. The red algal symbionts of *Spirolina* sp. liberated by gently breaking the foraminiferan's chamber. Magn. 1280 x.

that the symbiotic association manages to better resist rapid and drastic changes in the environment.

The presence of a symbiont may also alter the behavior of an organism. The pondwater ciliate shown in Figure 14 was observed to live aerobically, normally. After ingesting *Chlorella* cells and tolerating their multiplication, this protozoan frequently visited anaerobic strata in its Michigan pond, as can be seen from the simultaneous presence of gas vacuolated purple sulfur bacteria. Presumably the photosynthesizing algae produced enough oxygen for both the ciliate and themselves.

Aggregation of many cells of the same kind in order to withstand stress conditions can be observed quite often. Pollution and the occasional presence of oxygen may have caused the purple sulfur bacteria in Figure 15 to aggregate into huge masses. The same holds true for the *Zoogloea* sp. shown in Figure 16, a polymer-producing bacterium found in characteristic aggregates in sewage mud. *Sphaerotilus natans* cell filaments present in urea-polluted waters were always covered with smaller, rod-shaped bacteria (Figure 17). At lower pollutant concentrations these *Sphaerotilus* filaments grew better without other forms attached. Similarly, when *Hyphomicrobium* cells are found in sewage mud flocs, the mother cells are usually embedded within the mud, while hyphae radiate outwards and release the buds (Figure 18). The cover of a bacterial cell with other bacteria or detritus is probably advantageous in the frequently changing sewage environment.

One other type of interaction may consist of the removal, by one organism, of a toxic compound from the system. One such example here shows the gas-vacuolated, filamentous bacteria *Leptothrix pseudovacuolata* specifically oxidizing and depositing manganese compounds, which then collect on the filament's surfaces (see dense layers, Figure 19). By this process all of the manganese can be removed completely from the liquid phase of the system.

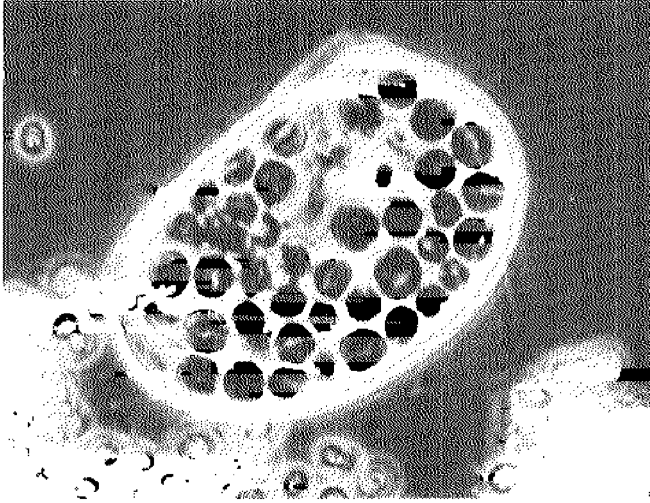

Figure 14. A pondwater ciliate carrying *Chlorella* endosymbionts which when exposed to light alter the ciliate's behavior. The coccal organisms in the periphery are purple sulfur bacteria containing gas vesicles. Magn. 1280 x.

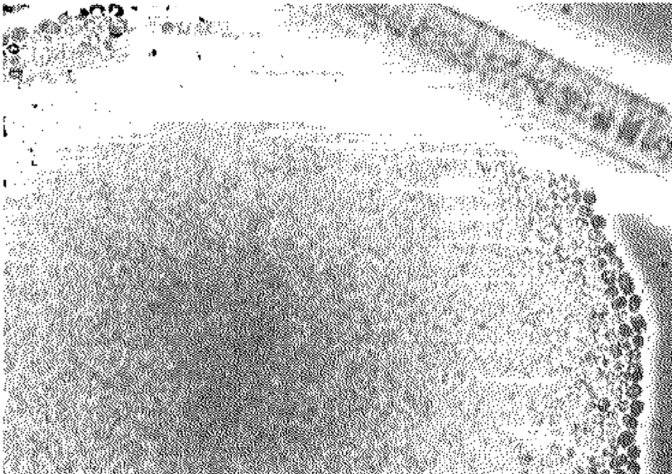

Figure 15. An aggregate of purple sulfur bacteria from the sediment surface of a polluted river, Schwentine, Holstein. Magn. 1280 x.

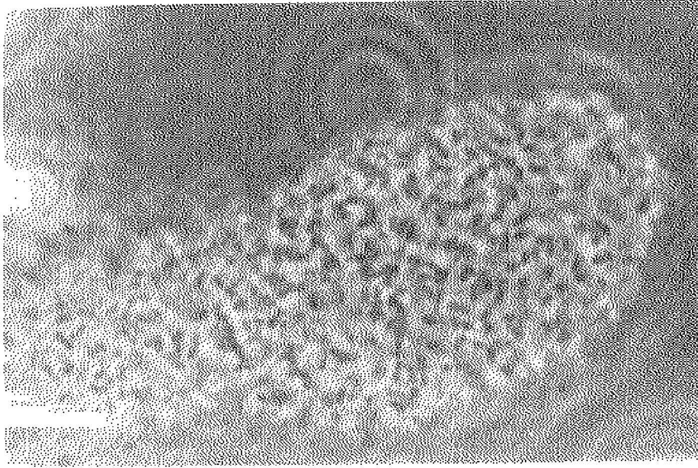

Figure 16. *Zoogloea ramigera*, an aggregate from active sewage sludge. Magn. 1280 x.

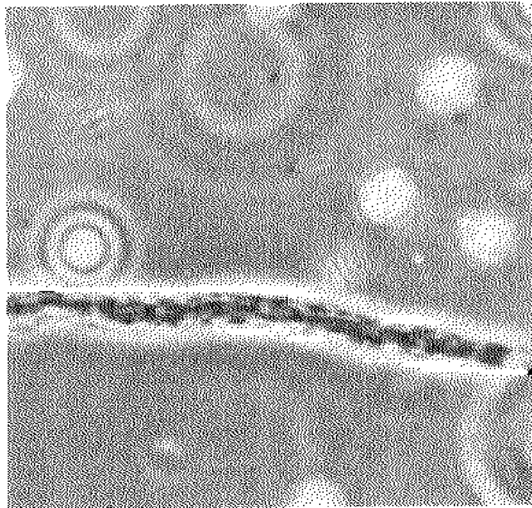

Figure 17. *Sphaerotilus natans* cell chain from sewage plant effluent and covered by bacteria that are only present in effluent of high concentration. Magn. 1280 x.

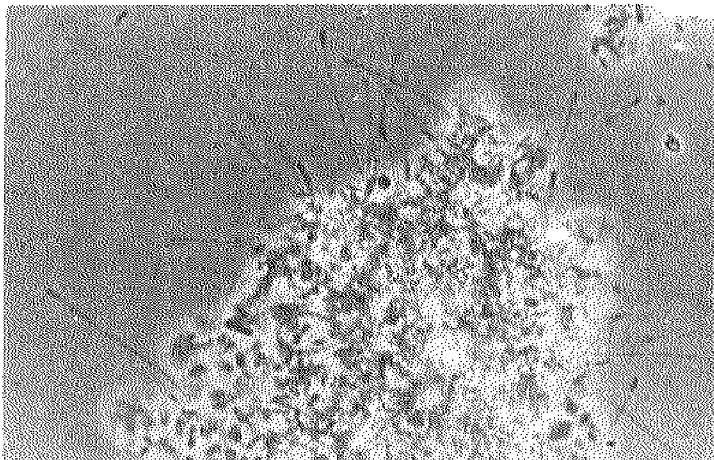

Figure 18. *Hyphomicrobium* sp. from a sewage floc. The hyphae radiate outward while the mother cells are buried (protected by?) the sewage material. Magn. 1280 x.

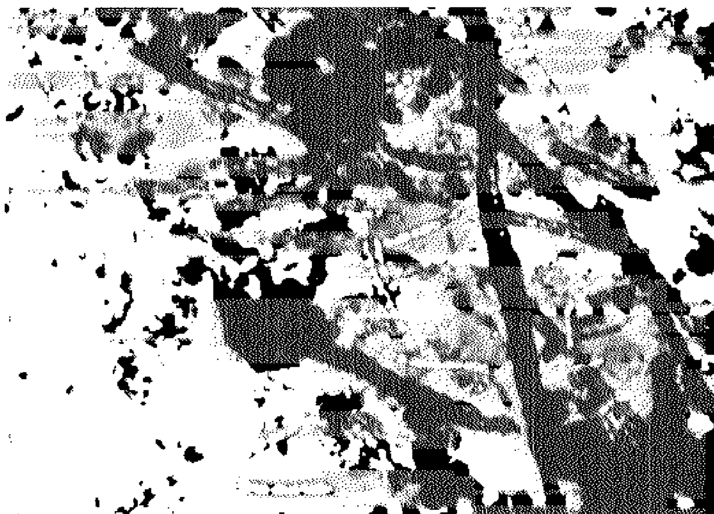

Figure 19. A sediment surface sample from a polluted pond in Holstein, Germany, showing manganese-depositing trichromes of *Leptothrix pseudovacuolata*. Magn. 1280 x.

Manganese-sensitive organisms can now thrive and possibly stimulate growth of the Mn-oxidizers.

Last but not least, and in contrast, there is a rarely studied organism that apparently does not need to associate with others, but still manages to live in a very hostile environment. It is *Achromatium oxaliferum*, a huge bacterium living in acid bogwater swamps at a pH of 3.5 and yet maintaining masses of storage granules of $CaCO_3$ in its cells (Figures 20 and 21). One also finds some sulfur storage granules (Figure 21). It is of course highly speculative to assume that the initiation of an association procedure may be facilitated by a more "leaky" (communicative) cell boundary, which is not the case with *Achromatium*.

These are, in summary, a few examples of microbial associations with varying degrees of interdependency and which may arise under quite different conditions and for different reasons. Some of these associations may be ephemeral, even nonfortuitous, such as the attachement of anaerobic bacteria to gas-vacuolated forms that may then rise to the aerobic water surface. Other associations that turn out to be more beneficial may result in increasing interdependency. From an evolutionary point of view, one could assume that accidentally affiliated organisms, when subjected to selective stress conditions, eventually join to form relatively stable associations, which become ever more stable in the course of co-evolution of the association's partners.

Finally, I would like to comment on Dr. Mitchell's last item, the possibility of biological control. One technique to be explored is the study of the unwanted organism's natural microflora, as was done in the case of a grease ant pest in a Michigan building. Isolation of natural components of this microbial population and a search for mutants with pathogenic effects are possible. Also, modern gene manipulation techniques may allow a piece of genetic information to be introduced to one of these bacteria, resulting

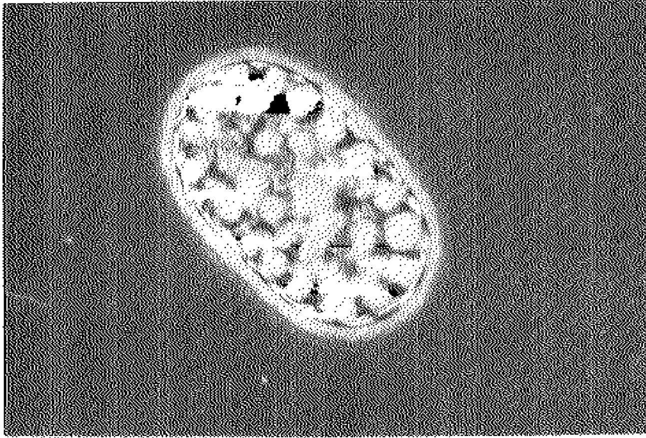

Figure 20. *Achromatium oxaliferum*, one of the largest bacteria, living in acid bogwater swamps at pH 3.5. The bright particles inside the cell are $CaCO_3$ crystals. Magn. 1280 x.

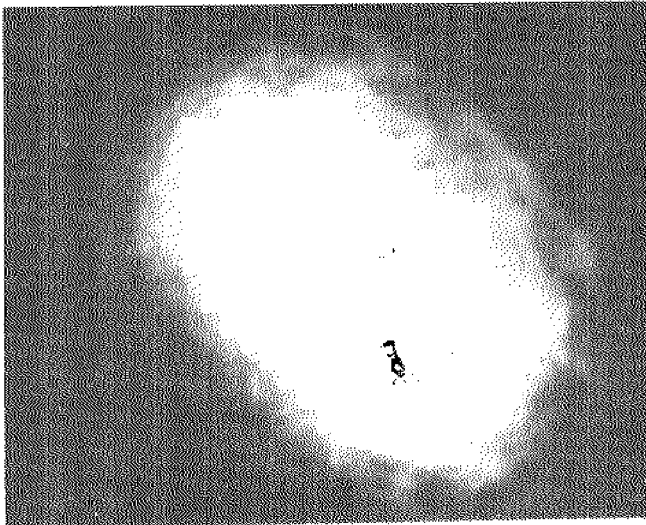

Figure 21. *Achromatium oxaliferum* at higher magnification shows $CaCO_3$ crystals and smaller sulfur granules. Magn. 1920 x.

in pathogenicity against the unwanted organism.

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Diversity of Aquatic Heterotrophic Bacterial Communities

JAMES T. STALEY

One of the concerns of aquatic microbiologists is how to measure the effect of environmental stresses on the microbial community. There are two types of response expected of a community subjected to stress: a change in the *activity* of the community and a change in the *species composition* of the community. Both of these responses can, at least in theory, be measured.

In the case of sudden, severe stress, such as a rapid and dramatic change in pH, one would expect the community response to be correspondingly rapid and dramatic. The measurement of almost any activity, such as respiration of heterotrophic potential, would provide a satisfactory assessment of the degree of stress applied. Likewise, gross measurements of community structure, such as biomass measurements, would adequately reflect the extent of stress. However, in their more subtle forms,

stress effects are more difficult to assess. For example, what effects would a temperature increase of 1 or 2 degrees above the normal high have upon the community? Perhaps this would impart no lethal effects on any of the organisms. The measurement of activities and biomass might not adequately reflect the changes this type of subtle stress has caused. Changes in community composition may prove to be useful in mirroring the effects of stress in such circumstances. The response to stress may not be immediately reflected in a change in the community species composition, but within a generation or so, i.e., usually in a matter of a few hours, altered growth rates and death rates would be expected to result in a changed species composition.

In measuring species composition or diversity, one needs to determine the number of individuals of all species of a particular trophic group (e.g., heterotrophic bacteria) in the habitat. Since it is impossible to process all the individuals in an environment, in practice a manageable number, say 100 or 1,000 individuals, are identified. For heterotrophic bacteria there are in theory two approaches that can be used to accomplish this. One can use either direct microscopic identification procedures or viable count identification procedures. There are technical difficulties with either approach. Perhaps the best way to illustrate the problems encountered is to describe some results we have obtained using these two types of procedures.

Samples were aseptically collected from a pulp mill oxidation pond. A portion of the material was fixed with Lugol's iodine solution, and the remaining portion was used for plating on various growth media. The fixed sample was used for electron microscopy. Cells concentrated by centrifugation were resuspended and placed on 300-msR Formvar-coated electron microscope grids. These were examined in an electron microscope,

and electron micrographs were taken of grid sectors. After printing, the sectors were examined visually, and morphologically identifiable types were grouped into "form species" based upon their size, shape, and other characteristics. About 100 bacterium-sized organisms were included in each determination. This information was then used to determine the "species composition."

Obviously, there are a number of difficulties with this approach. First, some of the organisms may not have been alive or, if they were alive, may not have been indigenous and active in the community. Second, it is not possible to determine by this electron microscopy procedure whether an organism is a heterotrophic bacterium or an autotrophic bacterium or, in some cases, even a cyanobacterium. Third, it is impossible to differentiate many species solely on the basis of their appearance by electron microscopy. Within a given species there may be sufficient morphological variation to make identification impossible. For example, in the swarmer stage *Caulobacter* spp. do not have prosthecae, so they cannot be identified as belonging to a given species of *Caulobacter* or even to the genus. Also, involution forms complicate identification. Conversely, two rod-shaped bacteria of different species may appear identical when observed in an electron microscope. Therefore, the data obtained from this approach provide only an estimate of the bacterial diversity of a sample.

Viable count procedures were also tested. By plating organisms on different media, one can determine which medium provides growth of the most organisms. Indeed, one could incorporate replica plating into this to determine whether the number of bacteria can be increased because some may grow on one medium but not on another. If so, then a combination of media would be preferable. In pursuing this approach, one identifies and enumerates colonial types on the basis

of their morphology. There are several limitations to this procedure. First, though it is true that one is identifying bacteria that are viable, this does not necessarily include *all* that are viable, because the medium or media used may not support growth of all of the heterotrophic bacteria. Indeed, it may allow growth of some organisms that are not indigenous or active in the community but are present and viable. Second, the technique, as described, does not permit one to identify different species because colony morphology may differ within a species (i.e., for different strains) and different species may have similar colony morphology. Of course, by subcultivating all colonies and identifying them to species one could actually determine the species that are present (or at least growing), but this modification of the technique would require much additional processing work.

It should be readily apparent from the foregoing discussion that it is currently impossible to determine accurately the species diversity of the heterotrophic bacteria in typical aquatic habitats because of identification limitations and enumeration problems. What can be determined is an *estimate of strain diversity* of heterotrophic bacteria.

In the pulp mill oxidation pond, which has numerous organic substrates, some in high concentrations (Stanley and Staley 1977), most of the bacteria are heterotrophic; therefore, the problem with autotrophs is minimized. Furthermore, there was close agreement between the numbers determined by microscopic counting and viable counting procedures; thus, the estimates of community diversity obtained by these two separate and independent procedures could be directly compared. Total numbers of bacteria determined by Petroff-Hausser count were actually lower than those obtained on the plating medium (Table 1). This unexpected result is

Table 1. Total and viable concentrations and community composition of heterotrophic bacteria from a pulp mill oxidation pond

Procedure	No. of bacteria/ml	Community diversity	
		Shannon	Raw data
Total	2.14×10^7	3.89	4(1), 3(2), 2(3), 4(5), 1(6), 1(8), 1(9), 1(10), 1(11), 1(13)
Viable	3.30×10^7	3.73	6(1), 5(2), 2(3), 2(4), 1(5), 1(6), 1(7), 1(10), 1(17), 1(18)

[a] Each of the raw data terms expresses the number of "strains" that contain that number of individuals, i.e., 4(1) would indicate that four strains contain one individual each; the total number of terms indicates the number of strains observed in the sample.

explained by the difficulty encountered in phase microscopic counting when appreciable numbers of organisms are aggregated together or attached to wood fibers. Nonetheless, the data do indicate close agreement between the two counting procedures.

The estimates of strain diversity are presented in two different formats. In the first instance the data are provided as calculated from the Shannon index (Shannon and Weaver 1963). This measurement is often used by ecologists, though not without some reservations (Hurlbert 1971). The other format provides simply a listing of the raw data. Although the Shannon index suggests that there is similar diversity by both techniques, it does not provide as much information as the raw data. The disadvantage of the raw data, however, is that it is difficult to assess readily the diversity of the sample. One possible alternative means of presenting the raw data information would be to use a graph such as that used by Patrick (1964). (In her procedure for diatoms, some 10,000 individuals were identified; with only 100 individuals, trends may not be as apparent from graphs.)

The measurement of heterotrophic bacterial diversity is still novel when compared to species diversity measurements for plants, animals, and algae. The major reason for this is the difficulty associated with the identification of bacteria directly on the basis of their organismal and colonial morphological attributes. Perhaps by appropriate modifications of the procedures described heretofore or by development of entirely new ones, this difficulty can be overcome. If so, this measurement may be useful in complementing activity measurements in assessing the effects of stress on communities. In addition, information on bacterial diversity will be useful as a means of characterizing communities and following changes in community composition during

successions. Furthermore, it may assist in the resolution of the long-standing problem concerning the discrepancy between total and viable counts of heterotrophic bacteria. For these reasons, it is hoped that additional research on techniques in this area will be conducted by aquatic bacteriologists.

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Low Temperature, Energy, Survival, and Time in Microbial Ecology

RICHARD Y. MORITA

Most of the biosphere is cold, and within this cold environment there exist very unproductive waters (e.g., the deep sea) as well as extremely productive waters (e.g., Eastern Bering Sea). From a microbiological viewpoint, the ecology of this cold environment is far from being understood (Baross and Morita 1978; Morita 1975). In fact, it is probably the most neglected area of microbial ecology.

In the cold marine environments which are productive, the availability of energy and nutrients does not pose a major problem, whereas in some open-ocean and deep-sea waters the lack of energy and nutrients is a major problem in the growth and metabolism of microorganisms inhabiting the waters. Much research has yet to be done on these areas so that we can understand all the ramifications on the microbe's role in the ecosystem. In nutrient-poor systems of the open ocean and the deep sea, the amount of dissolved and particulate organic carbon is approximately

0.35 to 0.70 mg/liter and 3 to 10 μg/liter, respectively (Menzel and Ryther 1970). The maximum total organic carbon is 0.71 mg/liter, based on the above data. Much of this organic matter in the deep sea is resistant to microbial degradation (Barber 1968). The free amino acid concentration in deep-sea water is in the nanomoles per liter range (Lee and Bada 1975). These data indicate that there is very little energy available for the microbial life present in the deep-sea ecosystem.

The age of the dissolved organic carbon in water samples taken from 1,880 and 1,920 m in the Northeast Pacific Ocean was found to be 3,470 ± 330 and 3,350 ± 300 years by radiocarbon dating (Williams, Oeschger and Kinney 1969). The apparent age of various masses of water in the ocean can vary from a few years to 925 years (Broecker 1963). By ^{228}Ra dating of the age of a benthic clam (*Tindaria callistiformis*) taken from a depth of 3,800 m, it was found that it takes 50 to 60 years for gonad development and 100 years for the clam to reach a size of 8 mm (Turekian et al. 1975).

With the recognition that the amount of organic matter available to the heterotrophic bacteria in the sea is minimal, the lack of energy and proper nutrients becomes an important ecological factor. In most ecological systems, the lack of energy and nutrients is the normal state that microorganisms must deal with except in eutrophic conditions, or where dead plants or animals have added energy and nutrients to the eco-system. How then do the heterotrophic bacteria obtain their nutrients in a nutrient-poor system? Past data obtained by others indicate that there is a low metabolic rate of bacteria in the deep sea, whereas other data indicate a fairly decent rate of metabolic processes (for an overview, see Morita 1976). It seems that those organisms associated with an energy source (gut of animals, epiphyte on macroorganisms)

have a respectable metabolic rate, whereas those that are associated with the water and sediment have a slow metabolic rate. This latter situation may result from the lack of nutrients in the environment. It may also be the result of the normal metabolic rate of the indigenous microflora under hydrostatic pressure. This slow rate of metabolism is considered a "blessing in disguise" by Morita (1976) since a rapid metabolic rate would degrade all the organic matter in the ecosystem of the deep sea and result in no energy being left for other forms of life as well as the bacteria. Thus, life would perish in the deep sea. This slow metabolic rate may, then, be "in tune" with the environment. Yet this slow metabolic rate may not actually be slow if one takes into consideration the time element that the organisms in the deep-sea ecosystem must be in. Therefore, if a low metabolic rate is indicated, then the residence time (apparent age) of a body of water must be taken into consideration. Time then represents a span of hundreds of years, not laboratory time.

It should be recognized that no free methionine has been detected in the deep waters of the ocean (Lee and Bada 1975). Bacteria are associated with fecal pellets, detritus, etc., that sediment to the botton of the sea. These attached and free bacteria can be the food source for many protozoans and meiofauna, which in turn are eaten by other forms. Higher organisms, including the protozoans, require essential amino acids, and methionine is one of these essential amino acids. Translation cannot occur without methionine. Hence, bacteria (dead, dormant, or living), when ingested as food, play a very important role in the bioenergetics of the sea by providing methionine and a nutritive food source (Morita 1979*a*; Morita 1979*b*).

Survival (preservation of the species) is also a very important ecological factor in

microbial ecology. The succession of various physiological types in ecosystems (e.g., Winogradsky column) represents survival of certain bacteria until the ecological conditions are favorable for them to express themselves. Mechanisms and means of survival have been investigated quite extensively in plants and animals. The mechanisms of survival of vegetative bacterial cells other than spore formation were addressed in the 26th Symposium of the Society for General Microbiology (Gray and Postgate 1976), but only a few bacterial species have been investigated. The procaryotic cells have evolved for approximately 2.3 billion years longer than other forms. Therefore, there probably are many mechanisms of survival for bacterial forms other than the formation of spores in certain species of bacteria. Survival of the genome is very important so that the microbe can play its role in any ecosystem when the environmental factors favor a specific species' growth and metabolism. In oceanography we recognize that upwells (diverged waters) are very productive and that microbes in them are quite active. This represents a situation where the indigenous bacteria may have been in a survival state and do not express themselves until the environmental conditions become correct for a particular species. Survival occurs with pathogens also. In addition, time must be considered in survival studies.

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Discussion

EFFECTS OF ENVIRONMENTAL STRESSES ON MICROORGANISMS

COSTERTON: Since in today's discussion of aquatic microbial ecology we have examined consortia, it may be interesting to compare what is happening in a parallel field of microbial ecology. When a cellulosic cell wall forage material is injected into a cow's rumen, the cellulose surface is colonized within the first 7 to 15 minutes by very large numbers of cellulothic bacteria. In fact, to pick the cellulothic bacteria out of a rumen, you just dump in cellulose fibers and spin the fibers out and you've got most of the organisms with this capacity. Morphologically, these are one type; they are rapidly backed by a different morphological type--a very thin little bacterium that is easy to distinguish. The concept that we have hundreds of species in the pelagic population, but that when we add a very specific surface, be it one of Ralph's coral heads or rumen or whatever, we get specific colonization may be very important in aquatic microbiology. This phenomenon is caused, I think, by an affinity between the surface and the exopolysaccharides of these organisms. And I

would like to raise the possibility that these preexisting species (I've got them sort of labelled here in the general population) could be called forth from the basic organization by an affinity--the first organisms by an affinity for the substrate and the second organisms by an affinity for the exopolysaccharides of the first organisms. The question in the aquatic system is how many, what proportion of aquatic bacteria exist in communities like this. John raised it, and we know that these consortia happen, but how many specific bacteria take part in these consortia? Fosberg has just done some work in the rumen where he enumerated, using ATP measurements (perhaps somewhat imperfect), the number of bacteria associated with particles in this system and found them to be 70% of the entire rumen population.

So perhaps this allied field of microbial ecology first of all shows us consortia and secondly gives us some idea of the proportion of organisms in the environment that are involved in consortia at any given time. I don't think this concept is foreign to concepts we have had presented to us today.

So much for that small personal digression. What we are going to do now is invite you to ask questions--preferably integrated questions.

BROCK: There was a point at the end of Jim Staley's talk that I would like to comment about. I agree with him that you need to culture organisms to determine species diversity, but I am concerned about the kinds of culture media most commonly used. These media are often selective for the "weed" species, but may not provide a good indication of the species diversity of the habitat as a whole. The best way to get an idea of species diversity is to carry out direct microscopic examination of the habitat, as illustrated by the talk of Peter Hirsch. Many of the organisms seen by the microscope do not appear with the usual culture procedures. An interesting approach, and one

that deserves to be widely adapted, is that of Meyer-Reil (published in September 1978 *Applied and Environmental Microbiology*). He combined fluorescence microscopy with autoradiography, thus determining whether the organisms seen under the microscope were active. He used tritiated glucose and showed that in Kiel Bay between 10 and 50% of all bacteria that could be seen were capable of taking up this substrate. This suggests to me that the organisms we are seeing under the microscope are not dormant, but are really active in the system. The reason they are active is that if they were not, they would be destroyed by lysis, grazing, predation, or sedimentation. So the things we are seeing with the microscope are important. I think this technique has tremendous potential for studying the activity of diverse microbes in aquatic systems, and is probably more effective, as well as easier, in the long run.

COSTERTON: And I think perhaps the use of fluorescent antibodies as used very elegantly by Schmidt's group also offers promise.

MORITA: Tom, I don't think that the number of active bacteria in the environment was that high. Meyer-Reil perfected the method in my laboratory when he was on leave from Kiel, so I am quite certain that the number of active bacteria in the environment was closer to 10%. Even if the number of active bacteria in sediments was 50%, there may be a survival mechanism among the nonactive cells.

I would also like to ask Ralph Mitchell whether he investigated the perturbation effects on macroorganisms and whether this perturbation effect might not bring out the endosymbionts which, in turn, might indicate that the disease comes from within the organism.

MITCHELL: Yes, I think that's a very good point. A lot of what goes on in the interaction between the macroorganism and the microorganism

has to do with opportunism. There are certain opportunistic microorganisms waiting in the host organism for an opportunity to take off. I have no doubt that in the large percentage of cases where we see disease, it's that opportunist, who has been sitting there all the time. When the physiology changes, he takes off and goes to town.

While I'm on my feet, I might comment on the diversity index question that Jim Staley raised. We've tried this in our lab over about a year or year-and-a-half without any success whatsoever. Trying to use Shannon indices, particularly with bacteria, is a very hairy thing to do. You are really dealing with biochemical diversity--not what the Shannon index was made for--and all you have to do is change the physiological conditions and you come up with a different group of organisms and your index changes quite dramatically. I think, unless you control your conditions very tightly, you will have a lot of trouble with diversity indexing.

STALEY: As I said, I don't advocate this as a procedure, but I think it ought to be explored much more. In answer to what Tom said, it's still not certain whether, in fact, many organisms are alive, when one looks at them microscopically. That question is still really up in the air. So microscopic diversities, which I think ought to be done also, on a trial basis, still leave this problem. It is a difficult question.

COSTERTON: I don't think we were limited in our terms of reference here, but Jim was the only speaker this morning who addressed our topic. Our topic was, "Can we assess environmental effects on bacteria?" And I think he made a very brave attempt to disclose two methods that might work. If I can just postpone the questions that are already in the air, can I ask a general question of the audience? Do we have to tell the regulating agencies that at this time we have absolutely no handle on

the microbial ecology of natural systems and we can't tell whether or not they are stressed. . . Do we have to tell them that? It's sure going to cut down the grant money. I think Jim's raised the two possibilities. Is there anybody who has a positive statement to make?

WRIGHT: I think the first of Jim Staley's two sorts of indices of change ought to be given more attention. I know there are a few studies of some data available that show, for example, heterotrophic activity as indeed affected by pollution or by the addition of toxic substances. I can't think of anything that would be quicker and easier to work with to get a general response from the microbial community than using radioisotopes. I think this has a lot of promise and has not been used very much. So I would answer NO, we can't tell the regulatory agencies we have no possible way to assess the effects of environmental stress on microorganisms. I think we have a number of ways of measuring bacterial activity, including heterotrophic uptake, including the method of using INT reduction, for example, as a measure of the active bacteria. Getting back to another thing that Jim Staley mentioned, I think that we certainly have abundant evidence now that the number of active bacteria in the environment far, far exceeds the number of viable bacteria that you can plate out. I don't think that's even a debatable point at this time.

IVANOVICI: I wish to draw attention to a biochemical method that has been studied in recent years that may be useful as an index of change. The method uses measured concentrations of ATP, ADP, and AMP to calculate a ratio known as the adenylate energy charge. Studies with micro- and macroorganisms indicate that high values (0.8-0.9 in a range of 0 to 1) correlate with organisms which are in optimal conditions and are metabolically active and reproducing. When lower values are found, reproductive and growth rates are

lower. These lower values are generally associated with nonoptimal or stress conditions. There are several problems with the method, but overall the results do suggest that the energy charge may provide a tool that can be used to evaluate stress. It is a technique that offers a lot of potential.

HIRSCH: I would like to raise the question of how one defines a stress condition. I am not sure I know what a normal condition is so that I could judge what stress is like. Now let us take environmental pollutants. I agree if one pours phenol into a pond, the pond and the bacteria are stressed, but what about mercury, for example? Recent reports by Siegel seem to indicate that mercury also comes off from volcanoes, so there may be some natural mercury sources. Would that be a stress situation?

ALEXANDER: I am glad you raised this point, because it is, to a great degree, the motivating question behind the organization of this meeting. We are not now in a situation where testing is mandated by law, and many of us are here, directly or indirectly, for this very reason. It is very important for us to look at realistic responses, changes in community structure and community function, and to assess what can be done quickly and cheaply, and to develop tests that will have some real impact on EPA. Unfortunately, it is common in regulatory agencies, in this country and abroad, to require microbiological tests and then essentially ignore the results. I can't think of a single case in the area of testing pesticide effects on microorganisms where an inhibition of microbial processes has led to canceling the registration of a chemical. This is to a great degree because we microbiologists are talking to ourselves and not responding meaningfully to the regulatory agencies. We have to show that a particular change in an aquatic habitat is significant in the real world as well as to microbiologists.

COSTERTON: I think this doesn't preclude that we debate the usefulness of these techniques, but we have to arrive at a synthesis, as Marty says. The diversity index is debated by the invertebate people, endlessly, but it is still used, and used to quite good effect. And I think that is what we have to come to.

SHILO: I would like to mention a new technique that might be useful in measuring pollutants and toxicants in the environment and which might lend itself to the task of monitoring. The method is based on an interaction, between the bacterial predator *Bdellovibrio* and its prey organisms, which is very sensitive to changes in the environment. Using luminous bacteria as prey organisms, it is now possible to follow their interaction with *Bdellovibrio* by direct measurement. Upon penetration of the luminous bacteria by *Bdellovibrio,* light emission stops within seconds. The light emission pattern is highly characteristic and its dependence on a variety of changes in the environment leads to change in the typical kinetics of drop in the light emission caused by *Bdellovibrio.* We have already looked at the effect of a number of stress conditions, including adding small amounts of toxicant, and obtained preliminary results.

COSTERTON: Perhaps these concerns split into two areas as they do in other fields of ecology. One looks at species diversity (can we pull the species out of a given environment?); the other looks at overall activities, physiological activities. And they are of course not incompatible.

ATLAS: I wanted to return to the diversity question for a minute. My lab has been working on diversity indices, using Shannon's diversity, based on numberical taxonomic studies such as Rita Colwell's NT studies. We find that one can use diversity indices as an index of environmental

stress--both natural and pollutant stress. Admittedly, we are looking at stresses on particular segments of the population. Since we are getting cultures off of viable plate counts, we are not able to study the whole community, but we can show responses, we can look at seasonal responses where temperatures change. We work in the Arctic where severe winter stress with ice formation and lack of light give a combined environmental stress. During that winter period, the diversity drops significantly, and then comes back up in the spring, peaks in the summer, and comes back down. The same cycle repeats the next year. Whether or not bacterial species are involved, the community structure returns. So while we can identify different particular species, the diversity goes back to the second decimal place, using Shannon's index, during the following summer and back down again. If we spike the system with a pollutant, we can show a decrease in species diversity. This technique has its limitations, but I think it has a real value here. In fact, I think it has more value in microbiology and microbial ecology than in macro ecology because the microorganisms respond so rapidly to environmental change. The rapid generation times of microorganisms allow them to respond quickly, colonizing and degrading materials, both of which show diversity drop caused by toxicity; whereas, I think the macro ecologist looks at only one response to toxicity. Therefore we have the advantage. I don't think the microbial ecologist faces all of the pitfalls that the macro ecologist faces in interpretation of diversity indices.

SULLIVAN: One question that I would like everyone to consider is: Regardless of what stress we are talking about, or what method of assessing it, do we consider the time function between the onset of environmental stress and the time we do the sampling? It seems from work of Dr. Azam at CEPEX, using heterotrophic assay, that after the insult of adding various concentrations of mercury

or copper to a population, there is a rapid depression in the heterotrophic uptake; but it returns to normal. So with any of these indices that we are looking at, along the lines that Dr. Atlas mentioned, we may actually miss the direct effects if our sampling period isn't timed very closely to the original insult. Whether that means that the population bounces back to a similar state, but adapted to the insult or . . . just what that means I think is very difficult to say.

COSTERTON: . . . Very graphically illustrated in the Arctic oil spills in soils.

FLOODGATE: My problem when talking about species diversity is I am not very sure what a species is. There are two as far as I know. One is called *E. coli* and the other is called "not known in Bergey's Manual." I think this is a very real problem when it comes to talking about things like species diversity, because obviously one turns up a lot of bugs which one just doesn't really label properly. The result is species diversity in any wide sense is of very little use. You can, I think, get a certain species diversity index for certain very specific situations. Ron mentioned the oil one. Perhaps in those sorts of circumstances, we have something which is very well defined and maybe we can talk about it, but talking about species diversity in the whole of the aquatic environment seems to me at this point rather meaningless.

COLWELL: Numerical taxonomy is very useful in providing a generic index. The fluctuation of genera in a given system can be monitored and can provide a means of detecting effects of pollutants, etc. A very real problem is the lack of knowledge about what species are found in a given ecosystem. Work we have done in my laboratory indicates that, in deep sea habitats, the intestinal flora of fish, sediment, and water column in which the fish are found are individually characteristic.

Indeed, there is a commensal flora of the gut of fish, a specific skin surface flora of the fish, a water column population and a sediment population. An extensive numerical taxonomy analysis of these populations permits separation of isolates into clusters. Thus, more baseline work in the area of defining natural populations of bacteria is needed.

We have only begun to realize that bacterial taxa, i.e., generic groupings and species, demonstrate seasonality in occurrence and distribution. In the past, we have not really considered seasonal fluctuations of bacterial populations. We have accepted such concepts for algae and higher animals, but the occurrence of population variations occurring with season have not been considered in depth. A good example is *Vibrio parahaemolyticus*, which shows a very distinct seasonality, from the work of T. Kaneko in my laboratory. Thus, part of the problem is that we are groping for information because we simply haven't done the hard work of characterizing the natural populations of bacteria.

COSTERTON: I must mention the work of the Franklins in this respect as well, in which seasonal changes in the St. Johns River are beautifully documented, with some good speciation.

PFAENDER: I want to say a word about diversity and then make a point about something else. We have looked at diversity in some flow-through systems with oil stress using marsh microcosms, and we find that the diversity index stays just about the same; but if you isolate the organisms and identify them, you find the species composition of the community has changed, even though the diversity looks the same.

The other point I wanted to make is that we all seem to be looking for one magic technique that we can use to evaluate stress, but in about three years of looking at a lot of natural systems, we have found that you can't use any one technique to give a valid picture of what's going on. You

have to use a combination of techniques.

PASSMAN: Two comments, one, regarding the discussion Dr. Slater had about diversity index and what Ron Atlas said about essentially the same system: If you use base diversity on colony morphologies instead of actual taxonomic identification, such as the Colwell scheme (approximately 312 tests), you get a much different sort of picture. Frequently, once you pick out isolates and then do taxonomic identification and cluster analyses, isolates with slightly different colony morphologies will turn out to be essentially identical organisms. Two, the stresses: We used the parameter specific biomass (LPS biomass/epi-direct counts) to look at a waste discharge stream (pharmaceutical wastes). We observed a very nice decrease in specific biomass within the plume. After 15 to 20 hours there was a return to higher specific biomass levels. I think Dick Wright has done similar work using specific heterotrophic activity, where net activity doesn't change significantly, but activity per unit cell itself does change, providing a very nice reflection of short term perturbations.

HOLDER-FRANKLIN: Our work was done in cold rivers in New Brunswick, Canada. There is very little work done in running cold rivers, perhaps because coral is a much more attractive habitat. It would be almost impossible for me to answer every question regarding our work, which has been going on about seven years and is quite comprehensive. So I will speak entirely on the subject of species diversity. It's an interesting concept, and we have recently been able to use it for some of our observed population changes. We certainly can show without any question that numerical taxonomy is a valuable tool for showing changes in species, and these are bonafide species. But what you are measuring in this diversity index primarily are the correlation coefficients you get when you

look at stacks of physiological reactions and compare them. This kind of a matrix is very deceptive in that you may not be looking at a particular or unique species. You may be fooled into thinking that it is a species, but that may not be genetically sound. However, we have looked at cluster analysis very closely. We have performed DNA analysis on the entire cluster, as well as DNA hybridizations on these clusters, and have shown that about 60 to 75% of the cluster is genetically sound. That is, it will hybridize. Therefore, perhaps, there is some measurable species diversity. I'll be presenting a poster in Los Angeles on this if anybody wants to come and have a look at the details of it. However, we are convinced that population shifts in microbial ecology can be studied using the techniques of numerical taxonomy.

COSTERTON: We have been talking about what we can legitimately and accurately measure. Now what I am going to do is just mention the ones that come to my mind and have you give us just short comments about what else can be done. What can we use to measure biomass? We can do direct epifluorescence counting. A great many people do it. We can do LPS in cases where we have largely gram-negative populations. What else can we do for total bacterial biomass?

AUDIENCE: We can measure muramic acid. We can measure lipo-phosphate in mud, but we had better be careful with LPS. LPS leaks out of organisms--that's why people die of meningitis.

COSTERTON: Right, and that's why people pick up bladder infections when there are no bacteria and LPS in the urine, right. O.K. for bacterial biomass. Nobody said ATP yet. Do we still believe in ATP?

FLOODGATE: I'll tell you all about it tomorrow.

COSTERTON: O.K. In deference to George Floodgate and his talk, we *think* we can get bacterial biomass quite accurately that way. Speciation: we can grow them out on plates, but we know we are missing a fairly large number of organisms on the media we use, and we also know that the counts come out a little bit low, partly because they are clumping. What else can we do with speciation?

AUDIENCE: Use a wide variety of media. . .

COSTERTON: . . . including ranges of concentration. When working in low natural waters, use low nutrient concentrations. I think that is a well-established point. Any further points on how to get the species out? How about fluorescent antibodies--do people believe in fluorescent antibodies? Although Schmidt's method is very laborious, it seems to me to be an elegant technique.

HIRSCH: I want to make a point about the most logical way to identify bacteria. It is our experience, in the aquatic systems that we have looked at, that at least 10%, if not 20% of bacteria can be identified morphologically to the genus. So I think if people learn how to do good microscopy with the phase microscope, they would not have problems. We could perhaps ignore the cocci and the rods.

COSTERTON: Beautifully put. We can clearly identify *Sarcina* and a great many of the stalked organisms and particularly among the aquatics a number of others. Now if we are talking about what kinds of organisms are present down to the genera, we go to physiological measurements. And if we were talking to a funding agency right now, what could we say about accurate physiological measurement processes for bacteria as they exist in aquatic ecosystems? We know that the energy charge, AMP-ATP ratios, and the amounts of storage compounds like PHB in the bacteria are going to

indicate what physiological shape the organisms take. We have the heterotrophic potential measured, perhaps. Dick Morita's point is that AMP (that's the energy charge), just AMP concentration, is very good for looking at cells that are not active.

ALEXANDER: The comments are very valuable, but we are forgetting one important point related to community function--somebody else has to care. They may not care about a lot of the things we are talking about concerning community function. So let us also consider very carefully that the test will have to be sold to someone else--not only to regulatory agencies, but to ecologists who are concerned with the quality of aquatic ecosystems. And these ecologists may not get too excited about species diversity or a particular process. I suggest we bear that in mind. I have no preconceived notion as to what ought to be done, except that it has to be capable of being sold.

COSTERTON: We had respiration mentioned among physiological processes as well, and just to sort of complete the list, are there short additions anyone else wants to make?

AUDIENCE: One of the things we overlooked is the use of earth-specific substrates, for example, aldehyde, which only certain organisms can utilize, can give us some idea of the total population by using one specific member of that population for the general things, and nitrification, again restricted to a few species. I think we should use very specific biochemical transformations to measure these higher populations.

COSTERTON: Your point is, I think, that very specific biochemical transformations, like nitrate fluxes, etc., should be used to indicate the rates of those processes and not necessarily extrapolated. Sometimes the rate of a given process is

particularly important. So there are a number of things that we can actually do in terms of physiological measurement.

Carol you had a point? Carol Litchfield says we can use the INT method in connection with the quantification of electron transport in a given system.

There is one other hand I saw down here. I will have to summarize that for the tape. The energy charge is questionable once adaptation of the bacteria to the stress has taken place and the surviving organisms are at a compendium of the charge.

O.K. I think we have arrived at a sort of a compendium of the things we can do. Perhaps for the rest of the conference we will be able to put these in relation to our topic, which was, first of all, the assessment of stresses, environmental stresses. We would like to thank the speakers very warmly for their excellent presentations.

Session II

EFFECTS OF MICROORGANISMS ON ENVIRONMENTAL PROCESSES

Microbial Roles In Aquatic Food Webs

LAWRENCE R. POMEROY

Classical ecological food web theory relegates to bacteria the role of degrading dead and refractory organic materials, an essential but presumably minor role compared to predator-prey and grazer-herbivore processes of macroorganisms (Elton 1927). There is growing evidence, however, that bacteria mediate a substantial part of the energy which moves through aquatic food webs. Much nonliving material, in a wide variety of particulate and dissolved forms, is available to be exploited, even in purely pelagic food webs which begin with the production of microscopic plankton (Andrews and Williams 1971; Derenbach and Williams 1974; Pomeroy 1974; Pomeroy 1979; Saunders 1976; Watson 1978; Williams 1970; and Williams and Gay 1970). Certainly, any coastal, estuarine, or freshwater system in which macrophytes are dominant producers supports a major flow of energy and materials through microbial pathways. Mann (1972) has said,

> When we try to generalize about the functioning of ecosystems, I wonder

> if it would be more correct to say that plants are usually consumed by microorganisms, which in turn are consumed by animals, rather than to give the usual story about plants, herbivores, and carnivores.

Microbiologists have shown little interest in providing the observational and experimental basis for the major revision of food web theory which seems to be needed. Rapid progress toward a better understanding of food webs is now possible because of recent advances in methods of aquatic microbiology.

Populations and Communities of Aquatic Microorganisms

The standard and rigorous culture techniques for identifying populations of microorganisms in many branches of microbiology have not been satisfactory to deal with questions about the abundance and ecological roles of aquatic microorganisms (Jannasch and Jones 1959). Advances in direct visualization of bacteria by light and electron microscopy together with assays for specific cell components, ranging from adenylates to cell wall lipids, have provided for the first time an emerging picture of the structure and function of aquatic microbial populations and of the taxa of bacteria in them (King and White 1977; Menzel and Ryther 1970; Watson et al. 1977; White et al. 1977; White et al. 1979). Each species has a distinct niche, or set of niche dimensions in the sense of Hutchinson (1965). As in phytoplankton, niche dimensions are determined by the biochemical interactions of the environment with the genotype (Pomeroy 1975), but some of the consequences of these modes of niche selection are complex and cryptic in cases of aquatic bacteria. For example, pleomorphism occurs in response to changing

conditions in the microenvironment (Wiebe and Pomeroy 1972). Therefore, some of the morphologically and functionally different populations of bacteria we now recognize in natural waters may be different phenotypic expressions of genetically similar organisms. In other cases, they do indeed represent distinct taxa, but it is not always evident which case we are observing.

Distinctive communities of bacteria occupy specific parts of the pelagic microenvironment (Sieburth 1979; Sieburth et al. 1977; Sieburth et al. 1978). An important recent discovery is the existence of relatively large numbers of free-living bacteria of very small size in virtually all natural waters (Figure 1). Ranging from 0.2 to 0.5 µm in size, they have been called minibacteria by Watson et al. (1977), and they are the numerically dominant bacteria in both fresh and salt waters, sometimes becoming the major component of total microbial biomass, including even the phytoplankton (Watson 1978). Presumably they are nonexacting heterotrophs which utilize dissolved organic compounds. It is a curious observation, recently confirmed again by this writer, that when water is held for 24 hours in a bottle or on a culture slide, the minibacteria become larger (Wiebe and Pomeroy 1972). Whether this transformation is the result of some subtle change in the microenvironment or the result of reduced grazing pressure is not known.

Fecal matter supports a distinctive microbial community in which the bacteria are larger, almost by an order of magnitude, than the free-living minibacteria (Figure 2). Large, motile rods tend to develop within 24 hours, and within 48 to 72 hours they have degraded much of the less refractory particulate matter in the feces, and are themselves being consumed by protozoans. The chitinous casing of crustacean fecal pellets is degraded by chitinoclastic bacteria in about 72 hours, and the

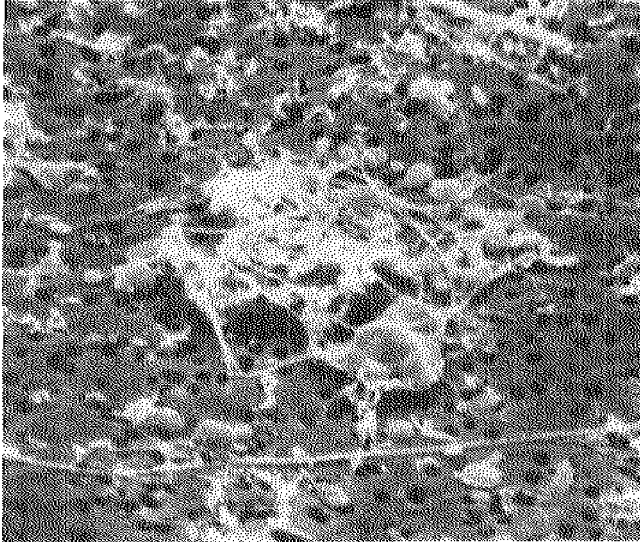

Figure 1. Free-living minibacteria collected on a 0.2 μm filter. Some of them have developed extensive extracellular processes, of which some have adhered to form a small aggregate in the center of the field. Scanning electron micrograph by Kenneth Kerrick.

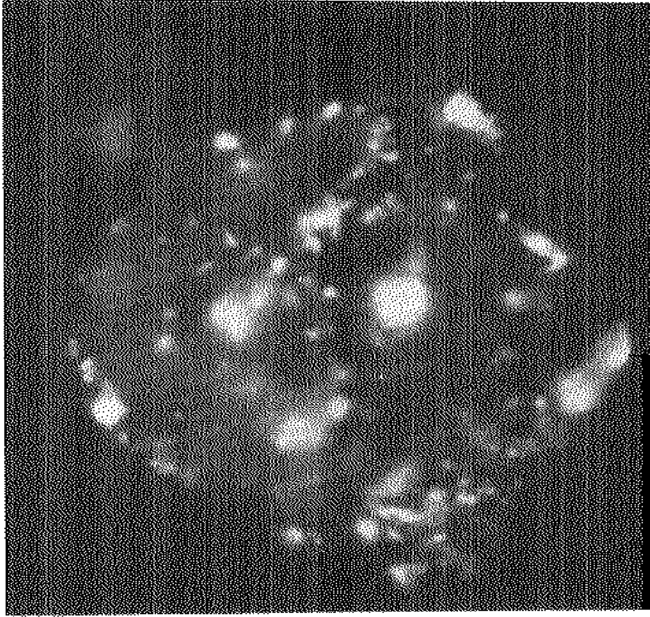

Figure 2. Fecal mass, 48 hours old, produced by the salp, *Thalia democratica*. Epifluorescence photomicrograph of acridine orange stain shows growth of bacteria within the mass. The entire particle is approximately 25 μm in its longest dimension.

pellet is then rapidly dispersed (Johannes and Satomi 1966). The fragmentary remains of old fecal pellets are largely devoid of bacteria (Wiebe and Pomeroy 1972). Whether this is because of the refractory nature of the substrate or because of the grazing pressure on such particles is not known.

Some other populations of bacteria occupy the surfaces of living phytoplankton and macrophytes (Sieburth 1979). They are in a favorable position to utilize low molecular weight dissolved organic compounds that are released from the plants.

The classification of microbial populations into a rational scheme of community structure proves difficult and challenges classical ecological theory. While it is relatively straightforward to organize the classical plant-grazer-carnivore structure into trophic levels, the microbial community, living at the expense of a variety of particulate and dissolved substrates, does not fit that scheme. Although there are several energy transfer steps between substrate, bacteria, and grazers, both protozoan and metazoan, they cannot be separated into neat, distinct trophic levels. This would be a trivial problem had not ecologists given the trophic level concept such a strong sense of reality. It must be remembered that the trophic level is a simplifying abstract concept that is, in this case, too simple to be useful (Pomeroy 1979).

Substrates for Bacteria in Natural Waters

Most of the open waters of lakes, estuaries, and oceans are aerobic and are the domain of the nonexacting heterotrophs. The bottom waters of stratified lakes, the Black Sea, the Cariaco Trench, and the sediments of shallow, eutrophic systems are anaerobic. These anaerobic zones and

the interfaces with the aerobic zones provide habitats for chemolithotrophs and other metabolically specialized bacteria. Although the anaerobic zones represent only a small fraction of the total area of natural waters of the world, bacterial activity in them probably has a significant impact on the planetary cycles of nitrogen. However, the mediation of energy is dominated by the communities in aerobic waters.

The largest single pool of organic matter potentially available to bacteria is that dissolved in natural waters. Total dissolved organic matter varies from less than 1 mg/l in the open ocean to more than 10 mg/l in some estuaries and lakes (Duursma 1965; Menzel and Ryther 1970). The long controversy about the availability of this dissolved organic matter to organisms of all kinds has now been largely laid to rest (Jørgensen 1976). Most of that fraction of it which can be utilized probably is degraded by bacteria. However, it is clear that much of the dissolved organic matter of the ocean is highly refractory. The mean radiocarbon age of marine humates is around 3000 years (Williams, Oeschger and Kinney 1969), and this sort of refractory material makes up most of the standing stock. Therefore, the available standing stock of dissolved material is probably less than 1% of the total. In reality, there must be a continuum from totally refractory compounds to those like glucose, glycolate, and amino acids which require no extracellular degradation, and for which permeases are likely to be mobilized. Therefore, such compounds, which are most readily available and most actively used, will have very short residence times in the water. Estimates of the residence time of amino acids in the surface waters of the ocean are on the order of days or weeks in most cases (Andrews and Williams 1971).

The sources of dissolved organic matter are what is released by living plants (including

phytoplankton), the products of defecation and excretion of animals (quantitatively mostly from zooplankton and protozoans), and the products of extracellular degradation of particulate substrates by bacteria. The rate of production of dissolved organic matter from these various sources is much less well quantified than is the standing stock. The release of dissolved material by phytoplankton and other plants is highly variable. Under the stressful conditions typical of the central gyres of the ocean and oligotrophic lakes, nearly half of the carbon fixed may be released as glycolate and other low molecular weight compounds. In the relatively favorable conditions in coastal waters, coastal upwellings, and eutrophic lakes, less than 5% of the carbon fixed may be released (Fogg 1971). However, the absolute amount of dissolved matter released by phytoplankton is greatest in coastal upwellings and eutrophic lakes. The production of dissolved materials might be on the order of 50 to 100 mg/day, while in an oligotrophic lake or a central ocean gyre it might be on the order of 10 mg/day. Since these compounds do not accumulate beyond concentrations of a few micrograms/liter, they must be utilized by bacteria, principally the free-living minibacteria. Organic excretory and fecal products also will be produced in greatest amount in eutrophic waters and probably will amount to several times the rate of production of products released by phytoplankton; in oligotrophic waters they will not be more than the amount released by phytoplankton. While these rates are not well quantified, apparently the production of dissolved materials available to free-living bacteria is greater in eutrophic waters by an order of magnitude, and might be greater by several hundred mg/day.

The primary and principal source of particulate matter available to bacteria is from the growth of plants. In pelagic systems this means phytoplankton. Although it has often been

suggested that phytoplankton are consumed quantitatively by zooplankton (Steele 1974), both examination of naturally occurring detritus and studies of the feeding of zooplankton indicate that a substantial, if variable, fraction of phytoplankton is not eaten by zooplankton. Some species are noxious, notably cyanobacteria and some spiny or armored species, and these are but rarely eaten (Porter 1973; Porter 1977). The feeding efficiency of zooplankters varies with the size and species of food organisms available. While some species are ingested with very high efficiency, others, such as large, chain-forming diatoms, tend to be broken, with substantial losses of fragments. Those not eaten by zooplankton for one reason or another become substrates for bacteria. Quantification of the rate of production of this primary detritus is lacking.

Those phytoplankton which are ingested by zooplankton pass through the gut, with about 30% ultimately released as feces. Some zooplankton, such as crustaceans, have grinding mechanisms to break up food materials. Even so, it is commonly observed that some phytoplankters come through into the feces alive and well (Porter 1973; Porter 1977), while others are dead but little digested. Other organisms, such as salps, lack any grinding mechanism, and digestion is even less complete. Such fecal matter is a rich substrate for bacteria (Figure 2).

In shallow water environments, large, rooted vegetation, either submerged or emergent, is the major source of plant biomass production. In the coastal zone, sea grasses, kelps, and other macroalgae have very high production rates (Mann 1972); on coral reefs, a thin, heavily grazed mat of red and blue-green algae is responsible for more primary production than are the zooxanthellae of the corals themselves (Johannes et al. 1972). In terrestrial ecosystems generally the direct

consumption of plants by grazers amounts to about 10% of total primary production, the rest becoming litter and humus. Where it has been measured, the proportion of direct grazing of aquatic macrophytes also amounts to about 10% of primary production (Teal 1962). This means that most primary production goes directly into the detritus food web to be mediated by bacteria, and therefore, the flow of energy through detritus and bacteria is in fact the predominant one in most, if not all, ecosystems. It is therefore important to understand and to quantify the roles of bacteria in food webs.

Although a case is made in this review for the importance of bacteria in food webs, it must be emphasized that bacteria alone do not make a community in which varied substrates are utilized quickly and efficiently. It is necessary to have some higher organisms in the community as well to provide some functions of processing substrates. It has been known for many years that the degradation of litter, and even animal carcasses on the forest floor, depends on the activity of small invertebrate animals that chew, grind, and partly digest the material, making it more readily available as a bacterial substrate. Material enclosed in exclosure cages, which eliminate the activity of the small invertebrates, remains whole and relatively undegraded for a much longer time than does material not so protected. We now know that the same is true in aquatic environments. In streams, where the principal input is leaves, there is a guild of insect larvae which processes leaves, rapidly rendering them into fine detritus, and facilitating attack by both bacteria and fungi. Experiments similar to those done in forests have shown that in broad terms the community of the deep sea bottom functions in much the same way. They were inspired by the finding that the lunches which went to the sea bottom in the sinking of the ALVIN were little decomposed after many months (Jannasch et al. 1971; Jannasch and Wirsen 1973). While the unusual

conditions of high pressure and low temperature at the bottom of the ocean certainly contribute to the slow rate of degradation found there, it also appears that the action of benthic invertebrates is important in facilitating degradation (Sieburth and Dietz 1974).

Not only does the rate of degradation of large particulate material depend upon the action of invertebrates as well as bacteria, but so does the regeneration of essential nutrient elements, such as nitrogen and phosphorus. If conditions remain strictly aerobic, as they do in most natural waters, bacteria appear to retain all phosphate they assimilate as polyphosphates. It is released only when the bacteria are eaten and digested by protozoans or higher animals (Buchler and Dallon 1974; Johannes 1964; Johannes 1968). Under anaerobic conditions, where the excess metabolic energy necessary for synthesizing polyphosphates may not be available, phosphate is regenerated by bacteria. The fate of nitrogen may differ from that of phosphorus because of its different structural and functional roles. There appears to be no open-ended storage mechanism for nitrogen, as there is for phosphorus, in bacteria. Therefore, an excess might be released as ammonia, even under aerobic conditions. At the same time, there may be nitrogen fixation by specific bacteria and cyanobacteria. In anaerobic environments, where excess ammonia is being produced, much of it may be denitrified.

Bacteria in Aquatic Food Webs

Because they utilize a variety of dissolved and particulate substrates, bacteria occupy many positions in aquatic food webs. Distinct populations are associated with particles, including feces, while free bacteria utilize dissolved substrates from both primary and secondary sources.

The reality of any food web is much more complex than that shown in Figure 3. However, that is in itself much more complex than, and fundamentally different from, an Eltonian food chain. What would be recognized as the food chain of this generalized aquatic food web is connected by dashed lines in the upper right. All the rest is ignored by most aquatic ecologists, and most of it is the domain of bacteria, together with the specialized metazoans which feed on bacteria.

It has been argued that this part of the food web is not of interest to ecologists who are concerned with the total flux of energy and materials. One school of thought maintains that very little energy flows through these pathways. There is now substantial evidence that a relatively large proportion of the total flux of energy is mediated by microorganisms, however (Pomeroy 1974; Pomeroy 1979; Sieburth et al. 1977; Watson 1978; Table 1). Another school of thought maintains that even if more than half of the total flux of energy is through microbially mediated pathways, these pathways lead to a dead end so far as fishes and other terminal consumers are concerned, and are therefore of limited interest. While we do not have data from the real world to evaluate quantitatively the ultimate fate of energy that passes through microbial biomass, simple heuristic modeling exercises demonstrate that we cannot exclude the possibility that virtually all terminal consumers may be able to utilize energy from microbial pathways (Pomeroy 1979).

The principal basis for the conclusion that microorganisms create dead-end food webs is the assumption that there are too many transfers of energy between so-called trophic levels. Ecological dogma has it that the transfer of energy between trophic levels is about 10% efficient, and therefore can never go more than four or five steps before all of the energy from primary sources has

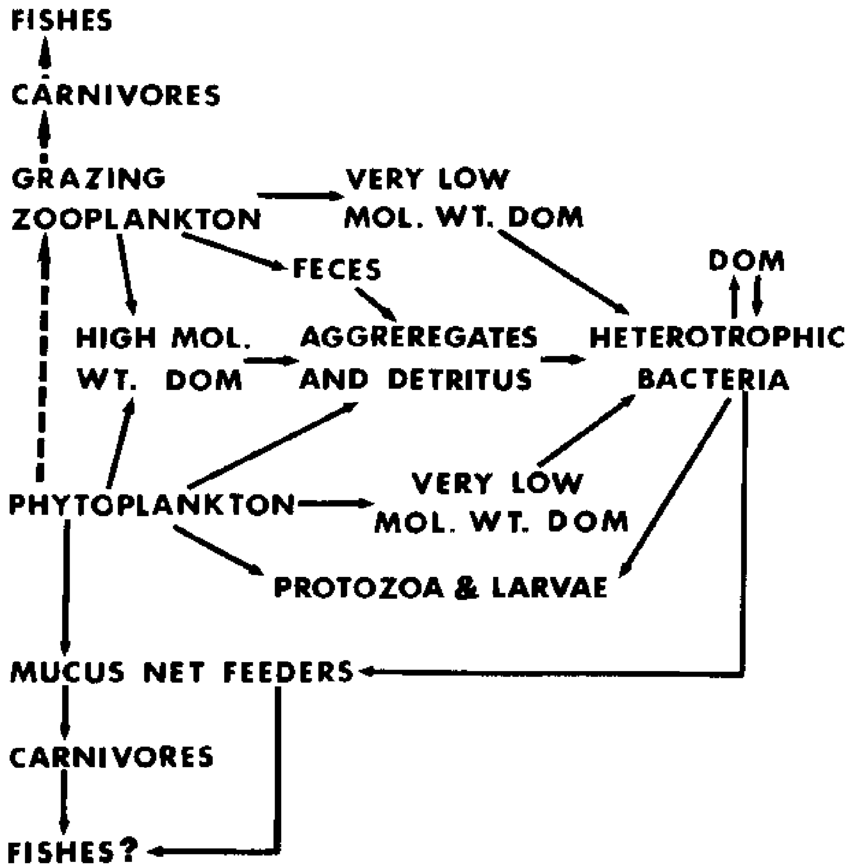

Figure 3. A generalized and simplified diagram of aquatic food webs. The compartments in the upper left joined by the heavy, dashed lines are the classical food chain. The remainder shows other compartments and pathways now recognized to exist, and illustrates the role of heterotrophic bacteria in utilizing a variety of dilute, dispersed, or somewhat refractory materials, while in turn becoming a food source for a variety of higher organisms.

Table 1. Bacterial growth rate in natural waters. In some cases mean values were calculated and units of reference changed for uniformity.

Location	Production Rate $mg\ C\ m^{-3}\ day^{-1}$	Production Rate $mg\ C\ m^{-2}\ day^{-1}$	Method	Reference
Arctic pond		5*	direct count	11
Lake Biwa, Japan	11.5		dark HCO_3	27
Lake Biwa, Japan, summer		900	direct count	27
Lake Biwa, Japan, winter		100	direct count	27
Frains Lake, Michigan	300		respiration	34
Dalnee Lake, Kamchatka	1,530		respiration	40
Ryblinsk Reservoir	57	457	dark HCO_3	24
Narragansett Bay	202		direct count	38
Butaritari Atoll Lagoon		410	respiration	40
Continental shelf water behind Great Barrier Reef		810	respiration	40
Black Sea, euphotic zone	2		respiration	40
Black Sea, chemosynthesis zone	6		respiration	40
N. Africa, coastal upwelling	3-30		rate of natural substrate production	43
Peru, coastal upwelling	11	1,600	respiration	40
N. Atlantic E. of Azores	120		direct count	38
Sea of Japan	45		respiration	40
N. & S. Pacific, mid and low latitudes	0.6	126	respiration	40

* Assuming 60 day active season

been dissipated as metabolic heat. In a recent analysis (Pomeroy 1979), the writer has shown that most of the transfers of energy between producer and consumer populations exceed 10% efficiency, and there is evidence that transfers involving microbial consumers operate at an efficiency of around 50%. Longer and more complex food webs appear to be possible, and observations of real communities of organisms tells us that they are the rule. What appeared to Hutchinson (1959) to be a paradox in classical food chain theory is probably a major stabilizing influence for complex communities (Pomeroy 1975; Pomeroy 1979).

If bacteria are metabolically active and responsive to the availability of substrates in natural waters, as they now appear to be, are they indeed links in the food web or merely energy sinks, as some ecologists now believe? If their supply of energy is intermittent, and they continue to respire when external energy sources are not available, bacteria may respire most of the organic matter they assimilate. On the other hand, if bacteria are consumed rapidly and efficiently by grazers and filter feeders, their excess production will be utilized efficiently (Pomeroy 1974). The standing stocks of bacteria in most natural waters usually are not great (10^4-10^6/ml), and do not seem to vary greatly (Ferguson and Rublee 1976; Watson et al. 1977). Yet, there is evidence that their production rate is substantial. This suggests that bacteria are consumed down to some refuge concentration at the same rate that they grow. We know that a wide variety of aquatic organisms, from protozoans to prochordates, are capable of consuming bacteria. While some uncertainty exists about the fate of bacteria in the food web, their assimilation efficiency appears to be high. A number of studies of assimilation efficiency of bacteria show it to be 50% or greater (Ho and Payne 1979; Payne 1970), but most of this is experimental work at substrate concentrations that are several orders of

magnitude greater than those found in natural waters. However, relative assimilation and respiration of defined substrates by natural assemblages of bacteria, at natural concentrations of substrates, also show efficiencies better than 50% in most cases (Hobbie and Crawford 1969). Therefore, food webs with a bacterial link may be as efficient as those without one, because of their relatively high efficiency coupled with their versatility in assimilating dilute and refractory substrates.

Contemporary techniques of microscopy and biochemistry make it possible to enumerate bacteria in natural waters reasonably well (Hobbie, Daley and Jasper 1977; King and White 1977; Watson et al. 1977; White et al. 1977; White et al. 1979), but measuring their rate of production remains a difficult problem. Direct counts on confined water samples over time are likely to be flawed by the induction of a latent period as the result of handling. It is also difficult to exclude all grazers, especially the small protozoans. Indirect measures of production, based on respiration and uptake of organic or inorganic substrates, depend upon conversion factors usually arrived at through laboratory manipulation of bacterial populations quite unlike those found in natural waters. Such factors are not really constants, even if they are carefully determined. In the end, one gets almost as good an estimate of production by inferring it from a known rate of substrate production (Watson 1978). Because of these uncertainties, the production values in Table 1 must be viewed as provisional ones, particularly since they involve the entire range of methods. While they may not be absolutely correct, they produce a credible picture of the production rate of bacteria in natural waters, which varies over six orders of magnitude. The arctic pond is, not surprisingly, lowest by an order of magnitude. The disparity between the Peruvian coastal upwelling and Narragansett Bay

indicates that the latter is not one of the most eutrophic of estuaries. Lakes have a wide range of bacterial production values, with smaller eutrophic lakes actually approaching sewage oxidation ponds in their level of activity. The larger oligotrophic lakes are not well represented in the table.

Bacterial biomass may at times equal or even exceed that of phytoplankton, and bacterial production may sometimes be equivalent to a significant fraction of primary production (Sieburth et al. 1977; Watson 1978; Watson et al. 1977). Therefore, it is potentially a major food chain link. Of course, not all higher organisms can consume bacteria, especially the free-living minibacteria. They must be utilized either by protozoans or by mucus net feeders, such as salps and appendicularians. We must then ask what organisms consume the protozoans and mucus net feeders, and what is the efficiency of assimilation of energy in such a pathway. It is possible that the bacteria are efficiently used by organisms that are themselves more or less dead ends. If this is so, an environment which promotes production of bacteria might stimulate secondary productivity, but at the expense of other secondary pathways that lead to the production of fishes. There are many gaps in our knowledge of food webs, not only at the level of bacteria and protozoans, but at higher levels as well. A clear overall picture of aquatic food webs has not emerged, in part because there has not been enough interaction between aquatic microbiologists and aquatic ecologists. Neither alone can see the entire system and how it works.

Questions not Answered

Aquatic microbial ecology is a fast growing field in which there have been some notable recent successes. Substantial progress has been made in bringing the best existing techniques in

epifluorescence and scanning electron microscopy to bear on aquatic problems. There has also been rapid development of applications of biochemcial methods to the problems of microbial ecology through the use of adenylates, lipopolysaccharides, and muramic acid as parameters of microbial biomass. However, it is still difficult to make clear distinctions between major taxa. Adenylates do not distinguish taxa at all. Muramic acid does not distinguish cyanobacteria from other bacteria. Because direct counts, while very useful, are terribly time-consuming, there remains a need for rapid, relatively straightforward analytical methods to measure the biomass of distinct taxa of aquatic microorganisms. Although the methods mentioned appear not to be sufficiently specific for major taxa, immunofluorescence appears to be too highly specific for most of the kinds of questions addressed here.

Measures of such seemingly well understood processes as photosynthesis and respiration do not always yield values which can be rationalized (Joiris 1977). The standard method for measuring photosynthesis in aquatic systems appears to underestimate the rate by a highly variable amount. There is no accepted method for measuring respiration in oligotrophic natural waters, and one would be especially useful if it were possible to use it to distinguish the respiratory rates of at least the autotrophs from the heterotrophs.

Surprisingly little is known about the food webs of aquatic systems and least of all is known about the microbial aspects of them. Most models of the flow of energy and materials through aquatic ecosystems either do not include bacteria at all, or include them implicitly in some other category, such as feces. Most aquatic ecologists and fisheries biologists are not convinced that bacteria play a significant role in the flow of energy through aquatic food webs. At the same time, most aquatic

microbiologists are not interested in the interdisciplinary questions concerning the production of substrates for microorganisms or the consumption of microorganisms by grazers and mucus net feeders. Many of these problems will yield only to team research,of which microbiology must be an integral part.

During the decade of the 1970s, the microbial populations of pelagic waters were identified by direct observation; their biomass was determined by both direct observation and biochemical means, and their metabolic activity was estimated using tracer methods. On the basis of these and other studies of pelagic ecosystems, a paradigm of the microbial pelagic food web was developed out of earlier concepts of the detritus food web of shallow waters. In the coming decade the details of this microbial food web will be revealed and quantified. If the rates of microbial consumption and production of energy-containing organic compounds prove to be as great as present indications suggest, several basic ecological concepts will have to be reexamined, not only in natural waters, but in ecosystems generally. The paradigm of a short, direct food chain from plants to grazers to carnivores with relatively low assimilation efficiency will have to be fundamentally modified to encompass the varied activities of microorganisms and their relatively high assimilation efficiency.

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Helpful, Harmful, and Fallible Microorganisms: Importance in Transformation Of Chemical Pollutants

MARTIN ALEXANDER

Considerable information exists on the role of microorganisms in the transformations of individual compounds in natural waters, and it is not the purpose of the present discussion to review these data. Instead, my purpose is to highlight some of the general issues that relate to the role of microorganisms in chemical transformations in aquatic environments and to point to some of the areas that I believe deserve high priority in research. The paper is, therefore, highly subjective, but it is with a view to provoking colleagues to think that I embark on this endeavor. To a degree, individuals who propose research priorities must serve as devil's advocates, often espousing views which they may or may not hold, but doing so to be deliberately provocative. Omission of a particular topic in such a list of priorities is either an oversight or a deliberate downgrading of a specific phase of investigation. Which it is will not be stated because the function of the devil's advocate would thereby be compromised. If you are

provoked or disagree and are thereby led to react, then the purpose of this discussion will have been fully served.

Microbiologists are usually not used to talking in generalities, and they want to react, even in the establishment of priority lists for research, to particular studies and individual data points. To satisfy our common yearning for numbers, numbers, numbers, I present data to illustrate individual points. The fact that these data come from investigations conducted in my laboratory is not an indication that these studies are the best or even that I think that they are the best, but rather that most of us know more about what is going on in our own laboratories and are in a better position to discuss these data than the findings of other research groups.

Microorganisms are prominent in chemical transformations in three ways: they are helpful, they are harmful, and they are fallible; that is, they help by destroying toxic chemicals and causing a reduction of environmental pollution, they do harm by generating toxicants from previously innocuous compounds, or they are fallible as shown by their inability to modify certain molecules at a significant rate, if at all, so that the pollutant persists and is subject in some instances to distant transport or biomagnification.

The helpful characteristics of aquatic microfloras are evident in their remarkable capacity for mineralization. Mineralization refers to the conversion of an organic compound to inorganic products. A necessary consequence of mineralization of toxicants is their detoxication. With but a few exceptions, mineralization in marine and inland waters probably is the result solely or largely of microbial activity.

Given the tens of thousands of compounds

that are now potentially subject to regulation under the Toxic Substances Control Act, it is surprising that the mineralization of remarkably few of the environmentally important synthetic chemicals has been investigated. In only a small number of instances is there information indicating that these synthetic products are destroyed in natural waters, or even in another environment. The limited information that is available is largely derived from studies of pesticides, but pesticides make up a small percentage of the total number of compounds and of the tonnage of chemicals that are introduced deliberately or inadvertently into natural waters. Hence, a critical area for research is *the establishment of the susceptibility to mineralization of synthetic organic compounds*.

The investigator concerned with the fate of chemicals in natural waters encounters several major obstacles. First, the concentration of importance is not at the percent or even parts-per-million level, but often the concentration to be tested is in the range of parts per billion or even lower; there is ample reason to believe, moreover, that different organisms and, hence, possibly different metabolic pathways might be functioning when a compound is present at low and high concentrations. Second, the molecules of environmental concern are frequently exotic, and biochemical precedents or even clues on the likely pathway cannot be found in the literature; therefore, the microbial ecologist must become a biochemist to understand what transpires in nature. Third, nature does not contain pristine cultures but rather a diversity of populations, and the organisms responsible for a transformation may be subjected to predation by protozoans, parasitism by other microorganisms, or competitive pressures such that the concentration of the nutrients that they require is too low for maximal or even good activity. Finally, analytical methods often need to be

developed or refined because of the unknown products or the minute levels that are present. As a consequence, it is common to initiate investigations of chemical transformations with individual cultures of microorganisms in clean, aqueous systems with high concentrations of the organic compound. These, then, serve as the prelude to tests in models of the natural ecosystem in which more reasonable concentrations and natural microfloras are present. Such an approach is illustrated by our investigations of 2,4-dichlorophenoxyacetate and related phenoxy compounds, first in culture (Loos, Roberts and Alexander 1967), then by enzymes (Bollag et al. 1968; Duxbury et al. 1970; Tiedje et al. 1969), and finally in studies involving models of natural ecosystems (Alexander 1974).

The good that microorganisms do by mineralization is well known but still requires iteration. Microorganisms are the major detoxifiers for many chemicals in natural habitats, and often they destroy synthetic compounds and all of the products of partial decomposition before the concentrations build up to hazardous levels. Unfortunately, this beneficial facet of microbial transformation has deluded many environmental scientists as well as microbiologists. Mineralization by microbial communities is widespread but does not apply to *all* molecules, and the concern with environmental quality and a proper assessment of the role of bacteria and fungi in nature require that attention be directed to the harm that they do.

The potential harm to humans is expressed in the synthesis and subsequent excretion of either products of incomplete breakdown or of conjugates or derivatives of the original chemical. These metabolites are of practical significance for four reasons: (i) some are more toxic than the original chemicals, (ii) some cause injury to entirely different organisms than

the initial compounds, (iii) several are less susceptible to microbial attack and hence persist longer than the parent molecules, and (iv) a few differ from the original chemical in being subject to biomagnification or bioaccumulation such that they may appear at hazardous levels in species higher in aquatic food chains. It is important to stress that toxicology, like other scientific disciplines, is in a constant state of flux, and compounds tested by current procedures, on re-examination by methods of the future, may be found to be harmful in ways not considered previously. In this light, the microbial formation and excretion of a metabolite from a chemical introduced into fresh or marine waters must be considered of potential concern inasmuch as subsequent inquiry may reveal the existence of an environmental hazard not initially apparent from the toxicology testing protocol. Hence, a critical area for research is *the identification of products arising in the microbial metabolism of chemicals introduced into natural waters*.

It is easy to find examples of the environmental conversion of chemicals of little or no toxicity at the levels in nature to highly toxic products. In not all instances is it clear, however, what role microorganisms play. Activation is the term to describe the conversion of an innocuous molecule into one that is toxic or of a substance with low potency into a product of greater toxicity. Among the examples of activation is the conversion of mercuric ions to the methyl mercury derivatives (Jernelov and Martin 1975).

A more recent example of activation, in part microbial and in part probably nonmicrobial, is the formation of nitrosamines. These compounds are of considerable importance because they are carcinogenic, mutagenic, and teratogenic. The immediate precursors of nitrosamines are nitrite and any one of a variety of secondary amines.

The nitrite level need not be high, however, as it is continually generated from ammonium during nitrification or as nitrate is reduced. Secondary amines, or the tertiary amines which are converted to them, are present in the algae of the phytoplankton, they may be generated by heterotrophic microorganisms, and they are deliberately introduced into waters as industrial or other wastes. Coupled with the ubiquity of the precursors is the facility for the formation of the carcinogen. Thus, our studies have shown that several dialkyl nitrosamines can be formed in either fresh- or wastewaters from secondary or tertiary amines (Pfaender and Alexander 1972). The nitrosation, which is the final phase in activation, may be microbial (Ayanaba and Alexander 1973) or it may be nonenzymatic, although possibly the reaction is abiotic but involves microbial cells or microbial products (Mills and Alexander 1976). However, the formation of nitrite and the genesis of at least some of the amines is entirely the result of microbial activity (Tate and Alexander 1976). Even quaternary ammonium compounds may give rise to the secondary amine precursors during their degradation provided that such compounds are subject to microbial attack (Dean-Raymond and Alexander 1977). These considerations clearly point to another major research need, namely, that *microbial activation and the products of that activation require exploration.* This recommendation is particularly important inasmuch as the kinds of compounds that may be subject to activation in aquatic environments and hence the identities of the products have received almost no attention.

Nitrosamines illustrate another problem for microbiological inquiry: the inability of microorganisms to destroy rapidly the product of a compound that they begin to metabolize. Although the secondary amines, at least those that have been studied to date, are good substrates, the nitrosamines that are formed from them are quite

persistent (Tate and Alexander 1976). They are not readily attacked by natural communities of microorganisms and hence will probably endure for considerable periods of time. This indicates that a nitrosamine that is formed at one location, for example, at a site of sewage discharge, may move for considerable distances and could affect humans drinking the water at a considerable distance from the site of formation of the hazardous chemical. In this regard, it should be pointed out that little information exists on the microbial biosynthesis of persistent organic compounds.

A growing body of evidence exists that microorganisms are involved in nature in the transformation of compounds that they cannot use for energy or as nutrients. This phenomenon is defined as cometabolism, that is, the ability of a microorganism to metabolize a compound that it cannot use as a source of energy or of any of the nutrient elements (Alexander 1979). The evidence for cometabolism in nature comes from observations that chemicals are modified in environmental samples that are not sterilized whereas the comparable reaction does not occur in sterilizaed samples, and an organism growing on the chemical cannot be isolated. The evidence for cometabolism in axenic culture is more direct, and numerous examples can be cited to show microbial reactions that provide the responsible organisms with neither energy nor nutrients. Our studies have focused on the cometabolism of DDT (Pfaender and Alexander 1972) and chlorinated benzoates (DiGeronimo, Nikaido and Alexander 1979) in wastewater as well as actions on the same types of molecules in microbial cultures (Horvath and Alexander; Subba-Rao and Alexander 1977).

Cometabolism has several important environmental consequences. Thus, chemicals that are subject to this type of process are not mineralized and organic products are generated, and because the parent molecule is not modified enzymatically to a major extent, it is likely that toxic precursors

will give rise to toxic products; that is, they represent similar chemical classes. Moreover, the lack of conversion of the substrate to microbial cells will be reflected in a lack of an increase in microbial cell numbers; hence, those compounds acted on by cometabolizing populations that are initially small will persist for long periods as a result of the absence of a rise in the biomass of organisms containing the responsible enzymes. Given the accumulation of products, some of which may be toxic, and the slow rate of disappearance of chemicals acted on by cometabolism, obviously, *research needs to be carried out to establish the significance of cometabolism in nature.* Moreover, inasmuch as analogs or other nutrient additions may promote growth of the cometabolizing populations, it is important to *seek means for enhancing cometabolism in fresh and marine waters.* Furthermore, the kinetics of a process that results from cometabolism likely will not be approximated by the logarithmic growth typical of bacteria and probably will not be the same as the classical kinetics for chemical reactions. Hence, *information is required on the kinetics of cometabolic processes in natural ecosystems.*

Even given the few examples cited, I hope that it is now clear that microorganisms are important because they do good and they do harm. Why are they important because they either do nothing or are unable to carry out reactions at significant rates? The persistence in waters of pesticides, polychlorinated biphenyls, synthetic polymers, alkyl benzene sulfonates, chlorinated phenols, and a variety of other organic compounds widely used in industry demonstrates that heterotrophic communities are not omnivorous, that they do not contain enzymes to catalyze reactions leading to the destruction of each of the multitude of organic molecules of current economic significance, and that they are not infallible. This microbial failing becomes even more acute in light of the role of microorganisms as the chief or sole agents

of mineralization. If the good that microfloras do is often associated with their capacity to destroy totally certain classes of molecules, then blame must be placed on them for their inability to destroy others. The duration of some of these persistent materials, which have been termed recalcitrant, may be weeks, months, years, or even decades in natural waters. The mechanism of recalcitrance may be linked with an inherent property of the persistent molecule such that no enzymes exist to bring about either mineralization or cometabolism, or the longevity may rest on the invariably slow rate of the enzymatically catalyzed reaction. Some of the chemical properties associated with such resistance have been considered elsewhere (Alexander 1973).

Some molecules are potentially subject to microbial attack, but environmental factors or the concentration of the substrate may alter their susceptibility to utilization by the indigenous microflora. For example, polysaccharides and hydrocarbons are characteristically good substrates for bacteria and occasional fungi, but the same polysaccharides in peat under water or the hydrocarbons in an anaerobic site are rarely destroyed quickly, and certain of the polysaccharides may endure for thousands of years. Certain other chemicals, such as the organophosphate insecticides or nitrogen-containing compounds used for the control of algae and rooted plants, persist because they are bound to inanimate components of the environment. Other than the effects of temperature and possibly pH, which have received much attention, *the environmental factors correlated with the protection of chemicals from microbial attack in waters have not been adequately defined,* and this subject too is deserving of far more research.

Consider, for example, the lack of a convincing explanation for the massive accumulations in nature of organic materials in anaerobic sites, such as in peat deposits. The slow attack or the

absence of degradation may be attributable to the absence of the O_2 needed as a reactant for the degradation, or it may result from the lack of a utilizable electron acceptor. Another explanation is the accumulation in the oxygen-free zone of substances that are toxic to microorganisms having the requisite enzymes. In this connection, we have been endeavoring to explain why even simple carbohydrates are resistant to attack in flooded soils or sediments and have correlated the accumulation of volatile fatty acids and the pH-dependent toxicity of these compounds with the lack of significant microbial degradation (Kilham and Alexander, unpublished data).

The concentration of potential substrates in water also greatly influences microbial decomposition. Jannasch (1967) first directed attention to this problem in an intriguing series of studies of marine bacteria. Typically, the tests performed by microbiologists in universities, regulatory agencies, and industrial laboratories to show microbial decomposition use substrate concentrations many orders of magnitude greater than those found in natural waters, and it is often tacitly assumed that the rate of mineralization or cometabolism of the compound at the low concentration in nature would be reasonably similar to that observed in laboratory studies using the far higher concentrations. However, even an undergraduate in the first course in biochemistry knows from Michaelis-Menten kinetics that the rate would be appreciably less at the lower substrate concentration, but how much less would be unknown. From the linear portion of the Michaelis-Menten plot of the influence of concentration on rate, one would conclude that the reduction in rate of attack would be directly proportional to the lowered level of the chemical in the water, but no conslusion of environmental significance could be drawn until the concentrations that fall on the linear portion of the plot had been established. We have recently assessed the effect of the concentration of serveral amines on

the rate of their mineralization in samples of natural waters. In several instances, the rate was directly proportional to the initial concentration of the chemical. On the other hand, with other compounds, a threshold was observed below which mineralization was not detected. The threshold was noted, moreover, for compounds which are toxic to lower animals and to aquatic plants (K. Boethling and M. Alexander, unpublished data). From these preliminary findings, one can conclude either that the rate of decomposition in nature may be less than that usually observed in the laboratory or alternatively that the excellent substrate for microorganisms in the laboratory is wholly refractory at the low concentrations present in nature. Given the inability to extrapolate from laboratory to field, therefore, it is of great importance to *establish the effect of the concentration of chemicals on the rate of their mineralization of cometabolism.*

Work in many laboratories has demonstrated a remarkable correlation between chemical structure and susceptibility of organic chemicals to microbial degradation. Compounds in a given class may be excellent substrates, for example, but the introduction of a particular substituent or a minor modification in the structure of a member of the class may render the molecule refractory. Because both the original and the modified chemical may have practical uses, substitution of one for the other may lead to the elimination of a serious pollution problem. Hence, *additional research is needed to establish the structural characteristics that account for or are correlated with resistance of organic compounds to microbial mineralization or cometabolism.* Such information will be of enormous importance to industry as it endeavors to replace polluting chemicals with other molecules that both have efficacy and do a minimum of environmental damage. The data also will be important to regulatory agencies as they endeavor to establish which of the enormous number of compounds that they are

obliged to regulate should be given high priority in their testing or regulatory programs.

An awesome aspect of research on these topics should be emphasized: the enormous number of compounds for which no information exists. Given this multitude of chemicals and our woeful lack of information, many of us would undoubtedly decide to retire to more satisfying but less significant problems. However, it is my belief that major progress can be made in overcoming our information gaps and that useful data can be provided to industry and government and for science itself if generalizations are sought and the investigations are not conducted on a chemical-by-chemical basis. By looking for the underlying microbiological and environmental phenomena and by seeking generalizations that will be applicable not to individual chemicals but to broad categories of compounds, significant contributions can be made to the solution of what are clearly major environmental problems. Work in this field will surely be frustrating to some, but I hope that it will be satisfying to those who are convinced that they are examining a topic of major importance to modern society, while at the same time making a modest contribution to the development of microbial ecology.

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An Attempt at Identifying Research Needs for Studies on Microbial Transformations

JAMES M. TIEDJE

Unlike ecologists and environmentalists, microbiologists begin by assuming that microorganisms play the major role in transformation of natural and man-made chemicals in natural ecosystems. The ubiquity and diversity of microorganisms dictate that they are the first lines of attack on these substrates.

Dr. Pomeroy has called for better methods--the proverbial challenge to the microbiological ecologist--for assessing the role of microbes in natural food webs and has identified another topic, efficiency of carbon assimilation, which I believe should be singled out as a particularly important but neglected topic. W. J. Payne's laboratory (University of Georgia) has made important advances toward a basic understanding of microbial assimiliation, but these concepts need to be extended to more natural settings, i.e., natural populations, very slow growth rates, and low substrate concentrations.

Dr. Alexander has pointed out examples of pollutant problems which illustrate research needs. Because I am more familiar with this topic, I am providing a detailed list of research needs as I see them, which generally augment his points.

Research Needs

Better methods to determine permanence of binding of xenobiotics to sediments and organic matter. The important question is, "When is a chemical essentially a permanent component of humus such that it will never again be released in any significant quantity as either the parent material or a similar product." A number of organic chemicals of issue bind to humic matter. If this is by covalent binding, my general opinion is that the parent compound can be considered permanently bound; it is in effect humus and can be dismissed as a chemical of no further concern. With weaker binding forces, however, the xenobiotic may be available for biomagnification, or could be released as a result of a change in the properties of the immediate environment. What is needed are methods to sort out the two cases reliably. This is of particular importance in studies with ^{14}C-labeled xenobiotics since the label can be followed beyond the point at which data can be interpreted.

Better understanding of the behavior of xenobiotic biodegradation at low substrate concentrations. To date, most work on pollutant transformation has been done at concentrations above those found or expected in nature. When this is the case, answers to questions on velocity of uptake mechanisms, induction of necessary enzymes, and the general pattern of kinetic response to very low concentrations become essential if one is to predict biodegradation in the habitat.

Better characterization of mechanisms and

kinetics of cometabolism. Cometabolism probably accounts for much of the xenobiotic degradation in nature since only a limited number of compounds serve as sole growth substrates, yet some slow disappearance with time is noted. If the mechanism (s) of cometabolism was understood for cases of importance, then one would have a better chance of identifying a practical method for enhancing degradation to remove the pollutant. Also, if one could clearly establish that degradation of a compound was due to cometabolism, as opposed to its serving as a growth substrate, selection of a more appropriate model for prediction of degradation should be possible. Because most cometabolism is due to the xenobiotic being a secondary substrate for a nonspecific enzyme, I would suggest that a high K_m and low turnover number might be used as a characteristic to identify cometabolic behavior.

Better understanding of the influence of starvation physiology on metabolism of natural and xenobiotic substrates. Since most organisms in nature are in a starvation or semidormant state as a result of carbon limitation, the ability of the organism to respond to natural or pollutant substrates might be quite different than for the more luxuriant state which is normally studied. If the metabolism requires an inducible enzyme(s), what is the source of energy for protein synthesis? If the uptake mechanism is active transport, what is the source of energy for transport? We know very little about the metabolism and capabilities of the prevalent microbes in nature in their *in situ* physiological state.

Better understanding of anaerobic metabolism of xenobiotics, its significance, and types of prevalent reactions. Many chemicals of issue bind to particulate material, both organic and inorganic, which finds its way to aquatic sediments. Because of the low diffusion coefficient of a saturated porous matrix, O_2 diffusion cannot keep pace with

the respiratory consumption of O_2, and the sediment becomes anaerobic. Thus, the potential for anaerobic metabolism of xenobiotics may be much greater than one might think. However, our store of knowledge about anaerobic metabolism of compounds, other than the standard fermentation substrates, is very limited. So we cannot predict which types of structures might be degraded, nor do we have an idea of the relative rates of degradation.

More use of assay systems which have the salient features of the receiving habitat. If assay systems are to be useful, their results must be reliable in predictin behavior. One must achieve a balance between including the significant features and excessive complexity. In our studies on alachlor degradation in soil, we compared the rate of degradation of the herbicide by 50 g of soil in 250-ml sealed Erlenmeyer flasks incubated in the laboratory---a simple system--with degradation of the same chemical in the same soil but applied by tractor sprayer in the field. In the latter case natural factors such as rainfall, temperature, and the presence of plants could influence degradation. The results (Table 1) led to the general conclusion that all systems showed roughly similar rates of degradation; thus, the simple laboratory flask assay adequately predicted degradation in the field. It is true that the two field incubations were slightly slower, probably as a result of cooler temperatures, but this difference is probably not of significance to most of the practical decisions that must be made. If, however, the chemical was long-lived, then the accuracy of the assay system becomes more important since the measured rate will be extrapolated over a much longer time and error could become significant.

The major point that I want to make is that assay systems should be verified, and if necessary calibrated to the environmental response, so that reliable predictions can be made.

Table 1. Comparison of biodegradation rates of the herbicide alachlor in soil measured in laboratory and field environments[a]

Assay description	Half-life (days)[b]
Standard laboratory assay; herbicide pipetted onto soil in a flask and then incubated in the laboratory	8.0
Sprayed in the field; soil collected and incubated in flasks in the labora-laboratory	8.4
Sprayed in the field; soil collected and incubated in flasks in the field	9.2
Sprayed in the field; soil remained in place but was subsampled periodically	10.5
Sprayed in the field; soil collected, placed in a flask, sterilized with propylene oxide, and incubated in the field	100

[a] From S. F. Chou, Ph.D. thesis, Michigan State Univeristy, East Lansing, 1977.

[b] Calculated as the first order decay constant from the curve of disappearance of parent herbicide with time.

More focus on accurately predicting the fate of xenobiotics in nature. Particularly in academia, we focus on demonstrating a phenomenon, but rarely carry it to the stage where its importance in nature can be evaluated. There are reasons for this: the experimental complexity becomes greater, and the chances for getting distinctive results become less. Our goal, then, should be to arrive at novel approaches that overcome these difficulties, yet yield results that provide the important answer, that of predicting fate in nature.

I shall take some risk and suggest one area in which I think less research is needed--this is the effect of xenobiotics on microorganisms, especially pure cultures of microorganisms. Why? First, the resistance and resilience of the microbial community is overwhelming compared to the active concentrations of chemicals to be encountered, even for the worst case. Second, experience now shows very, very few examples where this type of experimentation has indicated that a significant problem could be expected. Rather, I suggest that resources for toxicity testing be spent on eucaryotic organisms since they are much more sensitive. If toxicity is indicated, then procaryotic organisms should be examined.

Microbial-Meiofaunal Interrelationships: A Review

JOHN H. TIETJEN

The marine meiobenthos, as defined by Mare (1942), is comprised of microscopic metazoans. For the most part, it consists of representatives from the Hydrozoa, Turbellaria, Gnathostomulida, Gastrotricha, Kinorhyncha, Nematoda, Tardigrada, Oligochaeta, Polychaeta, Copepoda, Ostracoda, and Amphipoda; other groups are sometimes present (Coull 1973). On shallow (<100 m) bottoms, meiofauna densities range between 10^4 and $10^7/m^2$, generally averaging about $10^6/m^2$. Nematodes and harpacticoid copepods are usually the dominant groups present (Coull 1973; McIntyre 1969).

Trophic types represented within the meiofauna include predators, herbivores, and bacteriovores. The only groups thought to be exclusively, or nearly exclusively predatory are the Hydrozoa and Turbellaria; the other groups, therefore, impact directly on the benthic microflora. The small individual body size (~0.1 to 1.0μg, dry weight) has led to the speculation that the meiofauna may be about five times more metabolically

active per unit of body weight than the macrofauna (Gerlach 1971). Their roles in the ecosystem have been summarized elsewhere (Coull 1973; McIntyre 1969); here I will be concerned with the influence of the bacteria and microalgae on the growth, reproduction, and distribution of members of the meiofauna and the effects of the meiofauna on the microbial community.

Influence of Benthic Microflora on Meiofauna

Most meiofauna exhibit patchy spatial distributions, and an important factor affecting this distribution appears to be the distribution of benthic microflora. Coull (1973) summarized the work of Gray, Hopper, and Meyers, and others; more recently, Hummon et al. (1976) and Gerlach (1977) have demonstrated how meiofauna are attracted to *Limulus* eggs and decomposing fish. Less is known about the temporal relationships between the benthic microflora and meiofauna, although some evidence of a direct relationship between the seasonal distribution of benthic microalgae and herbivorous nematodes has been seen (Tietjen 1971; F.R. Cantelmo, Ph.D. thesis, City University of New York, New York, N.Y. 1978).

Quantitative studies of the amounts and varieties of benthic microflora consumed by the meiofauna have been few (Duncan, Schiemer and Klekowski 1974; Lee, Tietjen, Garrison 1976; Marchant and Nicholas 1974; Rieper 1978; Sibert, Brown, Healy, Kask, Naiman 1977; Tietjen and Lee 1973; Tietjen, Lee, Rullman, Greengart, Tropeter 1970). Results of these studies indicate that the dry-weight consumption of bacteria and algae by the meiofauna apparently clusters in the 0.1 to 10.0 μg/animal per day range. Ingestion of different species of bacteria and algae by the meiofauna is not necessarily indiscriminant. Selection of food appears to be

primarily a function of food cell size and shape and animal buccal morphology (Deutsch 1978; Tietjen and Lee 1977); chemical differences in food cells have also been mentioned (Tietjen and Lee 1977; Tietjen et al. 1970.

The assimilation efficiencies of ingested foods depend on the types of food ingested and the digestive enzymes present (Deutsch 1978). The relationship between assimiliation efficiency and trophic type is shown in Table 1 for three species of nematodes. *Chromadorina germanica*, which is primarily an herbivore, assimilates significantly less of the food ingested (diatoms and chlorophytes) than do *Rhabditis marina* and *Monhystera disjuncta*, which are bacteriovores. These observations are consistent with those made of other marine invertebrates (Kristensen 1972); lower assimilation efficiences are thought to be the result of more refractory materials present in plant cells (mainly in cell walls) than in bacterial cells. Also shown in Table 1 is the fact that adult female nematodes channel most assimilated food into production, mostly egg production (an average of 69% for the species given). The values in Table 1 compare favorably with those that have been obtained for the freshwater nematode *Plectus palustris* (Duncan, Schiemer and Klekowski 1974).

Impact of the Meiofauna on the Microbial Community

One impact of the meiofauna on benthic bacteria and microalgae is the direct effect of their grazing activities on microbial densities, species composition, physiology, and reproduction. No data on the above are available for the meiofauna; however, in systems grazed by protozoans, Barsdate et al. (1974) and Harrison and Mann (1975) found 2- to 10-fold decreases in bacterial numbers relative to ungrazed systems. Furthermore, evidence also

Table 1. Preliminary carbon budgets for adult females of three species of marine nematodes[a]

Determination	*Chromadorina germanica*	*Monhystera disjuncta*	*Rhabditis marina*
Body size of female (μg of C)	0.044 (dry)	0.04 (dry)	0.16 (dry)
Food ingestion (μg of C)	40×10^{-2}	14.4×10^{-2}	60×10^{-2}
Assimilation (μg of C), A	2.48×10^{-2}	2.63×10^{-2}	15.5×10^{-2}
Respiration (μg of C), R	0.53×10^{-2}	0.53×10^{-2}	0.53×10^{-2}
Production (μg of C), P	1.95×10^{-2}	2.1×10^{-2}	15×10^{-2}
Body growth	0.67×10^{-2}	0.5×10^{-2}	5.4×10^{-2}
Egg production	1.28×10^{-2}	1.6×10^{-2}	9.6×10^{-2}
Efficiencies (%):			
A/I	6.2	18.3	25.8
P/I	4.9	14.6	25.0
P/A	78.7	79.8	96.5
R/A	21.3	20.2	3.5

[a] Values given are based on data in the literature (Duncan, Schiemer and Klekowski 1974; Tietjen and Lee 1973; Tietjen and Lee 1977; Tietjen et al. 1970).

exists that the grazing activities of the meiofauna greatly stimulate the turnover rates of bacteria (Fenchel and Harrison 1976; Gerlach 1978; Johannes 1965). The grazing and locomotory activities of the meiofauna may stimulate bacterial growth in several ways:

Meiofauna may mechanically break down detrital particles, causing them to become more susceptible to increased bacterial colonization.

Meiofauna may directly excrete nutrients, such as nitrogen and phosphorus, into the medium for microbial usage.

Through the secretion of mucus, meiofauna may produce slime trails that attract and sustain bacterial growth (Riemann and Schrage 1978).

By their burrowing and swimming activities meiofauna may act as vertical conveyors of nutrients and oxygen within the sediments, and between the sediments and the overlying water column (Gerlach 1978 ; Coull, Ph.D. thesis, City University of New York, New York, N.Y., 1978).

That organic detritus breakdown occurs faster in the presence of benthic animals is known (Barsdate, Fenchel and Prentki 1974; Fenchel 1972; Johannes 1965; Lopez, Levinton and Slobodkin 1977; Tenore, Tietjen and Lee 1977). What remains unclear is whether animal excretion by small animals such as protozoans and meiofauna contributes significantly to the regeneration of nutrients in natural systems. In an experimental system in which the decomposition and remineralization of *Spartina* was examined, Johannes (1965) attributed elevated othophosphate levels in systems containing protozoans to direct excretion, whereas other investigators have concluded that protozoans do not significantly contribute to the regeneration of inorganic phosphorus (Barsdate, Fenchel and Prentki 1974; Fenchel and

Jorgensen 1977).

Evidence that the meiofauna may be contributing to nutrient regeneration is shown in Figure 1. In experimental flasks in which axenic cultures of the chlorophyte *Chlorococcum* sp. and the diatom *Cylindrctheca closterium* were grown, the addition of nematodes *(Chromadorina germanica)* significantly increased the levels of orthophosphate relative to nematode-free flasks. Whether the increase was the result of nematode excretion or the result of lysis of cells that passed through the nematode guts is not known. What is significant is that bacterial activity in these experiments was nil because the experiments were begun with axenic algal cultures and conducted in antibiotic-antimycotic-containing medium (penicillin, 10,000 U/ml; amphotericin B, 0.25 μg/ml; streptomycin, 10,000 μg/ml).

That nematodes may release soluble carbon and phosphorus metabolites has been demonstrated in the soil nematode *Caenorhabditis briggsae*, which may release as much as 40 to 50% of its ingested carbon in soluble form (Nicholas and Viswanathan 1975). More experiments are called for to determine whether meiofauna release soluble metabolites into the water and the extent to which bacteria and other microorganisms utilize these metabolites.

Meiofauna may also serve as a substrate for heterotrophic (and perhaps autotrophic) microflora through the medium of their carcasses, which have been observed to undergo rapid decomposition. In the presence of colorless euglenoid flagellates, Tietjen (1967) found that dead nematodes in culture were decomposed at rates 10 to 20 times faster than in the presence of bacteria alone, and that nematode decomposition by bacteria alone was never complete. He also observed that autolysis of dead nematodes was extremely slow (~2 months). The importance of heterotrophic flagellates in organic matter decomposition and remineralization processes, and the

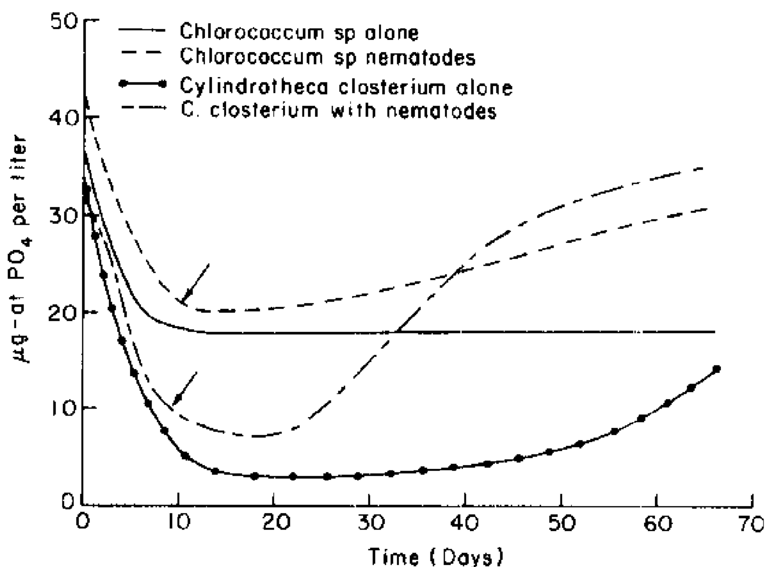

Figure 1. Concentration of dissolved inorganic phosphate in culture flasks containing algae alone (solid lines) and algae plus nematodes (dashed lines). Arrows indicate dates on which nematodes were added to algal cultures. Nematodes were added at initial densities of 25/10 cm^2; after 20 days, densities in flasks were ~1,000/10 cm^2.

value that meiofauna carcasses might have as organic substrates, are unknown. It is more than likely, however, that most meiobenthic animals never die of "old age," but rather are eaten by other animals.

Living and dead nematodes have also been observed to act as a substrate for diatoms (Tietjen 1971). In culture, dead nematodes become rapidly overgrown by diatoms and disappear within days; certain species of live nematodes commonly are covered with ectocommensal algae (Hopper and Cefalu 1973; Tietjen 1971; Wieser 1959), the ecological significance of which is not known.

The potential impact of the meiofauna on the benthic microflora, especially with respect to nutrient dynamics, has been recognized by marine microbial ecologists; it now remains for benthic animal and microbial ecologists to establish the quantitative significance of this impact.

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Some Considerations on the Microbial Transformations of Organic Pollutants: Microorganisms, Degradation, and Toxicity of Complex Mixtures

RONALD M. ATLAS

The task of evaluating the degradability and toxicity of the many organic chemicals and their transformation products that may enter aquatic environments is indeed immense. The task is even greater when we realize that organic pollutants often occur as complex mixtures. The interactions of organic compounds in a mixture influence the toxicity and the degradative fate of the individual contaminating organic compounds. Some interactions of organic compounds lower toxicity; others increase toxic effects. Some interactions accelerate biodegradation; others delay or prevent biodegradation.

For convenience, I would like to illustrate some of the considerations that must be given to environmental contamination by organic mixtures using examples from studies in my laboratory on the fate and effects of polluting hydrocarbons. Hydrocarbons often enter the environment in complex mixtures as petroleum or refined oil products. Each pollution incident represents contamination of the environment with a unique

mixture. The fate and toxicity of individual hydrocarbons in the mixture depend not only on the characteristics of that hydrocarbon but also on the overall characteristics of the mixture. The numerous works on the toxicity and biodegradation of pure hydrocarbons do not reflect what happens in the real situation.

Some processes accelerate microbial degradation within a mixture. Cometabolism clearly plays an important role in the degradation of hydrocarbons in a mixture. Some compounds would be resistant to microbial attack if they had entered the environment alone. For example, we have observed benzanthracene to be degraded by microorganisms in sediment within the context of a petroleum mixture, but not as a pure compound. One must also consider a phenomenon which can be termed "co-availability." The presence of one hydrocarbon may increase the availability of another hydrocarbon. Many hydrocarbons are solids at ambient temperatures, but these hydrocarbons are maintained in liquid form within petroleum by the presence of other hydrocarbons. The greater surface area of the liquid compared to the solid form, together with increased dispersion, favors accelerated microbial attack on these compounds within the petroleum mixture. The increased availability also results in increased toxicity over a greater area for toxic components. This increased availability accelerates mobilization through the environment and may enhance biomagnification through the food web. The addition of organic chemical dispersants to accidental oil spills has the same effect.

In contrast to effects that stimulate degradation, some compounds that normally would be degraded are preserved within a petroleum mixture. For example, the branched isoprenoid hydrocarbon

pristane is readily degraded by many microorganisms. Within the context of a petroleum hydrocarbon mixture, however, pristane frequently is not degraded or is degraded very slowly relative to the degradation of other aliphatic hydrocarbons. This appears to be caused in part by a sparing or diauxie effect. It is well known that microorganisms can preferentially degrade one compound in a mixture before synthesizing the enzymes needed to degrade other compounds in the mixture. Pragmatically, diauxie is the antithesis of cometabolism: cometabolism allows resistant compounds to be degraded; diauxie prevents metabolism of degradable compounds. The ecological significance of the diauxie phenomena has not received sufficient attention.

A hydrocarbon mixture may contain toxic chemicals that are microbicidal or microstatic. Such compounds inhibit biodegradation. We have found that numerous petroleum mixtures contain volatile toxic hydrocarbon fractions that delay the onset of hydrocarbon biodegradation. Many refined oils contain additives that inhibit microbial degradation. Biodegradation of some compounds within a mixture can produce toxic compounds that inhibit biodegradion of other compounds. We have found, for example, that the accumulation of fatty acids from the degradation of some hydrocarbons blocks further degradation of the petroleum mixture. Physical modification and binding can limit degradation. The formation of emulsions called mousse and the later formation of tar balls are a physical-chemical limitation to the availability and degradability of hydrocarbons in petroleum mixtures.

The interactions of organic compounds within a mixture are also interactive with environmental conditions. In the case given above concerning the occurrence of a toxic light hydrocarbon fraction, inhibition of biodegradation

would be important in cold environments where volatilization of this fraction is reduced, but not in tropical regions where volatilization would be expected to occur rapidly. We have observed the preferential utilization of *n*-alkanes over branched alkanes, such as pristane, in temperate environments such as the sediment contaminated by the spillage of the *Amoco Cadiz*. We have, however, found a lack of preferential degradation in cold Arctic marine ecosystems.

The above considerations are certainly not restricted to petroleum. We face a major problem with disposal of toxic organic wastes. Some such mixtures find their way into sewage systems, which already receive high loads of organic matter from domestic sources. We are continually finding sites where barrels of toxic organic wastes have been legally or illegally buried. Many of these sites are leaking toxic compounds into aquatic environments. The mixtures are ill defined, and the interactions that affect toxicity and degradation are totally unknown. Regulations based on scientifically sound investigations are needed to locate safe disposal sites and to restrict them to waste compounds which can be disposed of together. Some combinations of organic chemicals should be prohibited because they increase the potential of environmental threat.

In summary, more consideration needs to be given to the interactions of organic chemicals relative to their fate and effects. Codegradation and sparing effects require increased attention. Such considerations are complex and require multifactorial experimental designs and multivariate statistical analyses. There is a need to combine laboratory studies with field experiments and real situations in the examination of this topic.

Discussion

EFFECTS OF MICROORGANISMS ON ENVIRONMENTAL PROCESSES

MITCHELL: Despite the lateness of the hour, I have a suspicion that there are many people in the room who would like to comment, criticize and discuss.

PRAMER: I want to comment on the statement that aromatic compounds can follow one of two pathways as they undergo transformation: one is opening of the ring followed by degradation of the aliphatic product, and the second is polymerization of the starting substance to what Jim called a humus-like compound. Well, at Rutgers, in studies of degradation in soil of the pesticide propanil, we detected an aromatic intermediate, a dichlorinated aniline, which took the polymerization route, yielding at least 13 different polyaromatic products. Many were azo compounds. Some azo compounds are carcinogenic and others have different unpleasant effects on the human body. So what Jim described is true. You may get a polymerization of aromatic substrates. The products

of the transformation will be of a greater molecular weight and more complex in structure than the starting substance, and they may be humus-like in the sense of being aromatic polymers. However, we ended up with "hazardous humus" rather than with something desirable or beneficial, and we now are very concerned about the persistence in soil of these polyaromatic compounds.

STERN: I would like to raise an issue that Jim Tiedje brought up today. He indicated that he would like to see less work on the effect of pollutants on microorganisms. I don't think we can afford to say that. He indicated that there is no evidence of the ecological significance of pollutants on these organisms. If one considers the length of time that the biogeochemical cycles have been going on in nature and the relatively short time that synthetic materials have been added to the environment, it becomes apparent that there is not yet enough evidence to warrant the exclusion of such tests.

TIEDJE: Perhaps my choice of words was crucial here and maybe I didn't chose them all carefully. Rather than saying less, perhaps a more acceptable position would be to ask for no more expansion of testing effects on pure microbial cultures. My position also reflects my feelings on the difficulty of doing those kinds of tests, compared to the benefit. I would be more favorable towards microbial toxicity testing if we had some good tests that would reliability and sensitively detect the influence of important toxicants in nature over the long term. This may not be feasible, however.

ALEXANDER: This is a very important area inasmuch as considerable time is spent on the effects of toxicants on microorganisms. The field clearly is extremely important in a practical sense. One has to know, when a chemical is introduced, whether

it will have a significant effect. But after the third time its effect is measured, stop. Testing for effects on microorganisms is simple to do and some of our colleagues do the same thing over and over again; this repetitiveness serves as an excuse for people who do not advance new ideas for research but think they still are doing something ecologically important. The first two or three times the tests are run are extremely critical, but not the 15th or 20th.

WOLIN: I would like to address this question to Dr. Alexander. In considering strategies to deal with pollutants, I wonder if you would comment on the possibility of intercepting pollutants at point sources of discharge for treatment prior to their entry into the environment itself. What future research might be accomplished in this area?

ALEXANDER: That is a good point. From the viewpoint of environmental chemistry, there are three major kinds of aquatic environments: marine, freshwater and sewage. Sewage is a unique ecosystem which is important for the control of toxic wastes because many wates, unfortunately not enough of them, do go through a sewage treatment system. We have really little microbiological information on chemical transformations and the effects on the biota in the sewage ecosystem. It is an almost ideal system because it can be manipulated. The chemical exists in high concentrations, and one can manipulate cometabolism. One of the issues that was not addressed this evening is that many populations in nature require growth factors, and the concentration of growth factors for the organisms degrading a synthetic material may be so low that the degradation is limited not by the intrinsic availability of the substrate but by the scarcity of growth factors. The growth factor problem can be overcome in a sewage treatment system. Hence, this is an important ecosystem, one that we should not leave solely to the environmental engineers.

TIEDJE: Perhaps another point I should make on the issue that I was trying to stimulate you on is that I was assuming that tests on higher organisms also would be done. I think they are more sensitive, so the focus should be there, rather than on microorganisms. I was trying to be careful when I said, "in microorganisms or microbial systems," because I think Ralph Mitchell gave an excellent example of how stress on higher organisms allowed microbial disease to develop. Carl Fliermans also has some examples where higher organisms are stressed and microorganisms then assume a disease role.

STOTZKY: I think that one aspect that has not been considered in today's discussion is the ability of the environment, be it soil, waters, sediment, sewage, or what have you, to mediate--either to potentiate or attenuate--an environmental stress. We must consider the physicochemical properties of the specific recipient environment and how they may mediate a stress condition. I am convinced, as undoubtedly most of us are, that pollution is going to continue and that we are in a trade-off situation between the maintenance or increase of our material comfort and the reduction of pollution. Consequently, one of the choices that we should be seriously considering now--not ten or twenty years from now--is to build new energy-producing and other polluting industrial complexes in locations where the ambient terrestrial and/or aquatic environments can tolerate higher levels of SO_2, mercury, cadmium, or other pollutants; i.e., where the environment will potentiate the toxicity of these pollutants by buffering, precipitation, chelation, transformation into less toxic species, etc. I will present some data on such environmental mediation of pollutant toxicity tomorrow.

FLOODGATE: I think one area that has only been touched on is the effect of pollutants on

particulates. I think this also needs to be looked at. We tend to think the answer to pollution is dilution--that's the phrase that is often used. But I think we have also got to wrestle with the fact that if you dilute too much, and get down below the level at which bacteria can attack the material, then we may get the pollutant reconcentrated by being absorbed onto particulate matter, possibly on the bottom, perhaps being deposited over a very wide area of ocean. I think we have to look at what happens when material is absorbed and reconcentrated onto particulate matter and look at the dynamics of degradation in the soil-water interface.

ALEXANDER: I agree completely, George. This is one of the factors I chose not to consider in commenting on environmental factors. We know little about the impact of surfaces. If our extrapolation on 2,4-D nonbiodegradation in aquatic environments is valid, then the oceans should have in them, at low concentrations, every single organic compound that has ever been introduced. This may be true, but I doubt it. I believe that there is a time factor or a surface factor. We also have not seriously considered the chemical properties of molecules that enhance their retention on microbial surfaces. A chemical that is retained on a microbial cell may be more readily degradable, although it is not free in solution. Such factors need to be assessed as part of our evaluation of "environmental factors," which most people take to mean solely temperature, pH, and oxygen. These factors are not enough.

MITCHELL: I think the microbial ecology of surfaces is an area apart from the field of pollution that is fascinating and really we don't know much about.

WOLIN: Another comment--and I'm going to make this a little strong. I am not sure why microbiologists should always have to be concerned with

idiotic strategies for disposing of wastes that are dumped by other people who have absolutely no idea what is going to happen to these wastes microbiologically after they get into the environment. I also think that microbiologists should have significant input in establishing policy for what should and what shouldn't be disposed, and how wastes should be disposed, based on already known microbiology--without having somebody just dump something and then worry about it afterwards. A case in point is PCBs. If I had known what a PCB was before it started to cause a problem, I could have told somebody that it would cause a problem. Organic chemicals are defined, literally, as having tetrahedral carbon, but anything that is highly substituted with a halogen without giving the organism a carbon-hydrogen bond, a carbon-oxygen bond, or a carbon-nitrogen bond to work on has got to be a recalcitrant molecule--or whatever you want to call it these days. The dumping of PCBs, in hindsight, was purely stupid. And now we have to worry about it.

I hope there will be some management in the future that will keep these problems from appearing, because they are purely stupid. There will certainly be enough situations where there are no point sources, where we have to put pesticides on the land. And there will certainly be point-source contaminants, important industrial chemicals that we will have to discharge as trade-offs for the maintenance of our society. We will have enough of both to worry about, but if we assume that we have to deal with all of these monstrosities that are thrust upon us by poor management practices and poor regulatory practices, which I hope are going to be improving, we will not have time to devise new strategies for the future, and we will be relegated to the role of dealing with the unnecessary problems. I think this is something we should try to change our thinking about.

MITCHELL: I don't think that the poor management and regulatory practices come from

stupidity; rather, they result from the people dealing with public policy who are uninformed.

ALEXANDER: The real problem, as I see it, is that it is not stupidity as much as the absence of good science or good management. There are many circumstances in which an industry is doing what is best at that particular time.

Hence, we need to push for good science and to push for good management. At the same time, we do have an obligation as professionals to help clean up the mess that people made, inadvertently or deliberately, at an early phase.

Session III

BIOGEOCHEMISTRY OF THE AQUATIC ENVIRONMENT

Microbial Transformations of Elements in Aquatic Systems

THOMAS D. BROCK

Although energy is dissipated in ecosystems, the chemical elements of the systems usually are not lost, but undergo cyclic transformations. In some parts of the cycle, the element is oxidized, whereas in other parts of the cycle, it is reduced; thus, a biogeochemical cycle may be defined based on the changes in oxidation state which the element undergoes. Although these redox changes may occur spontaneously, they often occur at significant rates only when they are catalyzed by living organisms. Among the living organisms catalyzing redox reactions, microorganisms (especially bacteria) are generally the most important. When discussing biogeochemical cycles, an element of confusion can often arise between the redox cycle, just mentioned, and a transport cycle which can occur when an element moves from one place to another in the biosphere. A transport cycle often involves a redox cycle, since certain oxidation states of many elements are more volatile or more soluble than other oxidation states. Thus, a transport cycle may involve redox, but it need not.

Elements such as phosphorus undergo transport processes without undergoing redox.

Microbial Redox Cycles

We are all familiar with microbially catalyzed redox reactions. Of most significance in any discussion are the redox reactions involving the elements carbon, nitrogen, sulfur, and iron. Of secondary importance, but still of some interest, are cycles involving manganese, mercury, and selenium. I discuss only the major cycles here, since the principles involved are the same for all.

Microbes are only catalysts of chemical reactions. A chemical reaction will not occur in the presence of a microbe that would not also occur in the absence of the microbe, although the rates under the two conditions may be vastly different. Thermodynamic considerations come to the forefront here, and we can predict whether or not a reaction is feasible by carrying out simple and routine thermodynamic calculations. Since any redox reaction requires both an electron donor and an electron acceptor, and since a variety of potential donors and acceptors are present in natural environments, a wide array of reactions are possible.

Considering these donor/acceptor interactions, we come to the obvious fact that, if we are considering the microbial oxidation of a reduced compound, an appropriate acceptor must be present in the environment. Certain donor/acceptor combinations should not occur because of unfavorable free energy yield. Ferrous iron, for instance, will only be oxidized with oxygen as electron acceptor, so if oxygen is absent, ferrous iron should be stable in the environment, even if an appropriate microbial catalyst is present. On the other hand, hydrogen sulfide can couple with either oxygen or nitrate, so its oxidation is less restricted in

nature. Hydrogen gas is the most versatile electron donor, coupling with a wide variety of electron acceptors. Thus we can predict for a given environment whether or not a certain oxidation reaction will occur from an assessment of the electron acceptors that are present in this environment. This does not mean that the reaction will occur, since the situation may be such that an appropriate catalyst (i.e., microbe) is not present. On the other hand, it may be the case that an appropriate microbe is present, but that the environmental conditions necessary for its activity are improper.

It should also be noted that environmental conditions other than those just mentioned must be appropriate if a redox reaction is to occur. The pH of the environment may be inappropriate, the temperature may be too high or too low, or insufficient nutrients may be present for microbial growth. It is likely that no reaction will occur to any great extent unless the organism (or organisms) carrying out this reaction is able to grow in the habitat in question. Of course, reactions may be catalyzed by nongrowing organisms, but it is necessary that at some time the organism did grow and develop a population of sufficient size. The minimum population size that is necessary for a reaction to occur at an environmentally significant rate is not certain, but it seems probable that fewer than 100,000 cells per ml is unlikely to have any significant effect. If only a low population density is present, then it is likely that detrimental factors such as predation and starvation will eliminate the organism from the environment.

Biological Versus Nonbiological Reactions

Since microorganisms are only catalysts, it

seems evident that microbially catalyzed reactions will also occur nonbiologically. In fact, it is well known that many important biogeochemical reactions will occur at quite significant rates under sterile conditions. Two good examples are ferrous iron oxidation and hydrogen sulfide oxidation. At neutral pH, both of these reactions occur quite readily in the absence of microorganisms, although at acid pH values only the microbially catalyzed reactions are of any geochemical significance.

How can we distinguish chemical from biolo reactions? It is sometimes possible to make inferences from analyses of the products being formed (using knowledge gained from more defined systems to help in interpretation), or from measurement of fractionation of stable isotopes (making the assumption that, since fractionation is primarily a low-temperature process, it is likely that it will have occurred at a significant rate in nature only if it is catalyzed by microorganisms). However, the most direct way of studying biogeochemical reactions is by an incubation method, in which a sample of water or sediment is incubated for a period of time under conditions as close to natural as possible, and successive analyses are made to determine changes in a chemical consituent. Given a sufficiently sensitive assay method, a chemical analysis can be used, but in many cases it is necessary to add a radioactively labeled compound and measure its transformation to products. Incubation methods not only permit a direct study of biogeochemical reactions but also make it possible to deduce rates, so that the geochemical significance of a reaction can be determined. But, since chemical transformation in incubated materials may result from nonbiological processes, it is essential to have some method of sorting out biological from nonbiological reactions. The obvious choice here is some sort of poisoned control, in which biological, but not nonbiological reactions are inhibited, so that

the relative importance of the two kinds of processes can be assessed. I have discussed the poisoned control in some detail elsewhere (Brock 1978) and only point out here that the selection of a suitable poison is not simple, as many poisons are also chemically reactive and may either speed up or slow down the reaction under study, irrespective of their effect on microorganisms.

One of the best ways to show that a process is biological is to show that is has a temperature optimum. As temperature increases, the rates of purely chemical reactions increase proportionately, without showing any fall-off, whereas biological reactions show sharp temperature optima and fall off dramatically when the temperature is further increased. This property of biological reactions was first noticed by Arrhenius in the early years of this century, and it has been recognized as the hallmark of a biological process ever since. In a sense, temperature can be considered to be the "poison" that stops the biological reaction without having a significant effect on the chemical reaction. However, it is not the use of high temperature per se, but rather the demonstration of a temperature optimum for a process, that shows that it is biological.

Key Microbial Reactions

In thinking about the kinds of reactions that are almost exclusively microbial, certain reactions come immediately to mind. These are reactions which experience and research have shown do not occur to any great extent in nature chemically. Interestingly, most of these reactions are reductions rather than oxidations.

Reduction reactions that are predominantly microbial:

Carbon dioxide reduction to organic matter

Carbon dioxide reduction to methane
Reduction of nitrogen gas to ammonia (nitrogen fixation)
Nitrate reduction to ammonia
Nitrate reduction to nitrogen gas (?)
Sulfate reduction to hydrogen sulfide
Elemental sulfur reduction to hydrogen sulfate

Oxidation reactions that are predominately microbial:

Methane oxidation to carbon dioxide
Elemental sulfur oxidation to sulfuric acid
Sulfide mineral oxidation

It should also be noted that a number of important photochemical reactions are catalyzed primarily by microorganisms. Indeed, the phototrophic sulfur bacteria are able to catalyze an anaerobic oxidation of sulfide and elemental sulfur to sulfate, a reaction that in the dark will occur only if a highly oxidized electron acceptor is present (nitrate or oxygen). In fact, a completely anaerobic sulfur cycle can be carried out under microbial auspices, using the phototrophic bacteria to carry out the oxidative reactions and the sulfate-reducing bacteria to carry out the reductive reactions. Such an anaerobic sulfur cycle may be quite common in permanently anaerobic marine and freshwater environments. It should be noted, however, that a phototrophic oxidation of ammonia or nitrogen gas to nitrate under anaerobic conditions does not take place (phototrophic bacteria using ammonia or nitrogen gas as electron donors have not been found). Thus, a completely anaerobic nitrogen cycle does not exist in the same manner as the anaerobic sulfur cycle. It is also not clear whether a completely anaerobic carbon cycle exists, since the anaerobic oxidation of methane (an important component of such a cycle) is uncertain.

Conclusion

This necessarily brief presentation is intended merely to serve as a basis for further discussion. It is obviously important, if we are to predict the cycling of elements through the biosphere, to be able to sort out strictly chemical from biological reactions. In only a very few cases has it been shown that a particular geochemical reaction occurs at a significant rate only if a microorganism is present. Indeed, some of the most important reactions from an environmental point of view have not been shown to have a microbial component in nature. Even such a widely studied series of reactions as those involving mercury transformations have not been shown to be microbially catalyzed, although organisms capable of carrying out these reactions can be readily cultured in the laboratory. However, laboratory culture is not the same thing as activity under natural conditions. Biogeochemical cycles must be studied directly in aquatic systems. This is one of the themes addressed by this conference.

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Geobiological Cycles

C. C. DELWICHE

It is customary, in looking upon geochemical and geobiological cycles, to assume that conditions for the recent past represent a steady state that has prevailed for millions of years. The sedimentary cycle and the plate tectonic cycle, although long (with half-lives in the neighborhood of 2.4×10^8 years), are sufficiently short in terms of the total age of the earth that they too can be looked upon as a nearly steady state, although there is evidence of striking discontinuities in these cycles.

It is commonly recognized that the geobiological cycles, and for that matter the sedimentary and plate tectonic cycles, are energy driven. Our information regarding the geobiological cycles is uncertain, most of it gathered from limited field and laboratory observations and reconciled so that estimates of one process can be made to conform by inference with the rates of other processes, and so that the books can be balanced,

not only for a given element, but also for interrelationships of elements as they are cycled through the biosphere, the atmosphere, the hydrosphere, and the soil.

There are certain generalizations that are important to keep in mind when interpreting the processes of these cycles and when attempting to evaluate the significance that human activities may have had on the cycles.

The potency of the biosphere in regulating its own environment is frequently underestimated. It cannot be looked upon as a simple lumped reaction, wherein if the concentration of one or another reactant is changed by human activities, this change will be reflected directly in the concentration of the products at the other end of the reaction chain. The concentrations of oxygen and carbon dioxide in the atmosphere as well as the concentrations of some secondary constituents such as nitrous oxide (N_2O) and various other volatiles are largely determined (some would even say regulated) by the organisms of the biosphere and the totality of their reactions.

The reactions of the biosphere are strongly influenced by climate, and we know that the earth's climate has not been constant through geologic time. The extent to which changes in temperature, rainfall, and atmospheric composition influence biological processes and the extent to which these factors themselves are determined by biological processes are not clear. Although numerous explanations have been offered for recurrent climatic fluctuations, such as the occurrence of ice ages, none of the causes is known for sure; it is possible that the biosphere itself has been responsible at least in part for these episodes.

The "cycling" of mineral elements is

absolutely essential to maintaining the level of biological activity that now characterizes the earth. Were it not for continuing processes of decomposition, which liberate the mineral constituents of plants and animals, the binding of these constituents would soon limit biological activity. The quantity ofcarbon fixed annually by photosynthesis is about 10% of the atmospheric CO_2 content. A number of other elements are likewise closely retained and rapidly cycled.

Biological activity in the oceans and on its fringes is responsible for the production of volatile compounds, which return mobile elements to the land. One example is sulfur, which in the soil as sulfate ion is comparatively mobile, but must be transferred to the atmosphere as hydrogen sulfide or methyl sulfides. These are volatile and are oxidized in the atmosphere and returned to the soil in rain or dry fallout.

The quasi-stable composition of the atmosphere, with its coexistence of molecular nitrogen and oxygen, is biologically determined. Denitrification, which liberates molecular nitrogen to the atmosphere, counters processes that otherwise would transfer most of the oxygen and much of the nitrogen to the oceans as nitrate ion.

Oxygen

One of the remarkable expressions of the potency of this biological activity is the large oxidative capacity, which must have been generated by photosynthesis through geologic time. Starting with an atmosphere that was largely reducing, containing nitrogen as N_2 or ammonia, methane, water and carbon dioxide, an initial very low concentration of oxygen could have been achieved--about .01% atmospheric levels (PAL)--as a result of direct photochemical processes. This

concentration would then have shielded the atmosphere from ultraviolet radiation and limited further reaction. But before any furhter accumulation of oxygen in the atmosphere could take place, the enormous reducing capacity of ferrous iron, hydrogen sulfide, ammonia and other reduced compounds in the ocean would have to have been countered. Thus, the dissolved sulfate ion in the ocean, large deposits of hydrated ferric oxide and an unknown quantity of atmospheric nitrogen derived from ammonia would have to have been produced. This oxidizing power is summarized in Table 1. It amounts to approximately 100 times the equivalent of oxygen contained in the atmosphere (roughly 7 x 10^{19} gram atoms O). A corresponding quantity of more reduced material presumably had to be formed. This also is summarized in Table 1. The carbon of soil organic matter and deposits of coal and petroleum represent a comparatively small fraction of this total. The remainder must have been lost from the earth by escape as hydrogen. These figures emphasize not only the large role that biological processes have had in the shaping of the earth's surface and atmosphere, but also the fact that our present steady-state view of the characteristics of the biosphere and the geochemical properties of the earth in general can be applied with confidence only to conditions of the past 10^9 years or so.

Biological Oxidations and Reductions

The cycles of carbon, oxygen and nitrogen and to a lesser extent phosphorus and iron differ from other biological mineral cycles in that they are active participants in biological energy processes. They provide the coinage of energy transfer. In their reduced form in an aerobic environment, they serve as energy substrate; in their iodized form, they serve as electron acceptors for the

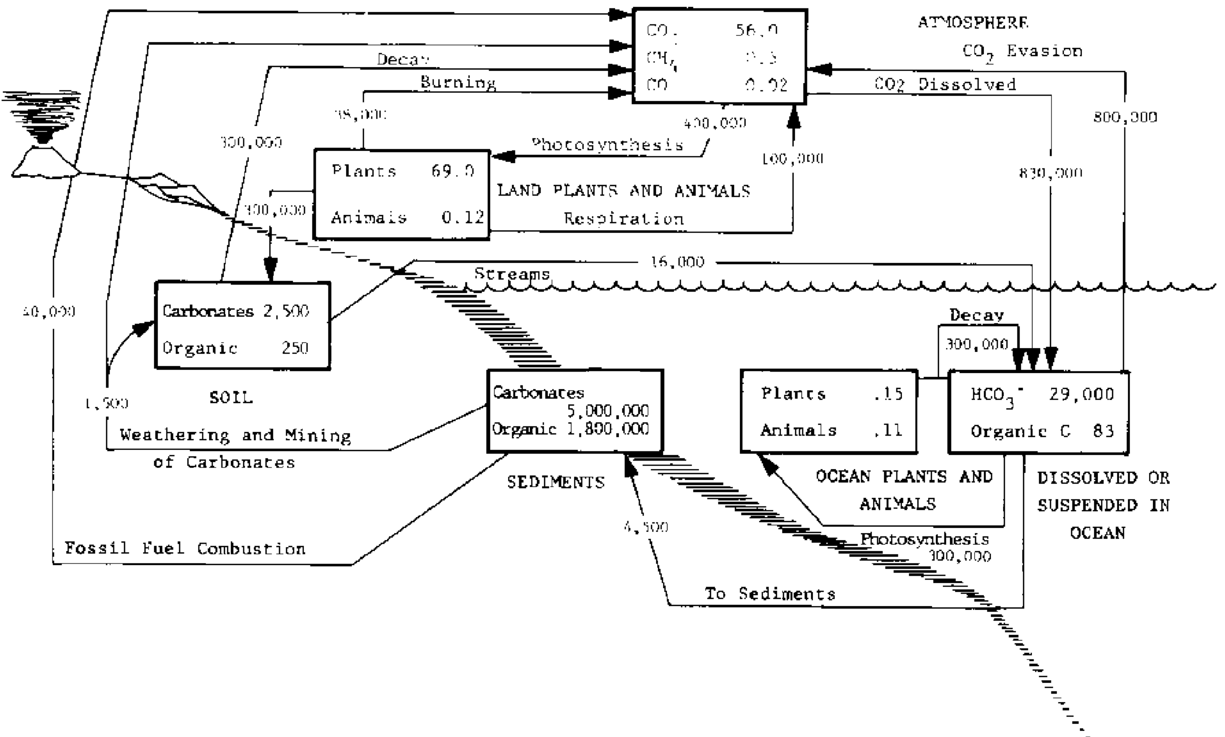

Figure 1. Compartments and annual transfer rates for carbon. Compartment contents are in units of 10^{10} g atoms C. Figures for photosynthesis are net primary production and do not include photorespiration. Figures for land plant and animal respiration are secondary respiration and do not include photorespiration. For ocean organisms "respiration" is included in "decay." The soil compartment includes all unconsolidated materials on the land mass not included in sediments.

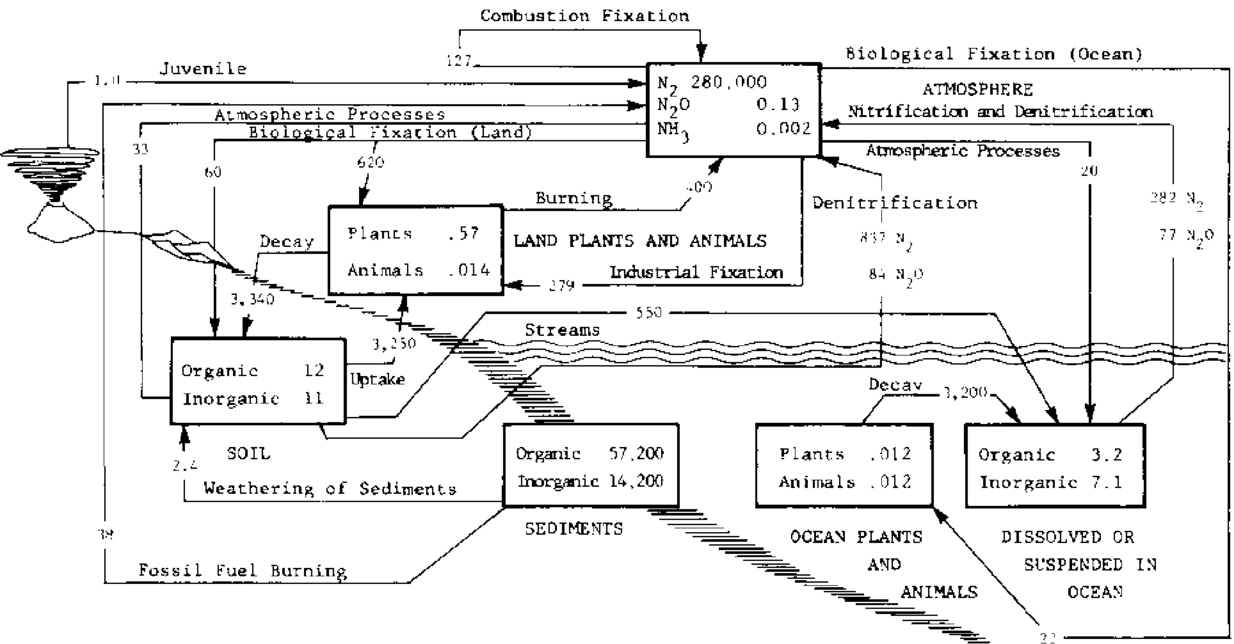

Figure 2. Compartments and transfer rates for nitrogen. Compartment contents are in units of 10^{15} g atoms N, transfer rates in units of 10^{10} g atoms N. The soil compartment includes all unconsolidated materials on the land mass not included in sediments.

oxidation of reduced compounds under anoxic conditions. As a result, their cycles are more active and more complex than are those of mineral elements whose biological roles are largely structural or catalytic. Moreover, carbon, nitrogen and sulfur all form volatile gaseous compounds at normal temperatures; consequently, the atmosphere is part of their cycles. In contrast, other mineral elements are liberated by the decay of organic materials to the soil and soil solution. There they again become available for uptake by plants. The coupling of the atmospheric CO_2 reservoir to the biosphere is close, whereas the atmospheric reservoir of nitrogen is about 10 million times greater than that contained in the living biosphere, and more than 100 million times greater than the annual biological turnover of nitrogen. Atmospheric content of sulfur compounds is comparatively low, but as cited earlier, through the atmosphere is the principal way sulfur is cycled from the sea back to the soil.

Human Influences

The cycles of carbon, nitrogen and sulfur are also those upon which human activities have had the most profound influence. The annual combustion of fossil fuel results in a discharge of carbon dioxide to the atmosphere equal to about 10% of the annual land photosynthesis. Nitrogen fixed industrially, by combustion processes, and by the use of legume crops approximately equals the rate of "natural" nitrogen fixation before human intervention. Sulfur discharged to the atmosphere by fossil fuel combustion is approximately 5 to 10 times that volatized by biological processes.

The significance of these human sources is difficult to evaluate, but some of the consequences can be seen directly andothers inferred

with varying degrees of confidence.

Atmospheric carbon dioxide concentration of approximately 326 ppm on a volume basis varies annually through a range of about 2% of this value, largely as a result of biological activity, but also has increased by about 4% in the past two decades. This increase is about half that expected to result from fossil fuel combustion were not the atmospheric CO_2 in equilibrium with other pools. The movement of carbon dioxide into the oceans in response to a higher atmospheric concentration is to be expected, but some oceanographers argue that movement into the oceanic sink is not rapid enough to account for the difference. It has been suggested that the biosphere has responded with an increased photosynthetic rate, thereby providing an additional sink. This argument has been challenged, however, and even countered with the suggestion that the biosphere itself is serving as yet another source of carbon dioxide because of the clearing of forest lands and their conversion to younger age stands. The carbon stored in mature forests constitutes one of the major pools, and the rate of forest harvest both for lumber and for fuel has greatly increased.

Biogeochemical Succession of Bacterial Activities in Aquatic Sediments

GALEN E. JONES

Bacterial activities and populations have not been studied as extensively in aquatic sediments as in the pelagic water column. This is surprising since the energy available and the potential for regeneration of nutrients from the sediments is higher than in the overlying waters (Bemer 1977; Fenchel and Blackburn 1979). This brief treatise conceptualizes the microbial succession in aquatic sediments.

Oxygen is the terminal electron acceptor during mineralization in aerated soils and the pelagic water column of most of the ocean and nonstratified lakes. In aquatic sediments, reduced microniches, and waterlogged soils where organic matter is deposited at a rate exceeding the supply of dissolved oxygen, mineralization processes become anaerobic (anoxic). This depth denotes the boundary between aerobic and anaerobic metabolism which may be above, but is usually below or at, the sediment-water interface. This aerobic-anaerobic boundary is preceded closely by a redox gradient of

+200 ± 50 mV as the boundary between oxidizing and reducing environments (Hallberg 1978).

Aerobic respiration of organic compounds is the most efficient metabolic process. Under anaerobic conditions, terminal electron acceptors other than oxygen are utilized during biodegradation of organic matter. Based on energetic considerations (Claypool and Kaplan 1974), nitrate, sulfate, and carbon dioxide are the terminal electron acceptors, utilized in order depending upon their availability (Table 1). The maximum energy yield from the metabolic process is indicated as ΔG^0 values (change in Gibbs free energy) for standard conditions (1 M concentration). Under natural conditions, actual free energy changes may differ somewhat. In addition to the respiratory processes (Table 1) where inorganic compounds are used as terminal electron acceptors, fermentation processes where various organic compounds serve in this capacity occur simultaneously. The local environment selects for the specific composition of the microbial population based on their ability to compete for the same organic substrate supply. Thus, a succession of microorganisms is detected in time regulated by depth and electron acceptor availability, with the most efficient microorganisms at the surface and the least efficient ones at depth (Table 1).

Fermentative processes are not competitive at the same level of substrate organization, but are preparatory to respiratory processes (Claypool and Kaplan 1974). It is at this lower level that the potential exists for the competition among microorganisms using different respiratory processes (Mechalas 1974).

Biogeochemical Succession

Carbon, nitrogen, and sulfur are similar to

Table 1. Succession of microbial metabolic processes in aquatic anaerobic sediments governed by energy-yielding, oxidation-reduction reactions.

Metabolic process	Biogeochemical zone	Prominent bacteria	Chemical equation	ΔG^a	Comment
Aerobic respiration	Aerobic zone	Aerobic heterotrophic	$CH_2O + O_2 \rightarrow CO_2 + H_2O$	-686	In organic-rich environments, this zone may be very thin (a few millimeters).
Anaerobic respiration	Redoxocline	Nitrate-reducing and denitrifying	$2CH_2O + 4NO_3^- + 4H \rightarrow 2N_2 + 5CO_2 + 7H_2O$	-579	The redox range varies from +200 mV ± 50 mV (Hallberg 1978). Denitrification occurs below this zone and is extremely narrow to nonexistant in the marine environment due to low NO_3^-.
Anaerobic respiration	Sulfate-reducing zone	*Desulfovibrio*, *Desulfotomaculum*	$2CH_2O + SO_4^{2-} \rightarrow S + 2CO_2 + 2H_2O$	-220	A dominant zone in marine anaerobic sediments due to high SO_4^{2-} concentrations (28mM) and toxicity of free S^{2-}.
Anaerobic respiration	Carbonate-reducing zone	Methanogenic	$2CH_2O \rightarrow CH_4 + CO_2$	-99	Below sulfate-reducing zone in the marine environment, but often predominant in lakes near sediment surface where SO_4^{2-} concentration is low.

[a] Expressed in kilocalories per mole of glucose equivalent oxidized. ΔG, change in Gibbs free energy.

one another in several respects. Assimilative reductions of the oxidized compounds of all three elements occur in the biosphere. These three elements participate in dissimilative oxidation-reduction processes. They are represented with valencies spanning eight oxidation levels: $Ch_4(-4)$ to $CO_2(+4)$, $NH_4^+(-3)$ to $NO_3^-(+5)$, and $H_2S(-2)$ to $SO_4^{2-}(+6)$, the energy potential of the couples decreasing in order.

The relationships between sedimentation and ecology result in three biogeochemical environments characterized by their respiratory metabolism. In an organic-rich marine sediment, these zones are the aerobic zone, the sulfate-reducing zone, and the carbonate-reducing zone (Claypool and Kaplan 1974). When oxygen is exhausted, obligate aerobic bacteria are excluded. Facultative anaerobic bacteria can function fermentatively or anaerobically, substituting nitrate or reduced inorganic sulfur compounds as alternate metabolic processes. Since these electron acceptors are limited, this biogeochemical zone is very narrow (Claypool and Kaplan 1974; Fenchel and Blackbum 1979; Jørgensen 1977). Sulfate-reducing bacteria become dominant under anaerobic conditions as a result of the concentration of sulfate (28 mM) in undiluted seawater and the production of the toxic hydrogen sulfide (Claypool and Kaplan 1974). Substrate limitations probably make an association with a fermenting bacterial population essential (Claypool and Kaplan 1974; Mechalas 1974).

Carbonate or carbon dioxide reduction should replace sulfate reduction when the sulfate concentration of the water in the pores of the sediments is low or depleted. Large amounts of methane do not exist in sediments containing dissolved sulfate, but methanogenesis commences as sulfate disappears (Claypool and Kaplan 1974). This phenomenon may be because of the toxicity of hydrogen sulfide to methane bacteria, or it may be that

sufficient hydrogen gas pressures cannot be maintained for methanogenesis during active sulfate reduction. Hydrogen and carbon dioxide are the preferred substrates for eight species of methane bacteria in pure culture (Wolfe 1971). Hydrogen-producing bacteria, therefore, become essential constituents of anaerobic sediments. Many fermenting bacteria produce hydrogen instead of a more reduced organic compound if hydrogen-utilizing species are available (Iannotti et al. 1973).

Substantiation for *in situ* bacterial sulfate reduction and methanogenesis in these biogeochemical zones can be derived by sulfate and carbon dioxide depletion, respectively, and relative enrichment in the heavier isotopes, ^{34}S and ^{13}C, compared to the preferential ^{32}S and ^{12}C removal in the sulfide and methane (Claypool and Kaplan 1974).

Thus, microorganisms mediate anaerobic mineralization processes in sediments that are dictated by the bioenergetics of the aquatic environment.

Acknowledgment

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Biogeochemical Cycles: Their Complexity and Their Importance in the Mineralization of Wastes

MEYER J. WOLIN

The biology and biochemistry of the biogeochemical cycles are very complicated. Specific reactions require special environments, the biological cycles are intertwined with purely chemical and photochemical reactions, and the overall reactions of the cycles often contain stepwise sequences and minicycles. For example, oxidation of ammonia to nitrate requires an aerobic environment and the interaction of microbial populations that oxidize ammonia to nitrate with populations that oxidize nitrite to nitrate. The presence of significant amounts of easily biodegradable organic matter antagonizes nitrification because heterotrophic rather than autotrophic growth is stimulated and oxygen becomes limiting for nitrification. For denitrification, however, organic matter and anaerobic conditions are exactly what are necessary. In the sulfur cycle, a completely anaerobic cycle occurs when sulfide is oxidized to sulfate by photosynthetic bacteria and the sulfate is reduced back to sulfide in the anaerobic

environment, but aerobically formed sulfate produced by thiobacilli has to return to an anaerobic environment in order to be reduced. There are bacteria that cannot reduce sulfate to sulfide but can reduce tetrathionate and elemental sulfur to sulfide and others that oxidize sulfide to sulfur. These organisms participate in minicycles, i.e., the incomplete oxidation of sulfide and the reduction of the product of incomplete oxidation. The most complex aspect of the cycling of the elements is the catabolism of organic compounds by microorganisms. The diversity of reactions, the microbial food chains involved, and the massive number of different populations and environments that support chemoorganotrophic metabolism represent an exceedingly complex process.

Not all of the reactions of the biogeochemical cycles are known, which is equivalent to saying that not all of the metabolic reactions carried out by microorganisms have as yet been catalogued. New potential for degrading organic compounds continues to be discovered. Recent studies of the sulfur cycle indicate that the ability to reduce elemental sulfur but not sulfate is probably not as uncommon as one would have guessed 5 years ago (Pfennig and Biebl 1976). Recently, Dr. Yoshinari at the New York State Health Department discovered that *Vibrio succinogenes*, which cannot reduce nitrate or nitrite to N_2, can reduce N_2O to N_2. The organism catabolically reduces nitrate or nitrite to ammonia. The significance of this partial reaction in denitrification will have to be determined. The catabolic reduction of nitrate to ammonia carried out by *V. succinogenes* may be more common among microorganisms than was previously supposed (Cole 1978), and the competition of this reduction with N_2-producing nitrate reduction should be investigated. More attention is being given to the significance of nitrous

oxide as a product of nitrification as well as denitrification (Bremner and Blackmer 1978), and we are still seeking reactions that will pin down the biological sinks for CO (Seiler and Schmidt 1976). Chemical reactions such as those from the combustion of fossil fuels and industrial chemicals, along with naturally occurring chemical and biochemical reactions, add to the complexity of the biogeochemical cycles. We will have to continue to study these and the biological systems in order to evaluate fully their impact on the environment.

Because of the complexity of biogeochemical cycles, more effort should be applied to studies that have as their goal the control of biogeochemical activities. In many cases environmental difficulties occur because we depend on microbial populations in the aquatic and terrestrial environment to respond in a more or less uncontrolled manner to whatever compounds we decide are least obnoxious, given a particular economic and environmental scenario. We do have strategies for biologically treating human wastes that attempt to mineralize them and prevent the entry of ogranic material into the aquatic environment. However, we rarely recover anything useful or attempt to solve completely the problem of the ultimate disposition of the elements entering waste-treatment systems. If we treat sewage, we bury the large amount of biomass product somewhere and hope it will go away. Solid wastes are handled in a similar manner if they are not dumped into the aquatic environment. It would make more sense to develop organic waste-treatment processes as a controlled method for mineralization of organic wastes, with accompanying recovery and controlled disposition of the products. There have certainly been some attempts to do this. Aquaculture and physiochemical systems are being developed for purifying effluents from sewage-treatment plants, as well as systems for land application of waste-treatment solid and liquid products and direct

land application of untreated wastes.

In all of these approaches, however, little attention is given to understanding the microbiology of the waste-treatment processes in order to effect better control and increased efficiency. Without knowing very much about the complex microbial ecosystems involved in waste treatment, engineers have been able to effect significant mineralization of input wastes in what has been considered to be an inexpensive process. Waste-treatment systems, however, have mainly been designed to prevent the spread of human intestinal pathogens and to prevent oxygen depletion in aquatic environments that were considered capable of carrying out all remaining purification steps without major disturbance to the environment or public health. The design goal has not been to construct biological factories that convert all input elements into useful products, with the return of pure water to the aquatic environment. A reorientation of the goals, to focus on waste treatment as a biological recovery process might lead to the development of radically new modes of treatment that would be less expensive and less energy intensive and would prevent many of the environmental problems inherent in the present methods.

Immense amounts of material are processed by waste-treatment systems. Human sewage produced in the United States each year would contain about 1.5×10^{12} g of carbohydrate, 8×10^{10} gram-atoms of inorganic and organic N, 1×10^{10} gram-atoms of S and 8×10^{9} gram-atoms of P if the entire population were sewered into conventional sewer systems. The total water flow that would accompany an entirely sewered population would be about 3.7×10^{13} liters per year. New technology based on a better understanding of the microbiology of waste treatment, which is essentially the understanding and control of the mineralization aspects of the biogeochemical cycles, could lead to more

effective use of microorganisms in the conduct of desirable reactions of the geochemical cycle and the development of completely controlled processing of wastes before they enter the environment with uncertain consequences. Strategies applied to mineralize human wastes can be applied to any other wastes such as organic chemical wastes that are point sources of pollution. Prevention of pollution may in the long run be much more cost effective and beneficial than understanding and dealing with the consequences of pollution. Research directed toward understanding the microbiology of mineralization of wastes should contribute as much understanding about basic biological and biochemical events of the biogeochemical cycles as research on uncontrolled environmental systems.

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Physicochemical Factors That Affect the Toxicity of Heavy Metals to Microbes in Aquatic Habitats

GUENTHER STOTZKY*

Industrialization and domestic activities have resulted in the increased mobilization and deposition of toxic amounts of heavy metal pollutants into natural habitats. Heavy metals enter aquatic environments in industrial effluents, discharges from sewage treatment plants, mine water wastes, leachates from landfills, and through wet and dry deposition from the atmosphere. Consequently, lakes, rivers, and estuaries surrounding industrial complexes, such as metal smelters, are frequently contaminated to varying degrees with heavy metals. The probable increased use of low-grade fuels in the current energy crisis will exacerbate such pollution.

Current interest in the state of the environment has stimulated research to establish and define more clearly both the overt and short-term and the covert and long-term (i.e., the invisible injury syndrome) effects of heavy metal pollution on the biosphere. In contrast to the numerous investigations with plants and animals, especially

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with human beings, there has been relatively little research to evaluate the responses of microorganisms to heavy metals, even though microorganisms are dynamically involved in many basic ecologic processes, such as biogeochemical cyclings, energy production (chemo- and photosynthesis), decomposition processes necessary to maintain the fertility of soils and sediments, and many homeostatic activities required for maintenance of the biosphere. Those studies conducted with microbes and viruses have been concerned primarily with determining the concentrations of various heavy metals that cause inhibitory and/or lethal responses in the test organisms, usually in synthetic media. Little research has focused on the effects of heavy metals on microbial activities in natural environments (*see* Babich and Stotzky 1974; Babich and Stotzky 1978; Babich and Stotzky submitted).

Despite the fact that the toxicity of heavy metals is dependent on the physicochemical characteristics of the environment into which they are deposited, there have been few studies on the effects of these characteristics in assessing the detrimental effects of pollution on the biosphere. This paper provides data that demonstrate that abiotic, physicochemical factors do influence pollutant toxicity to microbes and viruses--either by potentiation or attenuation. It will emphasize the importance of considering the characteristics of the specific environment into which pollutants are deposited when predicting or assessing the toxicity of these pollutants.

Environmental Factors

Inorganic Ionic Environment. The cationic and/or anionic components of the recipient environment may influence the toxicity of heavy

metals to microbes and viruses present therein. For example, the toxicity of lead (Pb) to *Tetrahymena pyriformis* was greater in soft (20 mg/1 $CaCO_3$) than in hard (400 mg/1 $CaCO_3$) water, whereas that of mercury (Hg) was greater in hard water (Carter and Cameron 1973), and the sensitivity of *Selenastrum capricornutum* to zinc (Zn) was inversely proportional to the ionic strength of the media (Foy and Gerloff 1972). However, it was not determined whether the toxicities of Pb, Hg, and Zn to the microbes were influenced primarily by the cationic, anionic, or both ionic components of the media.

The cation content of the environment may attenuate (i.e., antagonism) or potentiate (i.e., synergism) the toxicity of heavy metals. The reduction in the toxicity of a heavy metal cation in the presence of another cation, whether metallic or nonmetallic, probably reflects competition between the cations for sites on or uptake by cells. The enhanced toxicity, resulting from the presence of two heavy metal cations and that cannot be attributed to a simple additive effect of the toxicity of each cation individually, is a synergistic interaction. The mechanisms involved in these synergistic interactions have not been defined.

Magnesium (Mg) reduced the toxicity of cobalt (Co), cadmium (Cd), or nickel (Ni) towards *Escherichia coli* (Abelson and Aldous 1950), of Cd, Co, Zn, or Ni towards *Aspergillus niger* (Adiga, Sastry and Sarma 1962; Adiga et al. 1961; Laborey and Lavollay 1973), and of Zn towards the filamentous green alga, *Hormidium rivulare* (Say and Whitton 1977). Calcium (Ca) reduced the toxicity of Zn or Cd to *H. rivulare* (Say and Whitton 1977) and of Cd to *A. niger* (Laborey and Lavollay 1977) and to *E. Coli* (Ohta and Udaka 1977) and reduced the uptake of Cu by *Scenedesmus* sp. (Hutchinson and Collins 1978). The toxicity of copper (Cu)

to *Chlorella pyrenoidosa* was decreased by increasing the concentration of potassium (K) (Steemann-Nielson, Kamp-Nielson and Wium-Andersen 1969).

Ion antagonism also occurs between heavy metal cations. For example, manganese (Mn) reduced the toxicity of Zn to *Lactobacillus arabinosus* (MacLeod and Snell 1950); Zn reduced the toxicity of Cd to *A. niger* (Laborey and Lavollay 1967) and *Euglena gracilis* (Nakano et al. 1978); Cd protected *Physarum polycephalum* against Hg (Chin, Lesowitz and Bernstein 1978); Mn reduced the accumulation of Cd by *C. pyrenoidosa* (Hart and Scaife 1977); Cu and Mn reduced the toxicity of Pb (Christensen and Scherfig 1979) and Cd reduced the toxicity of Cu (Bartlett, Rabe and Funk 1974) to *S. capricornutum*; and iron (Fe) reduced the toxicity of Cu to *C. pyrenoidosa* (Steeman-Nielson and Kamp-Nielson (1970).

Synergistic interactions also occur with heavy metal cations. For example, the toxicity to *Klebsiella aerogenes* of Cd and Zn added together was greater than the sum of the toxicities caused by each heavy metal alone (Pickett and Dean 1976). Synergistic interactions were also noted with Cd and either Co, Cu, Pb, or Zn in the inhibition of *P. polycephalum* (Chin, Lesowitz and Bernstein 1978), with Cu and Ni in the inhibition of *Chlorella vulgaris* and *Haematococcus capensis* (Hutchinson 1973), and with Cu and Mn in the inhibition of *S. capricornutum* (Christensen and Scherfig 1979).

As several different heavy metals are generally emitted from a single pollution source, subsequent deposition of high levels of different heavy metals into a common environment frequently occurs; e.g., lakes surrounding the smelters at Sudbury, Ontario, had elevated concentrations of both Cu and Ni (Stokes, Hutchinson and Krauter 1973), and human activities have resulted in

elevated concentrations of chromium (Cr), Cd, Cu, and Pb in the water and sediment of the Chesapeake Bay (MacLeod and Snell 1950). Thus, recognition of the many possible interactions, both antagonistic and synergistic, among heavy metals is of particular importance in assessing and predicting the impact of heavy metal pollution on the microbiota.

The inorganic, anionic composition of the environment influences the chemical form, and hence the availability of heavy metals to the microbiota. Additions of phosphate (PO_4^{3-}) reduced the toxicity of Pb to *H. rivulare* (Say and Whitton 1977), *Chlamydomonas reinhardtii* (Schulze and Bond 1978), *Fusarium solani*, and *Aspergillus giganteus* (Babich and Stotzky submitted *a*) and of Cu to *Aerobacter aerogenes* (MacLeod, Kuo and Gelinas 1967), probably as a result of the formation of the sparingly soluble salts, $Pb_3(PO_4)2$ and $Cu_3(PO_4)2$. The reduction in the toxicity of Pb to *F. solani* and *A. giganteus* by carbonate (CO_3^{2-}) (Babich and Stotzky submitted *a*) and of Zn to *S. capricornutum* (Hendricks 1978) and of Hg to a mixed rumen microbiota (Forsberg 1978) by sulfide (S^{2-}) was also probably caused by the formation of insoluble salts of the heavy metals (i.e., $PbCO_3$, ZnS, and HgS). The toxicity of filter-sterilized sea water to *E. coli* was also eliminated by additions of S^{2-}, probably because of the precipitation of toxic levels of "heavy metal impurities" in the water (Jones 1964).

Heavy metals may also form complex coordination species with some inorganic anions. Each heavy metal cation has a differential affinity for anionic ligands, and different anionic ligands have differential affinities for the same heavy metal. For example, in sea water, where hydroxyl (OH^-) and chloride (Cl^-) ions are the predominant inorganic anions, Cu occurs as a mixture of Cu^{2+} and $CuCl^+$ (Krauskopf 1956), Cd

as a mixture of $CdCl^+$, $CdCl_2$, and $CdCl_3^-$, Hg as a mixture of $HgCl_3^-$ and $HgCl_4^{2-}$, Pb as $PbOH^+$, and Zn as $Zn(OH)_2$ (Hahne and Kroontje 1978). The different forms of these heavy metals appear to have differential toxicities to the microbiota. The toxicity of $ZnCl_3^-/ZnCl_4^{2-}$ mixtures to *Pseudomonas aeruginosa* and to T1, T7, Ø80, and P1 bacteriophages of *E. coli*, of $ZnCl^+/ZnCl_2/ZnCl_3^-$ mixtures to *A. niger* (Babich and Stotzky 1978), and of $Zn(CN_3^-/Zn(CN)_4^{2-}$ mixtures to T2 coliphage (Kozloff, Lute and Henderson 1957) was greater than that of equivalent concentrations of Zn as Zn^{2+}. However, no differences in the toxicity to *F. solani* and *Cunninghamella echinulata* were noted between Zn^{2+} and $ZnCl^+/ZnCl_2/ZnCl_3^-$ mixtures, and *Trichoderma viride* and *Rhizoctonia solani* were more sensitive to Zn^{2+} than to $ZnCl^+/ZnCl_2/ZnCl_3^-$ mixtures (Babich and Stotzky 1978). *Erwinia herbicola, Agrobacterium tumefaciens*, P1 coliphage, Ø11M15 bacteriophage of *Staphylococcus aureus*, and the marine bacteria, *Acinetobacter* sp. and *Aeromonas* sp., were more tolerant of $HgCl_3^-/HgCl_4^{2-}$ mixtures than of Hg^{2+} (Babich and Stotzky submitted *b*), and Cd^{2+} was more inhibitory to a "mixed microflora" from activated sludge than was $Cd(CN)_4^{2-}$ (Cenci and Morozzi 1977).

pH. The pH of an environment not only determines the physiological states of the indigenous microbial populations, which may affect their sensitivities to heavy metals, but it also influences the ultimate chemical form of heavy metals deposited into that environment (e.g., as the pH is increased, Cd^{2+} sequentially forms the hydroxylated species, $CdOH^+$, $Cd(OH)_2$, $Cd(OH)_3^-$, and $Cd(OH)_4^{2-}$). The different hydroxylated forms of the heavy metals may have both differential toxicities to the microbiota and differential affinities for the surfaces of viral particles, microbial cells, and other particulates, such as clay minerals, hydrous metal oxides, and humic acids.

The toxicity of Cd to *C. pyrenoidosa* decreased as the pH was reduced from 8 to 7 (Hart and Scaife 1977), suggesting a greater toxicity to the alga of Cd^{+} than of $CdOH^{+}$. Conversely, the toxicity of Cd to *A. tumefaciens*, *Alcaligenes faecalis*, *Bacillus cereus*, *Nocardia corallina*, *A. niger*, *T. viride*. and *Rhizopus stolonifer* increased as the pH was increased to alkaline levels, indicating that $CdOH^{+}$ had a greater toxicity to these bacteria and fungi than did Cd^{2+}; the toxicity of Cd to *Streptomyces olivaceus* was unaffected by changes in pH (Babich and Stotzky 1977). *C. pyrenoidosa* tolerated aluminum (Al) better in alkaline than in acidic media, which may have reflected the conversion of Al from a soluble (e.g., Al^{3+} or $Al(OH)^{2+}$) form in the acidic media to an insoluable (i.e., $Al(OH)_3$) form in the alkaline media (Foy and Gerloff 1972). *A. niger* and *T. viride* tolerated Pb better in alkaline media, suggesting a lower toxicity of $PbOH^{+}$ than of Pb^{2+}, which predominated in the acidic media (Babich and Stotzky submitted *a*). The toxicity of Cu and Hg to *Fusarium lycopersici* (Horsfall 1956) and of Cu to *C. pyrenoidosa* (Steeman-Nielson and Kamp-Nielson 1970), *Penicillium nigricans* (Singh 1977), and *Scytalidium* sp. (Starkey 1973) decreased as the pH was decreased, whereas the toxicity of Cu to *Thalassiosira pseudonana* (Sunda and Guillard 1976) and of Hg to *Phaeodactylum tricornutum* (Hannan and Patouillet 1979) decreased as the pH was increased.

Although the pH influences the form of a heavy metal, other inorganic anionic ligands (e.g., Cl^-, sulfate (SO_4^{2-}), nitrate (NO_3^-)) also have affinities for heavy metals and compete with the OH^- ions. For example, $Hg(OH)_2$ is the theoretical dominant form of Hg at pH 8.2, but mixtures of $HgCl_3^-$/ $HgCl_4^{2-}$ and not $Hg(OH)_2$ predominate in sea water as a result of the high concentration of Cl^- ions and the greater affinity of Hg for Cl^-. Conversely, Pb has a greater affinity for OH^- than for Cl^- ions, and $PbOH^{+}$ is the predominant species in sea

water (Haline and Kroontje 1973). In synthetic media containing numerous inorganic anions, a variety of coordination species probably forms, and it is, therefore, difficult to determine the predominant chemical form of a heavy metal at different pH levels.

E_h. Inasmuch as the oxidation-reduction potential (E_h) of an environment influences the form of some inorganic anions (e.g., S^{2-} vs. SO_4^{2-}), the solubility, mobility, and toxicity of heavy metals deposited into that environment can be affected by the prevailing E_h. The E_h also influences the valency of some heavy metals, and different valencies of the same heavy metal can exert differing toxicities to the microbiota. For example, Cu^+ was more toxic to *E. coli* than was Cu^{2+} (Beswick et al. 1976), and Fe^{2+} exerted a greater toxicity and mutagenicity to *E. coli* than did Fe^{3+} (Catlin 1953).

Temperature. Temperature influences both the physiological states and biochemical activities of microbes and the solubility and rate of diffusion of heavy metal cations and their salts. The inhibition of the growth of the alga, *Cyclotella meneghiniana*, by Cr was more pronounced as the temperature was increased from 5 to 25°C (Cairns et al. 1978), and the sensitivity of *Nitzschia linearis* and *Nitzschia seminulum* to Zn (Cairns, Buikema and Parker 1972) and of *Paramecium tetraurelia* to Cu (Szeto and Nyberg 1979) increased with increasing temperature. Conversely, the toxicity and mutagenicity of Fe^{2+} to *E. coli* was greater at 1 than at 37°C (Catlin 1953).

Hydrostatic Pressure. The effect of hydrostatic pressure on the toxicity of heavy metals to the microbiota of most fresh and estuarine water environments, which are relatively shallow, is probably negligible, as the increase in

hydrostatic pressure with depth is approximately one atmosphere (atm) per 10 m of depth. However, hydrostatic pressure appears to influence the sensitivity of marine bacteria to heavy metals. For example, increasing the hydrostatic pressure from 1 to 340 atm increased the toxicity of Ni to Bacterium BIII39, an oxidizer of Mn^{2+} isolated from a ferromanganese nodule collected in the Pacific Ocean; Cu was toxic to the bacterium at 1 and 340 atm, but no toxicity was noted at 272 atm (Arcuri and Ehrlich 1977).

Clay Minerals and Other Inorganic Particulates. At the pH of most natural environments, most clay minerals and hydrous metal oxides have a net negative charge and attract charge-compensating cations (generally protons (H^+), K^+, sodium (Na^+), Ca^{2+}, Mg^{2+}) to their surfaces. Heavy metal cations may displace the natural compensating cations from clays and hydrous metal oxides, possibly as a result of mass action (i.e., a high local concentration) or of a greater affinity between them and these particulates. Heavy metals sorbed to the exchange complex of such particulates are, at least temporarily, unavailable for uptake by the microbiota. Incorporation of the clay minerals, kaolinite or montmorillonite, into synthetic media (Babich and Stotzky 1977*a*) or soil (Babich and Stotzky 1977*b*) reduced the toxicity of Cd to a variety of organisms, including *Bacillus megaterium*, *A. tumefaciens*, *N. corallina*, *Fomes annosus*, *Botrytis cinerea*, *Phycomyces blakesleeanus*, *Scopulariopsis brevicaulis*, *T. viride*, *Thielaviopsis paradoxa*, *Schizophyllum* sp., *Chaetomium* sp., *A. niger*, *Aspergillus fischeri*, *Penicillium vermiculatum*, and *Penicillium asperum*. The reduction in the toxicity of Cd was correlated with the cation exchange capacity (CEC) of the clays, and montmorillonite, the clay with the greater CEC, provided more protection than did equivalent amounts of kaolinite. Incorporation of kaolinite, attapulgite, or montmorillonite into media reduced

the toxicity of Pb to *A. geganteus*, *F. solani* (Babich and Stotsky submitted *a*), *R. solani*, *C. echinulata*, and *T. viride* (Table 1), with the amount of protection also being correlated with the CEC of the clays (i.e., montmorillonite > attapulgite > kaolinite). There appear to be no studies on the effects of hydrous metal oxides in reducing the toxicity of heavy metals to microbes.

Organic Matter. Particulate organic matter also removes heavy metal cations from solution, probably also by cation exchange, and thereby reduces their uptake, at least temporarily, by the microbiota. The CEC of organic matter is usually greater than that of clay minerals (*see* Stotzky 1972). Particulate humic acid reduced the toxicity of Pb to *A. giganteus*, *C. echinulata*, *T. viride*, *Pencillium brefeldianum* (Babich and Stotsky submitted *a*), *R. solani*, and *F. solani* (Figure 1). The toxicity of Hg to the anaerobic bacteria, *Bacteroides* sp. and *Clostridium* sp., was removed by the incorporation of a sediment with a high organic matter into the media (Hamdy and Wheeler 1978).

Simple soluble organic matter reduces the toxicity of heavy metal pollutants, as the complexed forms of heavy metals are, in general, less readily accumulated by and, thus, less toxic to microbes. The toxicity of Cu to *Chlorella* sp., *Scenedesmus* sp. (Stokes and Hutchinson 1975), and *P. tricornutum* (Bentley-Mowat and Reid 1977), of Cd to *C. pyrenoidosa* (Hart and Scaife 1977) and *E. coli* (Ohta and Udaka 1977), and of Cd and Zn to *K. aerogenes* (Pickett and Dean 1976) was reduced by ethylenediaminetetraacetate (EDTA), a synthetic chelating agent. The rapid death of *E. coli* in filter-sterilized sea water was eliminated by the incorporation of EDTA or other synthetic chelators, such as 8-hydroxyquinoline, thioglycolic acid, and o-phenanthroline, into the

Table 1. Influence of kaolinite (K), attapulgite (A), or montmorillonite (M) on mycelial growth of fungi.

Treatment[a]	Mycelial growth rate[b] (% of control)[c]					
	Rhizoctonia solani		*Cunninghamella echinulata*		*Trichoderma viride*	
No clay; no Pb	7.1±0.33	(100±4.7)	8.0±0.36	(100±4.5)	17.2±0.24	(100±1.4)
No clay + Pb	0	(0)	1.8±0.09	(23±1.1)	1.9±0.41	(11±2.4)
1% K	7.3±0.38	(100±5.3)	7.1±0.11	(100±1.6)	16.4±0.15	(100±0.9)
1% K + Pb	0.6±0.18	(9±2.5)	2.1±0.08	(29±1.1)	2.3±0.25	(14±1.5)
1% A	7.0±0.32	(100±4.6)	8.8±0.25	(100±2.9)	15.4±0.18	(100±1.2)
1% A + Pb	2.0±0.29	(29±4.1)	5.1±0.39	(58±4.4)	2.6±0.19	(17±1.3)
1% M	7.2±0.49	(100±6.7)	8.2±0.42	(100±5.2)	18.0±0.35	(100±1.9)
1% M + Pb	2.8±0.22	(39±3.0)	7.7±0.32	(94±3.9)	4.5±1.10	(25±6.1)

a Pb, as $Pb(NO_3)_2$, was added to medium containing 1.0% glucose, 0.5% neopeptone, 0.1% NH_4NO_3, 1.5% Bacto-agar, without or with clay, and adjusted to pH 5. With *R. solani* and *C. enchinulata*, 250 μg/ml Pb was added; with *T. viride*, 1,500 μgl/ml Pb was added. Cation exchange capacity (meq/100 g clay) of K = 5.8, of A = 34.0, and of M = 97.7.

b Mean radial growth, in mm/day ± standard error of the mean.

c Mean percent of control ± standard error of the mean (control = no Pb + an equivalent concentration of clay).

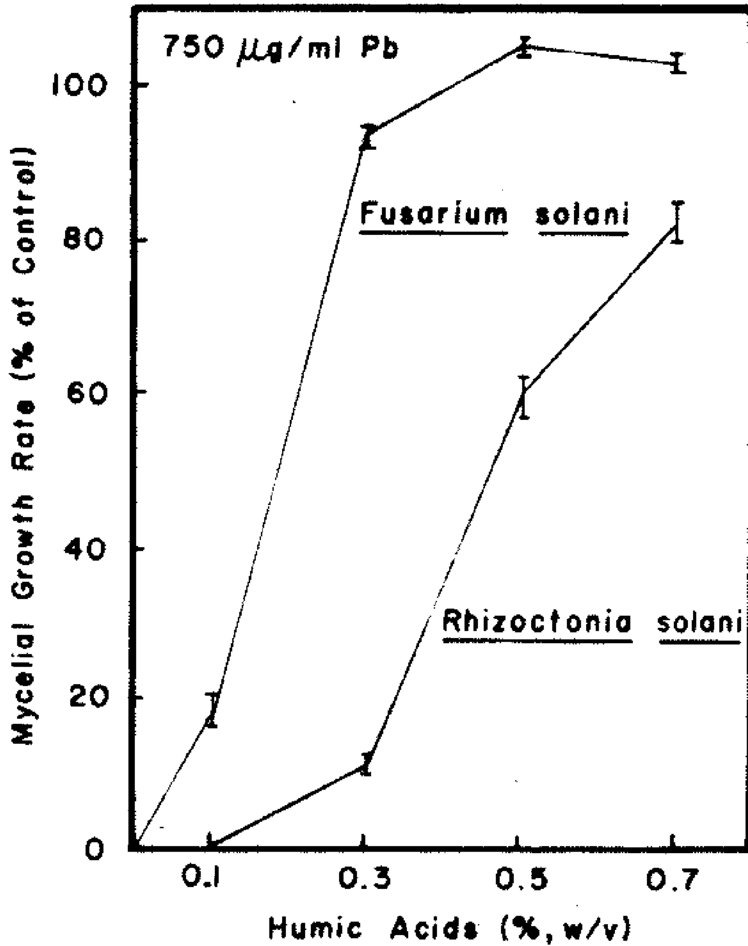

Figure 1. Effect of increasing concentrations of humic acid on the mycelial growth of *Fusarium solani* in the presence of 750 µg/ml Pb. Lead, as $Pb(NO_3)_2$, was added to an acidic minimal nutrient agar medium (see Table 1 for composition) containing 0, 0.1, 0.3, 0.5, or 0.7% humic acid (Aldrich Chemical Co., Inc.). Data are presented as the mean percent of control ± standard error of the mean, based on control plates which contained equivalent concentrations of humic acid but no Pb.

water (Jones 1964; Jones and Cobet 1975). Natural chelators, particularly the dicarboxylic acids, also reduce heavy metal toxicity: e.g., citrate and aspartate lessened the toxicity of Cd and Zn to *K. aerogenes* (Pickett and Dean 1976), malate reduced the toxicity of Cu to *Candida albicans* (Avakyan and Rabotnova 1971), and succinate reduced the toxicity of Pb to *R. solani* *F. solani*, *A. giganteus*, and *C. echinulata* (Babich and Stotsky submitted *a*). Cysteine reduced the toxicity of natural sea water to *E. coli* (Jones 1964; Jones and Cobet 1975; Scarpino and Pramer 1962), of Hg to *Aeromonas* sp., *A. tumefaciens*, and the Ø11M15 bacteriophage of *S. aureus* (Babich and Stotsky 1979), of Pb to *F. solani* and *A. giganteus* (Babich and Stotsky submitted *a*), and of Cu to *A. aerogenes* (MacLeod, Kuo and Gelinas 1967).

Complex soluble organic matter also interacts with heavy metals and, thereby, reduces their availability for uptake by the microbiota. Different complex organics have differing affinities for heavy metals, e.g., the capacity to complex Pb was casamino acids >> yeast extract > tryptone > peptone > proteose peptone (Ramamoorthy and Kushner 1975). The toxiticy of Pb to *C. echinulata* was greater in media containing peptone or neopeptone than in those containing equivalent concentrations of yeast extract or tryptone, probably reflecting the greater affinity of the yeast extract or tryptone for Pb (Babich and Stotsky submitted *a*). Yeast extract reduced the toxicity of Cu to *A. aerogenes* (MacLeod, Kuo and Gelinas 1967), tryptone reduced the toxicity of Hg to *Clostridium* sp. and *Bacteroides* sp. (Hamdy and Wheeler 1978), and proteose peptone reduced the toxicity of methyl Hg to *T. pyriformis* (Hartig 1971).

The studies reported herein were conducted primarily in synthetic media and show that the *in vitro* responses of microbes and viruses to

heavy metals are influenced by the physicochemical characteristics of the media. These studies also emphasize the importance of selecting proper media for the isolation and enumeration of microbial populations resistant to heavy metals from natural environments. The use of rich nutrient medium, amended with heavy metals, for maximum isolation of microbes tolerant to heavy metals may yield an overestimation of tolerant populations as a result of the complexing and detoxification of the added heavy metals by the inorganic and organic constituents of the medium. Conversely, the use of a nutrient poor medium, to avoid this masking of the effect of the added heavy metals by the constituents of the medium, may not be adequate to support growth of fastidious but heavy metal-tolerant microbes, thus yielding an under-estimation of those microbes tolerant to heavy metals.

For example, a concentration of 1,000 μg/ml Pb inhibited growth of *F. solani* and *C. echinulata* on an acidic, minimal nutrient agar (AMNA), but an equivalent concentration of Pb was not inhibitory when these fungi were grown on tomato juice agar (TJA), Sabouraud dextrose agar (SDA), or Czapek solution agar (CSA) (Table 2). *R. solani* was more tolerant of 1,000 μg/ml Pb on TJA, SDA, or CSA than on AMNA. This fungus tolerated Pb better on TJA than on SDA, the more acidic medium with less organic matter, and the toxicity was reduced most on CSA, an alkaline agar containing PO_4^{3-} amendments (Table 2). Studies evaluating the toxicity of Pb, Zn, and Cd to mycelial growth of the nematode-trapping fungi, *Arthrobotrys amerospora*, *Arthrobotrys conoides*, and *Monacrosporium eudermatum*, also showed that the type of medium used influences the toxicity of the heavy metals (Rosensweig and Pramer personal communication).

Table 2. Influence of the assay medium on the response of fungi to 1,000 μg/ml Pb.

Fungus	Treatment	Mycelial growth rate[a] (% of control)[b]: Minimal nutrient agar[c]		Tomato juice agar[d]		Sabouraud dextrose agar[e]		Czapek solution agar[f]	
Rhizoctonia solani	No Pb	8.4±0.46	(100±5.5)	13.0±0.35	(100±2.7)	12.2±1.08	(100±8.8)	11.4±1.02	(100±9.7)
	Pb[g]	0	(0)	10.9±0.84	(84±6.5)	6.7±0.13	(55±1.1)	12.3±0.55	(108±4.8)
Fusarium solani	No Pb	4.9±0.20	(100±4.0)	4.9±0.09	(100±1.8)	4.3±0.11	(100±2.4)	4.6±0.23	(100±5.0)
	Pb	0	(0)	4.7±0.10	(97±2.1)	4.4±0.09	(101±2.0)	4.5±0.13	(97±2.8)
Cunninghamella echinulata	No Pb	9.0±0.54	(100±6.0)	12.7±0.41	(100±3.2)	14.1±1.26	(100±9.0)	8.9±0.55	(100±6.2)
	Pb	0.6±0.29	(7±3.2)	12.9±0.07	(101±0.6)	13.9±1.88	(99±13.1)	9.0±0.33	(101±3.7)

a Mean radial growth, in mm/day, ± standard error of the mean.
b Mean percent of control ± standard error of the mean.
c Minimal nutrient agar consisted of 1.0% glucose, 0.5% neopeptone, 0.1% NH_4NO_3, and 1.5% Bacto-agar, adjusted to pH 5.0.
d Tomato juice agar (Difco) consisted of 2% tomato juice, 1.0% peptone, 1.0% peptonized milk, and 1.1% agar, pH 6.1.
e Sabouraud dextrose agar (Difco) consisted of 4.0% glucose, 0.5% casein peptone, 0.5% meat peptone, and 1.5% agar, pH 5.6.
f Czapek solution agar (Difco) consisted of 3.0% saccharose, 0.2% $NaNO_3$, 0.1% dipotassium phosphate, 0.05% potassium chloride, 0.001% ferrous sulfate, and 1.5% agar, pH 7.3.
g Pb added as $Pb(NO_3)_2$.

Conclusion

Although most of these investigations were performed in synthetic media, the data show that the physicochemical characteristics of an environment can attenuate or potentiate the toxicity to microbes and viruses of heavy metals deposited into that environment. Furthermore, it is apparent that the total content of heavy metals in an environment should not be used as the sole criterion for predicting the suitability of that environment for the microbiota; rather, such predictions should take into consideration the relative amounts of "free" vs. "fixed" metals, the chemical forms of the metals, and the availability of the metals to the microbiota.

These data also have broad implications for the criteria currently employed in establishing national pollution emission standards or guidelines. These guidelines are now based primarily on the maximum tolerable concentrations of a pollutant, without adequate consideration of the physicochemical (and biological) characteristics of the environment into which the pollutant is eventually deposited. Deposition of a pollutant into one type of aquatic environment may yield different biotic responses than deposition into another type of environment (e.g., consider the physicochemical differences between lakes, rivers, estuaries, and oceans). Consequently, guidelines based solely on emission concentrations are inadequate for all geographic regions.

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Discussion

BIOGEOCHEMISTRY OF THE AQUATIC ENVIRONMENT

IVANOVICI: Question for Dr. Stotzky. With the experiments you did, you demonstrated rather nicely that the addition of organic materials can, in fact, ameliorate the effect of heavy metals. Presumably these experiments were done with cultures. Have you done any of these experiments in the field? What would happen in a field situation?

STOTZKY: Most of these studies have been conducted in laboratory media with pure cultures. However, studies in sterilized soils with pure cultures have shown that the toxicity of cadmium and lead is reduced when the natural organic matter content of the soils is higher or when it is increased by additions of humic acids. Some of our current studies indicate that nonsterilized soils amended with humic acids or compost derived from sewage sludge can "tolerate" considerably higher levels of heavy metals than nonamended soils before microbial activity is impaired. Field studies in Germany with composts from sewage sludge indicated that even though the composts contain relatively

high concentrations of heavy metals, the metals are so tightly bound that they are not water soluble and therefore are not available for uptake by plants. For example, when the uptake of heavy metals by carrots, which are notorious accumulators of heavy metals, was compared in control soil, the same soil amended with sewage sludge, and the soil amended with composted sewage sludge, the levels of heavy metals in the carrots was even less in the compost-amended soil than in the control soil. This indicated that not only were the heavy metals in the compost not available for plant uptake but that the compost further reduced the availability of the heavy metals naturally present in the control soil. Although many more *in situ* field studies are obviously necessary, these preliminary investigations suggest that it may be possible to reclaim soils heavily polluted with heavy metals by the addition of compost or other highly stabilized organic materials, as well as by the incorporation of clay minerals with a high cation exchange capacity.

HIRSCH: I have a question for Dr. Wolin. You said that the majority of the carbon monoxide in the atmosphere is now thought to come from the photooxidation of methane. I would like to point to the increasing number of studies by Seiler (J. Geophys. Research 75:2217-2226, 1976) and others that indicate carbon monoxide comes from the ocean and has biogenic origins, for example, through the breakdown of compounds of heme, quercitol and flavonol. This is quite significant.

DELWICHE: I hope, Guenther, that you share with me the anxiety about the CO_2 in the atmosphere. I'm thinking of your comments in regard to the coal-burning power generating plants. I don't think we know which is the lesser of several evils, but burning a lot of coal and dumping a lot of CO_2 and SO_2 into the atmosphere I don't like either.

STOTZKY: If I had the option to choose between nuclear and coal-burning energy generating plants, I would opt for the latter because I think that the biota, including us human beings, has a better chance of adapting on ecologic and population levels to an increase in SO_2 and possibly CO_2 (in terms of the "greenhouse effect") than to radionuclides with half-lives in the thousands of years--which will not allow a helluva lot of chance for adaptation. I obviously share with you the anxiety about increasing the levels of SO_2 and CO_2 in the atmosphere. However, I am a realist about this, as I am certain that you are, and the popular trade-off choice is going to be in favor of continued energy generation and higher standard of living to the detriment of the environment. Consequently, we have to opt for the lesser of the evils, which, in may opinion, is increased levels of SO_2 and CO_2 rather than a wide and indiscriminante distribution of radionuclides. As I have suggested earlier, a possible partial resolution to this pollution problem is to site energy generating and other industrial plants in locales where the recipient environments can "buffer" the pollution effect.

WOLIN: Question to anyone. I think in terrestrial environments there is a tremendous amount of microbial processing of organic and inorganic materials in the intestinal tracts of both humans and animals. Ruminants do a lot, we do a lot, termites fix nitrogen and so on. I have never really seen this discussed in terms of the aquatic environment. Is there any perspective of the role of any aquatic animal intestinal tracts in the aquatic environment? Do they have intestinal microflora?

COLWELL: This answer is, of course, aquatic fauna do have an intestinal microflora. A lot of work has been done on fish microbiology and on bacteria found in and on marine and fresh water invertebrates over the last 40 or 50 years. Classic

work in this area was done in Aberdeen, Scotland, by Shewan's group. There they have demonstrated that *Vibrio* spp. often predominate in the gut of fish. A specific gut group *Vibrio* was described by Liston, who found these organisms in the gut of flat fish and related fish. Work done in my laboratory by J. Deming and P. Tabor, and J. Swartz shows that a pressure tolerant intestinal flora associated with amphipods in the deep sea is capable of functioning very well under deep sea conditions of high hydrostatic pressure. The problem is that not enough work has been done in this area of aquatic microbiology.

STALEY: I think there is an interesting tie-in here regarding the anaerobic metabolism that occurs in sediments, such as hydrogen utilization by anaerobic organisms, that is similar in many respects to what goes on in the rumen, for example.

I'd like to ask Tom Brock a bit more about this methane-oxidizing organism that presumably nitrifies. This is the first I've heard of this sort of thing. I've heard of nitrogen-fixing methane-oxidizing bacteria before, but not nitrifying.

BROCK: I don't think this is a new thing. As I understand it, every methane oxidizer will oxidize ammonia. This has been published by Roger Whittenbury. Additionally, Michael Higgins has shown that methane oxidizers are capable of oxidizing a wide variety of other substrates. The methane oxidizers do not grow on ammonia or these other substrates, but co-oxidize them. In ammonia oxidation, the relationship to methane is even closer because ammonia oxidation in nitrifiers is inhibited by methane, as shown by Suzuki. Thus, it is likely that ammonia and methane compete for the same enzyme systems. Although the phenomenon is not new, what is new is the possible significance of this in the aquatic environment. Both methane and ammonia are products of anaerobic decomposition and

accumulate in anaerobic environments. However, there is a lot more energy available from methane oxidation than ammonia oxidation, so methane oxidizers might form higher population densities and thus be able to coincidentally oxidize ammonia. In lakes, high population densities of methane oxidizers develop at the thermocline where, in Lake Menota, we see evidence for nitrification.

STALEY: It's amazing, considering the amount of methane utilization you showed on the graph, that you ever get any bubbles coming out of ponds at all.

BROCK: You don't see bubbles out of deep lakes, only shallow ones. I'm talking about deep lakes.

GEESY: I would like to comment on Dr. Stotzky's discussion of heavy metals. Metals which enter the aquatic environment are in many instances rapidly transported to the sediments. Within the sediment, the metals appear to be primarily associated with the particulate phase. In polluted sediments, organo-metal complex reactions are particularly important. In fact, the quantities of some metals that become associated with the particulate organic fraction are several orders of magnitude greater than the quantities of dissolved metal in the interstitial or overlying water. Sediment microbes quite likely contribute to the formation and fate of these organo-metal complexes. Studies in this area could greatly improve our understanding of the transfer of heavy metals through aquatic systems.

AUDIENCE: I can't fail to respond at this point to Guenther's remarks because we worked for a number of years on why nonmarine bacteria die off in sea water. There are numerous explanations for this, but the one I think is exceedingly important is heavy metal toxicity. As I mentioned earlier,

the amounts of metals in open water are only a few parts per billion for most of the transition metals, or 1 to 10 micrograms per liter. You might wonder if these metals are toxic. I think the reason they are toxic, or slightly toxic, to nonmarine bacteria is that there is little complexing because the level of organic matter is less than 1 milligram per liter. I think heavy metal ions react with organic compounds and with some of the major inorganic ions like chloride. Most of these things are being worked on with complex media and in complex systems, using peptone or triptone. It's very hard to unravel which of the many compounds in an organic milieu like tomato juice are going to complex a metal ion. But if you simplify the system to use a simple substrate like glucose, which has little kelating capacity, then you can work this out on reasonably stoichiometric grounds. These interactions are exceedingly important. With very small amounts of organic matter, the relatively lesser metals would become toxic. More organic matter takes a lot more metal to become toxic. Simplistically put, that is my concept of what is going on in sea water. A soil is even more complex, so maybe Guenther would like to comment on that.

STOTZKY: I don't think this is a simplistic situation, but rather an extremely complex one that requires not only intelligent experimentation but an appreciation of the many possible alternatives that can occur in natural habitats. For example, the form of a heavy metal in seawater is different from that of the same metal at the same pH but at a lower chloride concentration, or at the same chloride concentration but at a different pH. The relative toxicity of these different hydroxylated or chlorinated species varies depending on the heavy metal and its affinity for these and other ligands. In addition to this speciation, the type of organic matter present, whether living or dead, will affect the availability of the metals. Furthermore, as George Floodgate alluded to last

night, the presence of reactive surfaces, both organic, including living and dead cells and other organic debris, and inorganic, such as clay minerals, which act as either concentrating factors or as scavengers, can decrease the effective concentration of a toxicant in the aqueous phase. For example, a heavy metal in a positively-charged state will readily adsorb to the negatively-charged exchange complex of clay minerals, organic matter, and microbial cells. The adsorption to clays is reversible, but until desorption occurs, the amount of heavy metal available to do damage is reduced. Consequently, if the heavy metal is present as a negatively-charged hydroxylated or chlorinated ion, it will not adsorb, but its toxicity may be less than that of the divalent cation. Because of these many permutations, the biological effect of a heavy metal in a natural environment is difficult to predict.

BROCK: I'd just like to point out that talking about marine environments means talking about putting organic matter into sediments overlaid by water containing large amounts of sulfate. Sulfate reduction will occur, and sulfide will build up. Because of the solubility product of metal sulfides, things such as mercury, cadmium, zinc, and copper will be taken completely out of the system. I suspect that sulfide precipitation should be much more important than chelation in controlling heavy metals in marine sediments. However, in freshwaters the situation is different, because the sulfate concentrations are so much lower.

WHITE: There is a marked induction of poly-B-hydroxybutyrate (PHB) synthesis in the estuarine detrital microbiota, an interesting way of showing the binding of essential metals by fulvic acids in runoff waters. The binding produces unbalanced growth, i.e., inhibited protein synthesis, depressed PHB catabolism and rapid PHB synthesis, so despite the precipitation of the organic chelating

fulvic acids by the sea water, there is still sufficient chelation capacity to induce accumulation of PHB in the estuarine detrital microbiota.

KONOPKA: My question was prompted by Dr. Jones' slide. Typically one sees the hierarchy of organisms that are involved in anaerobic respiration based upon thermodynamic considerations. Can one come up with a scheme to explain the competitive advantage of one organism against another based solely upon these thermodynamic considerations, or does one have to consider the kinetics of substrate affinity of these organisms?

AUDIENCE: I'm glad you asked that. Obviously substrates are very important. In the case of sulfate-reducing bacteria, the geochemists are quite convinced that lactate, for example, or four-carbon compounds like isobutanol or something of this type are essential as substrates. I think Postgate's work would indicate that getting the pure culture, studying all the dehydrogenases of sulfate-using bacteria is exceedingly difficult. More work might be done on that. I think the geochemists are feeling restricted to lactate and four-carbon organic acids and this type of thing in the case of hydrogen-ion, sulfate-using bacteria because many strains can use hydrogen for energy, but they need some other growth factors as building blocks--in addition to needing hydrogen for energy. But those are the major substrates and obviously they must be present. That is an important point, but how many different strains of sulfate-using bacteria can knock hydrogen off of how many different types of compounds remains an interesting question. Perhaps not all the data is in on that.

BROCK: I think I can explain kinetics on thermodynamic grounds in such a situation. The more energy available from a compound, the higher the cell yield of an organism growing on the compound. Then, since the organism is only a catalyst,

if the reaction rate is catalyst-limited, the more catalyst the more rapid the reaction rate. Thus a thermodynamic scheme can be converted into a kinetic one.

DELWICHE: I'd like to comment on that element also. Many of these processes from which we can calculate thermodynamic yield still have a quantum phenomenon in them that consists of the least common denominator of exchange, which is ATP. You can turn the thing over and say, almost as a truth, that anytime there occurs ubiquitously in nature some reaction in which an excess of 15 kilocalories per mole can be extracted, some enterprising microorganism has arisen to exploit that asset. We see such things as cyanide being oxidized by organisms that are aerobic and have a cytochrome system. We see all sorts of elegant expressions of this and the only exceptions are things which we can explain away. Amorphous carbon, for example, persists in nature for a long period of time. It is exceedingly insoluble and therefore not very available. But enterprising organisms have devised means of solubilizing and getting some insoluble things, like sulfur, into the system. The second point with regard to the thermodynamic yield is that sometimes there are kinetic phenomena such as the activation of energy of a molecule involved. For example, the oxidation of atmospheric nitrogen with oxygen, under the right circumstances, is thermodynamically sound, but the activation energy of the dinitrogen molecule makes it a forbidden reaction. Even using hydrogen as an electron donor and nitrogen as an electron accepter for the oxidation of some organic compounds would make a lot of sense, but you have that barrier of the activation of the energy of the molecule and it doesn't go.

BARTHA: I would like to add a comment to Dr. Stotzky's and Dr. Brocks' talks about the relative toxicity of heavy metals in aquatic environments depending upon the nature of the environment. With

one of my students, Jim Blum, I looked at the effect of salinity and other environmental factors on the methylation of mercury in estuarine environments. Our most significant finding was that salinity very dramatically influences the rate of mercury methylation. As the salinity goes up, methylation goes down. We have not proven an exact mechanism, but I think it's reasonable to assume that high salinity together with high magnesium-sulfate concentration will give rise to H_2S. The H_2S will complex the mercury, the mercuric ions, and remove them from the methylation process. The issue may have been confused by the fact that the first dramatic mercury poisoning incidents took place in estuarine environments near Minamata and Niigata Bays. In these cases, there was direct methyl mercury discharge into the bays. It seems now that mercury potentiation by the biological methylation process will be much less of a problem in saltwater and estuarine environments than has been projected from freshwater studies.

WOLIN: I just want to add a few comments to that discussion. I think many of you are aware of Bryant's work which showed the interaction of *Desulfovibrios* with methanogens. The *Desulfovibrios* can grow independently on electron donors, such as ethanol, and electron acceptors, such as sulfate, but grow on ethanol and CO_2 only in the presence of a methanogen that can use the hydrogen produced by the *Desulfovibrios* from the ethanol. This pulls the ethanol-to-acetate reaction of the *Desulfovibrios* by removing hydrogen from the environment; that is, the methanogen removes hydrogen from the environment and reduces CO_2 to methane. The potential ATP available from sulfate reduction is probably greater than the ATP available from just going from ethanol to acetate for the *Desulfovibrio*. *Desulfovibrio* takes both the electron acceptor and its electron donor with ethanol and sulfate and the electron flow occurs internally, whereas when it interacts with the methanogen, it exports hydrogen.

The latter is a more difficult physical process. If the two species were in an ecosystem, sulfate reduction would probably be preferred by the *Desulfovibrios*. I don't really know what the competition would be like if H_2, CO_2 and sulfate were given to a hydrogen-using sulfate reducer and a hydrogen-using methanogen, where both had a chance to take in the electron donor and electron acceptor that each could independently use.

ZEIKUS: This is taken from published ecological data. When one studies the effect of carbon and electron flow in a freshwater sediment system with the presence of added excess hydrogen and excess sulfate, then the processes of sulfate reduction and methane formation occur concomitantly. Likewise, at the low SO_4 concentrations normally found in sediments, both methane production and sulfate reduction occur, driven mainly by hydrogen or acetate metabolism.

Session IV

MICROBIOLOGICAL METHODS FOR ENVIRONMENTAL ASSESSMENT

The Assessment of Marine Microbial Biomass and Activity

GEORGE D. FLOODGATE

In very general terms, the work of the ecologist is to examine a particular environment, count the number of individuals in each species that make up the population, and determine their activities and their interactions with both related species and the rest of the bios. Stated this way, the problems of the microbial ecologist are obvious and difficult. The uncertain state of microbial and especially bacterial taxonomy makes enumeration of each species almost impossible, except in a few well-defined instances. As a result, the microbial ecologist has to rely on methods which attempt to measure the biomass or activity of a very large conglomerate of organisms, such as measuring phytoplankton by chlorophyll or bacteria by muramic acid.

This paper is concerned with examining the methods available at the moment for estimating the activity and biomass of marine bacteria and, to a lesser extent, of algae. The problems that beset these estimations can be described as both logical and statistical and methodological and interpretive. The logical and statistical problems will be dealt

with together. The methodological and interpretive section will deal with techniques separately. No attempt is made to give a detailed review of the methods, but rather a critique of their value.

Logical and statistical problems. In the strict sense, microbial biomass is the weight of living microbes in a given volume of water or sediment (Gray 1967). The term is obviously imprecise, but becomes more closely defined if "weight of living microbial carbon" is substituted for "weight of living microbes." Since in this context microbes can only be delineated by size and since some of the methods do not distinguish between living and dead microbes, even this definition is ambiguous to some degree. And although the "given volume of water" may be exactly measured, the same cannot be said meaningfully of the "given volume of sediment," which can vary in composition from silts to pebbles. This problem is partly resolved by expressing the biomass in terms of weight of carbon per unit volume of "associated water" (Andrews, Floodgate and Pugh 1976). This at least makes possible some degree of comparison between the biomass in sea water and that in the sediments.

Because it is not possible to assess microbial biomass directly, to separate the microorganisms from a known volume of water or sediment and measure their carbon content, all practical methods are necessarily indirect. The general principle is that a property of the natural flora believed to exhibit a constant relationship to the biomass is measured, and then the biomass itself is calculated by means of a conversion factor. Unfortunately, the validity of the conversion factor must always remain in doubt. Its derivation involves estimating the measured property in cultures that have been grown under defined conditions and whose biomass can be directly evaluated, thus providing the ratio of measured property to weight of microbial carbon used to calculate the microbial biomass in the natural

environment. But there is no guarantee that the laboratory-determined relationship holds true, and no way of finding out! We can only check to see if the relationship holds true in the natural environment by measuring the biomass directly--but it is because we cannot do this that we have to resort to the indirect method in the first place. The philosophically minded will recognize this as an instance of the well-known problem of correlation, causation and induction. As Hume (1739) asserted, there is no logical reason to establish "that those instances of which we have had no experience resemble those of which we have had experience." Consequently, indirect measurements, no matter how frequently repeated, will always carry an element of uncertainty. Anyone who cares to follow up this line of thought in a modern text may find the essays of Popper (1972) interesting.

As already stated, a reliable conversion factor must bear a constant and preferably a simple linear relationship to the carbon content of the organisms. Our apprehensiveness is increased by the knowledge that none in fact do so. Some, such as adenosine triphosphate (ATP), are known to vary with the life cycle of the organisms and possibly with their metabolic activity; others, such as protein content, are difficult to apply because the ratio of living to dead protein is not known. Unfortunately, there is a tendency for a conversion factor to become sacrosanct once it has appeared in the literature, but it is unlikely that even the most carefully calculated factor will apply to that 70% of the earth's surface covered by the seas, to all depths and at all times. Continuous scepticism and examination of the validity of the conversion factors is urgently required.

Given the present state of the art, it can be concluded that all biomass quantifying measurements are indirect and therefore uncertain to some degree and that to reduce the uncertainty it is preferable

to use two methods in conjunction. When two methods give the same value, the reliability of the estimation is greatly increased. When they differ, it is because of the uncertainty in the methods or because some relevant, perhaps unknown complexity of the environment has not been taken into account.

Many of the problems, both theoretical and practical, that limit our knowledge of microbial biomass apply to the study of the activity of microorganisms also. The term biomass seems to have arisen from the realization that many microorganisms in natural waters are frequently in a resting or non-active state. Measuring their activity therefore amounts to assessing to what extent they have departed from this resting state. A wide variety of biochemical indicators have been used to do this, but oddly enough, the most obvious indicator of activity--growth--has only been examined to a limited extent in this connection, mostly by Russian workers. Sometimes it is possible to make direct *in situ* observations of activity by following chemical changes that can be reasonably attributed to microbial activity as it occurs in the environment.

More frequently, the investigation of activity involves either enclosing or often removing a part of the environment and trying to make observations under controlled conditions. Once enclosed, whether in a simple bottle, a plastic bag suspended in the natural water or a complex bit of machinery, the sample studied is in a new environment which is similar, but not identical to the environment which was the origin of the study. At this stage, Gibb's (1977) Uncertainty Principle begins to apply. The principle states that "the closer one's experimental model is to natural conditions, the more nearly impossible it becomes to measure the processes occurring." In other words, if the environment is pictured as a complex web of interacting dynamic processess--physical, chemical and biological--the greater the restraint put on one or more of the

processes in the model system (often called a microcosm), the greater the distortion produced in the whole model. Hence, the aim is to produce microcosms that mimic the natural environment very closely, which we can attempt to do only if we know what the properties of the natural environment are. But it is to discover those properties that the microcosm is built. We are back on the familiar "a priori a posteriori" roundabout.

The difficulty can be illustrated by a problem in growth kinetics. We know a good deal about the growth kinetics of pure strains of microorganisms in batch or continuous-flow cultures. So we have to ask if the microorganisms in our water body are behaving like the organisms in a batch or a continuous culture. In other words, which is the best model? We cannot study the growth of the bacteria *in situ*; we can only put a sample into a bottle, make a batch culture and so impose batch culture kinetics. Or we can put a sample into a continuous culture apparatus and so impose continuous culture kinetics. The position is confounded more when we admit the possibility that the bacteria in nature may behave on one occasion as a batch culture and on another as a continuous culture, or even as a mixture of both, as when, for example, the environment is made up of several subenvironments with different growth characteristics. Strictly, we ought to allow for the possibility that there is a third kind of kinetics that we do not know about. In addition, some important complicating factors in a situation may be affected in an unknown way in the microcosm. For example, the predation of bacteria by protozoans is likely to be important in most aquatic environments, but is ignored in most continuous-flow experiments.

It follows that the argument of much of the work on activities proceeds by "analogy." For example, if a substance is placed in a bottle of water, then the proportion of bacteria that can

degrade that material will increase; i.e., there will be enrichment. By analogy, the same enrichment will occur when the same substance is added to the natural water system. Referring back to the natural environment shows that this is so; hence, it is acceptable to conclude that the two processes are the same. But the analogy is not always so simple. Pugh and his colleagues (1974) studied an Anglesey beach for a two-year period, measuring the nutrient regime and the bacterial numbers. He then built a microcosm (Pugh 1975), a fairly complex model beach, which after a settling down period was found to have a bacterial and nutrient regime that was roughly the same as that for the natural beach (Andrews, Pugh and Floodgate 1976; Pugh 1976). The nutrients were known to govern the rate of oil loss, so it was argued that since the nutrient and bacterial regimes were the same in the natural and model beaches, the rates of oil disappearance should be analogous. The experiments were run on that basis. But later work on the natural and the artificial beaches suggested that the flow of water through the sand was different in each case. Since this was the route by which nutrients and oxygen were conveyed to the oil, doubt was cast upon the oil loss rate experiments.

This illustrates that the greater the number of factors that contribute to a phenomenon, the more difficult it is to build a microcosm that will enable us to follow all of the changes. Reference to the natural environment is always necessary to check that results obtained in the microcosm are close to those occurring in nature. Progress can only be made by proceeding on a hypothesis (e.g., that marine bacteria follow the same kinetic laws as those in continuous-flow cultures) and then attempting to refute that hypothesis. It follows that unexpected results from a microcosm can be as illuminating as expected ones.

Unfortunately, the element of doubt has been

ignored very often in the past. Consider the most common and simplest microcosm--a glass bottle. What positive reason can we have for believing that bacterial growth, oxygen consumption and loss of substrate will proceed at the same rate or along the same paths in water placed in a bottle as these would in water left in place in the environment? The best we can do is assert that we know of no reason why they should be different. Yet the marine chemist is concerned by whether his samples are taken in a glass or a plastic vessel. Could not analogous factors affect microbial biochemistry? The microecology literature affords plenty of examples where changes in someone's laboratory apparatus are reported as ecological facts, without any convincing evidence of why the data should be interpreted as referring to events in the sea, or even a village pond. Nor is it adequate to say that repetition of the data releases the experimenter from doubting his results. Repeating the same mistake does not bring it any nearer to being correct.

Some practical principles that follow from this discussion are: (i) Activity measurements in a microcosm, whether simple or complex, should take place over as short a time as possible. This allows less time for abnormalities to develop. (ii) All physical and chemical perturbations should match those expected in the natural situation as closely as possible. (iii) Attention should be paid to the effects of scale. What grounds have we for believing that a sandy beach is simply a sand column enlarged? What is the effect of using microcosms of different size? (iv) Reference must always be made back to the natural water body or sediment that is the center of the study program. Paradoxically, this involves a close study of what is going on in the microcosm to reveal the ways in which it may differ from nature. (v) As with biomass determinations, the more ways that can be used to study the activity of microorganisms in the aquatic world the better, especially if there are different assumptions in the methods.

Statistical considerations enter into the determination of microbial biomass and activity for two reasons. First, since the microorganisms of any defined body of water at any particular time are not uniformly distributed, some measure of their dispersion is required. Secondly, variations can occur because of seasonal, geographical, and tidal changes. Hence there are three sources of variation for the investigator to be concerned with: (i) Variation within the same sample of sea water or sediment. These should ideally show only a small variation; e.g., duplicate plate counts from the same bottle of water should be similar. (ii) Variations between samples taken from the same body of water or sediment at the same time. These can be expected to be greater than in source i because of patchiness within the water body. (iii) Variation attributable to different locations and times. Usually, this is the variation the study is concerned with, which should be significantly greater than the variations attributable to the other two sources. Statistical techniques are available to determine these variations and to assess their significance (see Elliott 1971; Jones 1973). Unfortunately, adequate analyses that take into account all the kinds of variation are rare in the literature, though when carried out they often reveal unexpected results. For example, Ashby and Rhodes-Roberts (1976) showed by this means that duplicated sample bottles giving numerically different spread plate counts could not be distinguished on the basis of differing responses to different counting media.

A related problem concerns the size of the sample taken for bacteriological analysis. The apparatus currently used to collect sea water vary in size from a few millilitres up to a litre or more. The question is whether the volume of a sample affects conclusions as to the biomass present or estimates of activity. The problem is similar in many respects to that of quadrat size in macroecology. If the microorganisms were uniformly distributed, then the

lower size limit of the sample would be set by the sensitivity of the measuring technique. Where the distribution is contagious, that is, patchy, the sample must contain all the relevant elements in the same proportion as they are in the parent water body. Hence for most surveys, the larger the sample is the better, so that it will contain the greater range of microenvironments. If, for example, we wish to survey a body of water for hydrocarbonoclastic organisms, then a large sample is more likely to include the range of bacteria in the locality that are capable of breaking down the multitude of hydrocarbon substrates. If, in contrast, we are concerned with a microenvironment, i.e., what goes on in one of the patches, then a much smaller sample is needed. Ashby and Rhodes-Roberts (1976) found evidence of a microdistribution in the significant differences among plate counts of 25 ml samples taken 10 cms apart. The optimal sample size, then, is determined by the task at hand.

The trouble is that aquatic microecologists are often trying to do two opposing tasks at once. On the one hand, we tend to extrapolate from a few samples to make generalized statements about what goes on in the "oceans" or "coastal waters" or "estuaries." On the other hand, we are aware that the organisms we are dealing with inhabit environments which are in fact too small for us to adequately study with the methods that are available. It should also be emphasized that adequate statistical planning before a series of measurements is begun markedly increases the effectiveness of the study and can eliminate a good deal of wasted effort.

Methodological and interpretive problems. In addition to the theoretical problems dealt with above, many practical complications in experiment design arise from methodological limitations--when it is desirable to ascertain the contribution made by different microbial groups to the total biomass such as the relative contributions of microalgae

and bacteria, for example. And it is very common when working in the field to find that a large number of samples have to be collected before any degree of reliability can be placed on the data. So the determinations must be fairly simple, quick, and cheap. In the case of marine research, these difficulties are compounded by those of working on small ships in rough seas. It is not very surprising that there is no universally applicable technique and that what is finally used in any particular situation is whatever is considered the least undesirable method, modified by practicalities such as the money and staff available.

Biomass Determination Techniques

The measure of biomass is currently based on counting techniques, chemical methods which estimate a substance common to all the microorganisms whose biomass is required, and the measurement of metabolic rates.

Counting methods. In certain specific cases, it is possible to use viable counts as a means of estimating the biomass of certain physiological types of bacteria. For example, Oppenheimer and his colleagues (1977) found that the ratio of the viable count of hydrocarbonoclastic bacteria to total heterotrophs was higher in the region of active oil fields in the North Sea than in areas away from the rigs. It is reasonable to assume that the bacterial biomass was in a similar ratio, though it must be noted that this is an assumption, not a fact.

However, the total microbial biomass is required more often than that of a highly specialized physiological fraction, and since it is impossible to grow all the microorganisms in a water sample under any one set of conditions, total counts have to be made using the microscope. Methods for estimating the microalgae biomass by microscopic techniques are

well established (Lund, Kipling and LeCren 1958). Iodine is added to the sample, the algae are allowed to settle, and then are counted and sized and often identified at the same time, using an inverted microscope. Biomass can then be calculated.

If samples for estimating the total bacterial biomass cannot be processed immediately, they are preserved with formaldehyde or glutaraldehyde, added as soon as possible after collection. There is perhaps a minor controversy as to which is the best preservative to use, but figures comparing the two are scarce. It is claimed that glutaraldehyde is less damaging. Nott and Parkes (1975) working on delicate specimens for electron microscopy, devised a mixture of 20 mls of 25% w/w solution of glutaraldehyde, 50 mls distilled water, and 140 mls of sea water. This solution has the same osmotic pressure as sea water (salinity = 35 ‰) and so is less likely to damage delicate organisms. The water sample is filtered through a polycarbonate filter, preferably with a porosity of 0.2μm. This must be done gently to minimize the destruction of the delicate cells. The bacteria are then stained on the filter. Simple stains such as erythrosin can be used, but it is difficult to distinguish the bacteria from organic detritus. Acridine orange, which fluoresces under U.V. light, is now the most frequently employed stain. This stain reacts with desoxyribonucleic acid (DNA) in a complex fashion recently reviewed by Lochte in her master's thesis and reproduced here with her permission.

"Acridine orange (3,6-tetramethyl diaminoacridine) interacts with the nucleic acids of a cell, forming a red-orange or a green fluorescing complex (Ranatiue and Korgaonkar 1960; Yamabe 1973). The ratio between nucleic acid and AO molecules, basically, determines which complex is being formed. Only a few AO molecules can be embedded into the rigid structure of double-stranded desoxyribonucleic acid (DNA); hence, the AO fluoresces as a monomer with a

green colour. When the DNA breaks up to a single strand or when AO attaches to the random coils of ribonucleic acid (RNA), many AO molecules can interact, so that in both cases the AO molecules form dimers fluorescing red-orange. Initially, Strugger (1948), who originally developed the method, proposed the use of these colour differences as an indicator for the viability of bacteria. However, as shown by Korgaonkar and Ranade (1966) and Yamabe (1973), the shifts in colour from green to red and the loss in viability do not occur concomitantly due to persistance of double-stranded DNA in dead cells. It also may be possible that highly active bacteria with high RNA contents fluoresce red as well as dead cells (Hoblin, Daly and Jasper 1977). Finally, the colour is influenced by the total amount of AO added by increasing the number of red bacteria with increasing AO concentration. Hence, the initial idea to distinguish between dead and living bacteria is questionable."

A number of authors have reported on the optimum procedure and conditions for staining (e.g., Zimmerman and Meyer-Reil 1974; Daly and Hobbie 1975; Jones and Simon 1975; Hoblin, Daly and Jasper 1977). After staining the cells must be counted and sized. It has to be assumed that the preservation and staining processes have not altered the volume of the cell, though there is some indication that substantial changes do take place (Troitsky and Sorokin 1967). The average volume can be calculated, and assuming density, wet/dry weight, and dry weight/carbon ratios, the biomass can be calculated. All the counting and sizing must be carried out in sufficient detail to satisfy statistical testing. Simply basing the calculation on an average value for the cell volume implies that the sizes are normally distributed, but this is not always the case. The sizing stage may be omitted, i.e., a further assumption can be made that all the bacteria are of equal volume and density, so biomass becomes directly proportional to numbers.

The method is difficult to apply to water containing a lot of suspended material or where bacteria are aggregated to any appreciable degree. For similar reasons it is not a very satisfactory method for sediments. In these circumstances it is especially difficult to see the very small bacteria, particularly when they are attached to organic matter that may "flare" in the U.V. light. For these reasons, the method is of greatest value in clean water with low bacterial numbers. Even then the method is time-consuming, so that throughput is slow. For accurate results the operator has to be fairly experienced, particularly if the samples contain a large number of very small organisms. However, since it is the most direct method available, it is probably the best technique for producing reliable data.

Finally, for some purposes a Coulter Counter, which both counts and sizes microorganisms, will give adequate results. However, very small bacteria (less than 0.5μ), which can be seen under the microscope, cannot be discriminated in the Coulter Counter, nor can detritus be distinguished from living cells. Nevertheless, when the biomass is dominated by healthy algae and the bacteria constitute only a small proportion of the biomass, the method is well worth consideration.

Chemical methods. To be successful, the chemical substance chosen as a means of estimating biomass must be specific to the microorganisms and present in a constant proportion to the carbon content of the cells. In addition, the estimating technique should be simple, reliable, and cheap. The most obvious candidate for this method is protein estimation. Unhappily, proteins are too ubiquitous; the suspended protein will include dead matter from phytoplankton, etc., as well as living material. When there is a lot of suspended material in the water, then the ratio of dead to living protein may be so high that variations in the living fraction are undetectable. And the proportion of protein is

not likely to be constant in all the live organisms, so even in favourable circumstances the conversion factor will be unreliable. Nevertheless, in some situations, where for example the flora is dominated by a single species, as during a dinoflagellate bloom, and the amount of suspended detritus is low, then protein estimations can give meaningful results.

Another widely distributed compound that has been used for many years to estimate the biomass of microalgae is chlorophyll *a*. Unfortunately, the proportion of pigment to total carbon varies by a factor of 5, from about 1:20 to 1:100 depending on the species that are present and the physiological state of the plants. Sesescence or heavy grazing can result when high concentrations of degradation products, such as phaeophytin and phaeophorbides, absorb at the same wavelength as chlorophyll *a*, and thus lead to inaccurate results. For detailed discussion of the problems and procedure (see Strickland and Parsons 1968 and SCOR-UNESCO Working Party of Photosynthetic Pigments 1966).

The composition of the cell wall of bacteria has a number of distinctive features, so it is not surprising that attempts have been made to utilize these special properties as a means of biomass determination. Several workers (Moriarty 1977; Moriarty 1978; King and White 1977) have attempted to employ muramic acid in this way, as it is found exclusively in the walls of bacteria, including the cyanobacteria. The amount of muramic acid per cell shows only comparatively small variations with growth conditions (Ellwood and Tempest 1972). Unfortunately, differences between species may be larger; gram-negative strains generally contain more muramic acid than gram-positive ones (Moriarty 1978). The main drawback to the method is the long extraction process, which severely limits the throughput. It involves an acid hydrolysis for several hours followed by separation of the muramic acid by chromatography. Lactate liberated from the muramic acid by alkaline

hydrolysis is then estimated colorimetrically or enzymatically, the latter giving higher values than the former (King and White 1977). The sample required for analysis is large, so the method is not sensitive enough when the bacterial biomass is low; in contrast, it may well be of value for finding the biomass in sediments, which are not amenable to other techniques. When Moriarty (1978) compared total direct counts to his muramic acid analyses in sediments, he found that only about 10 to 30% of the bacteria judged by the cell-wall method could be separated and counted by homogenizing.

Another cell-wall method is based on lipopolysaccharide (LPS) which is found only in the walls of gram-negative bacteria. Essentially, the estimation relies on an extract of the blood cells of the king crab, *Limulus polyphemus*, that contains an enzyme and a protein. The enzyme is activated by the LPS and causes the protein to precipitate. The increase in turbidity is followed spectrophotometrically (Sullivan and Watson 1974; Watson et al. 1977). Careful preparation of glassware and solutions is required because of the ubiquity of LPS. Other major drawbacks at the moment are cost and the fact that stocks of kingcrab must be limited, even though the blood can be removed without killing the animal. The method also fails to distinguish between living and dead bacteria, and there is the possibility that LPS remains intact long after the generating organisms have died. The conversion factor (Watson et al. 1977) has a value of about 6.35, a figure originally arrived at from determinations with *E. coli*. However, Watson and his colleagues (1977) show that if specific gravity, wet/dry weight ratios and dry weight/carbon ratios are assumed, the range of the LPS/C ratios for natural marine populations as estimated by the epifluorescence technique center at about this figure.

Another substance which may qualify as a biomass indicator is poly-β-hydroxybutyrate. This

polymer is found in a wide variety of procaryotes. Its concentration can be measured spectrophotometrically after a short extraction process with sodium hypochlorite and chloroform. Unfortunately, the polymer seems to be sensitive to environmental conditions, including starvation, as it accumulated in a freshwater *Spirillum* (Matin et al. 1979) grown under carbon limitation, and to lyophilization (Herron, King and White 1978). Interpreting the data is therefore the major problem in this case, and must probably wait until the role of the polymer in the metabolism of bacteria is better understood.

In a similar way, it has been suggested that lipids may be useful indicators, not only of biomass as such, but of the relative rates of growth of the different microfloral populations that develop on detritus (King, White and Taylor 1977; White et al. 1977). White and his co-workers have developed techniques for extracting various lipid fractions from estuarine sediments (1979) that are not excessively complex but are time consuming and which require operators with a degree of manipulative skill since strong acids are involved. Nor is it known how much lipid, associated with organically rich sediments, is free and not bound to living microorganisms. Nevertheless, since there is some evidence that lipids form a constant fraction of the biomass (Wilkinson, Morman and White 1972; White et al. 1979), this line of attack is promising.

However, the most widely used of the chemical methods for finding microbial biomass is that of estimating the adenosine triphosphate (ATP) present in the living cell. The method at first sight has many advantages, particularly for dealing with large numbers of samples, as the throughput rate is high. However, there are some problems, especially when the technique is pushed to its limits as is likely to be the case in marine research. The topic has been well-reviewed recently (White et al. 1979), so only a number of salient problems will be noted here.

The principle of the method is that luciferase will react with luciferin in the presence of ATP, though the chemistry is in fact complex (Afghan, Tobin and Ryan 1977). One photon of light is released for each molecule of ATP degraded, so that by collecting and measuring the light the amount of ATP can be calculated. Although the method is potentially very sensitive, when bacteria numbers are low, as is often the case in the oceans, a concentration step has to be included either before or after the extraction of the ATP. Concentration before extraction is usually done by means of collecting the organisms on nucleopore or millipore filters; the structure of the former makes them preferable. Since NaCl is inhibitory to the enzymic reaction, removal of the major part of the salt is an advantage where marine work is concerned. The disadvantage is that, even with slow, careful filtration using a minimum pressure difference, some of the delicate organisms may be broken up. If the proportion of these delicate organisms is small, then the error will not be important, but generally the analyst has no indication of the degree of anomaly introduced. When samples greater than 0.5 litre are filtered, losses occur, allegedly because of detrital interference (McElroy and DeLuca 1978).

Another possible source of error is the loss of ATP caused by the stress of filtration. ATP has a turnover time within the organisms of less than one second, and there is some evidence that centrifugal or filtration stress will reduce ATP very considerably (Sutcliffe, Orr and Holm-Hansen 1976), leading to an underestimate of the biomass. Again the error is impossible to quantify unless the ATP content of the cell is known. But this is what we are trying to find out. However, if it is assumed that the natural population will behave in the same way as a well-grown culture of say *E. coli*, and that dilution will not affect the ATP concentration by applying another stress, then it is possible to obtain some measure of the error by comparing the ATP per cell of an

unfiltered pure culture with that of a diluted (to approximately the same concentration as the natural one) filtered culture.

Following concentration, ATP is extracted from the microorganisms. This is done by disintegrating the cells in either boiling buffer, cold acid, or in solvents such as butanol and chloroform. The two extraction methods presently in vogue are buffer extraction employing either TRIS or glycine buffer, usually with Mg and some EDTA added, and acidic extraction, with sulphuric or nitric acids being the most commonly used. The buffers presumably differ in their extraction efficiency for reasons unconnected to their function as buffers. Possibly, they have an effect on inhibitory substances. Tobin and his co-workers (1978) indicate that humic acids as well as heavy metals can interfere with the analysis. Acidic extraction requires that the pH be increased at a later stage to a value around 7.5, at which the luciferase is optimumly active. This adjustment with alkali increases the volume and dilutes the ATP; in addition, the unbuffered nature of the solution results in exasperating swings of pH during the adjustment. And these extra steps in the process reduce the throughput of samples. Yet a further drawback is that manipulating acid, particularly at sea in rough weather, requires extra safety precautions.

Of the various solvent extractions used, 23% chloroform, as advocated by Tobin and his co-workers (1978) seems to be most successful. There does not appear to be any decisive evidence in favour of any one method of extraction. Possibly, the optimum technique varies with each particular situation. This creates the difficulty that if the worker uses the optimum method for a given set of circumstances, it may be difficult to compare the results from one place with those from another. If different techniques were used, it will not be possible to distinguish variation resulting from change in technique from variation resulting from change of place.

It is also possible to extract the ATP before concentration. Sulphuric acid is normally used, added directly to the water sample whose only previous treatment has been a coarse filtration to remove large detritus particles and zooplankton (Hodson, Holm-Hansen and Azam 1976). Passing the extract through a charcoal column separates the ATP from the interferring ions while the nucleotide remains on the charcoal and is next eluted with ammonical alcohol. The eluate is then dried in vacuo, in which state it can be stored at low temperatures until enough samples have accumulated for an analytical run. It is then dissolved in a small amount of buffer and the ATP is quanitified. Recovery from this method of concentration seems to be around 49%.

Extracted and concentrated, the ATP is now measured using the luciferin-luciferase reaction. Extra luciferin aids sensitivity (Seki 1970). Magnesium ion must also be present, but in our experience the major restraint in the analysis is the quality of the enzyme available. Poor quality enzyme is comparatively cheap, but the inherent light reduces sensitivity. The best quality material is about 50 times more sensitive, but is much more expensive; since a field trip may bring in several hundred samples to be analyzed, cost can be an important consideration.

The rate of mixing of the sample and the enzyme preparation is also an important variable, whether the sample is injected into the enzyme or the enzyme into the sample. In our experience, simply injecting enzyme into enzyme will cause a light response. The error can be standardized by using an injector system that causes the same turbulence on all occasions. Pure reagents and water must also be available. Obtaining water with a low ATP response can be tedious. Water from many deionisers found in the laboratory contain ATP in measurable quantity because of the growth of algae

and bacteria. However, some deionising systems are reported as being satisfactory (e.g., Lowry et al. 1971). Alternatively, the water may be acidified, autoclaved, and the pH brought back to 7.0 with NaOH. Similarly, low response water may be obtained by filtration through a 0.2µm filter to remove the bacteria and the filtrate passed through charcoal to remove any free ATP. Or, very pure water can be obtained by redistillation from a glass still after acidifying with phosphoric acid (Jones and Simon 1977). It is also prudent to check each batch of enzyme against known ATP standards, which themselves ought to be checked regularly. Very dilute ATP decays at room temperature and the temptation to keep old standards should be resisted.

Whether one uses the peak height of the light response curve or an integrated area under the curve for a period of several seconds depends largely on the machine available. On the whole it does not seem to make a great deal of difference.

The ATP method has not been applied to sediments with incontrovertible success, though extraction with cold sulphuric acid and EDTA (Smith 1972) is the most promising. The uncertainties appear to have two major sources. Firstly, inhibitors may be present which reduce the light produced and secondly, the sediment itself is apt to absorb the ATP so tightly that it cannot be eluted quantitatively. The second problem is probably insurmountable, as it is difficult to envisage a method which effectively prevents materials such as clays from mopping up the ATP. The obvious way is to separate the bacteria by agitation, but only a fraction of the total flora appears to be released from the substrata before the energy input into the system is high enough to destroy the organisms instead of releasing them from the surface. Adequate controls must also be run with the analyses. Spiking with known amounts of ATP can obviously help to monitor the recovery of the nucleotide once it has been released from the organisms. Monitoring the

extraction efficiency is very difficult, and it is usual to ignore any losses at this stage. It is very important therefore to be sure that ATP-ase is denatured when the cell is ruptured.

The final task is to interpret the data obtained from all these manipulations. Assuming that all the zooplankton has been removed prior to the ATP extraction, the nucleotide measured will be a function of ATP per unit of microbial biomass. Unfortunately, this will have been obtained from the microalgae and small flagellates as well as the bacteria, and each contributory organism will not necessarily contain the same amount of ATP per unit biomass. It is possible to assess the relative contribution of photosynthetic organisms if the chlorophyll concentration has been measured at the same time. From the information obtained in this department (Floodgate, Turley and Lochte unpublished), the ATP concentration in coastal waters is often dominated by phytoplankton, and the bacterial contribution is only a small fraction of the total. As a result, bacterial variation becomes lost in the "noise" of the system. The last step is to apply some sort of composite conversion factor to convert ATP values to biomass. The most popular one is that of Holm-Hansen (1969), which he computed from laboratory phytoplankton and bacterial studies and which has a value of 250 mg C μg^{-1} ATP. Where it has been applied, the general concensus seems to be that the biomass figure that results is not so far removed from what is regarded as plausible as to cause the user to distrust the factor. But if a greater degree of confirmation from other workers were forthcoming, then we would be on firmer ground. It will be clear that the ATP method has some uncertainties as regards the efficiency of extraction, the accuracy of the analyses, especially if inhibitors may be present, and the interpretation of the values obtained. Nevertheless, the method allows a large number of samples to be processed rapidly and is the most useful method

available for large-scale field work, particularly when data patterns rather than absolute values are required.

Metabolic methods. The third and probably least-used approach employed to measure biomass in aquatic systems is by means of metabolic studies. The uptake of a suitably labelled carbon source by the indigenous flora is compared to that of a pure culture of a named strain whose biomass is known by direct measurements. Other metabolic characteristics such as respiration can be employed in the same way (Holm-Hansen 1969). It is assumed that there is a simple relationship between biomass and metabolic activity, i.e., that none of the organisms are in a resting stage. The major advantage of the method is that it relates biomass to heterotrophism, thereby excluding the biomass associated with photosynthesis. It also distinguishes between living and dead cells. The disadvantages lie in uncertainty about how closely the conversion factor, generated by pure culture studies, relates to that of the mixed culture of the aquatic environment and to what extent the chosen substrate, usually glucose, is metabolized by the whole of the heterotrophic bacteria. Another problem with this approach as compared with other biomass measurements is that the sample cannot be preserved, but must be dealt with quickly before growth or decay has occurred.

Finally, it is worth noting that all methods of microbial biomass measurement, except for direct microscopic observations, involve either estimating the effect of metabolic function, such as the concentration of ATP or polyhydroxybutyric acid, or the concentration of a structural component such as muramic acid, LPS, or membrane-bound lipids. It is reasonable to consider the structural compounds as more likely to give a true estimation of the biomass because they are more likely to bear a constant relationship to biomass than the metabolic

substances.

Activity Determination Techniques

Activity measurements may be divided into those that record the general state of activity of the population, such as the energy charge or the rate of growth, and those that are dependent upon a particular metabolic function, such as the rate of uptake of a certain substrate or an enzymic reaction involving a specific biochemical change.

Rapid division has always been considered by microbiologists as an indication of a healthy, active culture. This increase in count does not always imply an increase in biomass. For example, the bacteria in a turbidostat are actively dividing though the biomass is kept constant, a situation perhaps analogous to nature, where predation keeps numbers constant. Novitsky and Morita (1977) have established that a pure culture of a vibrio may respond to starvation conditions by forming a large number of small organisms with presumably little or no gain in biomass. Not surprisingly, procedures for measuring the potential for protoplasmic increase have been developed.

The methods for measuring photosynthetic increases have received a lot of attention (Fogg 1975), but the bacteriological techniques are less well developed. Those available for freshwater (Sorokin and Kadota 1972) include comparing the difference in the increase in numbers of bacteria between filtered and unfiltered aliquots of a water sample after a given time interval. The method assumes that both grazing rates and bacterial growth rates are the same in the sample bottle and the natural environment. Wall effects are ignored. It is therefore impossible to relate the figures thus obtained to what happens in

nature, except to suspect that they are maximum values.

It is also possible to estimate heterotrophic production using gaseous methods, particularly CO_2 exchange and the uptake of O_2 (Sorokin and Kadota 1972). Bacteria of course vary quite markedly in their CO_2 uptake--from the complete heterotrophs which obtain only a small percentage of their carbon from this source, to the autotrophs, which obtain all of it by this route. In dark bottles facultative autotrophs can oxidize low molecular weight molecules. Hence the conversion factor to bacterial production is uncertain in its accuracy. Yet mathematical equations generated from these data (Sorokin and Kadota 1972) are claimed to apply to freshwater environments. This does seem a rather bold assertion in that it relates to an environment with a variety of mixed cultures and a great range of organic carbon sources in a wide diversity of nutrient, salinity, and temperature conditions. It would be interesting to compare the numerical and gaseous techniques to see if they give similar estimates of productivity. However, the greatest drawback to the bacterial productivity measurements is that they are very difficult to apply to a body of natural water. Nevertheless, in that they represent potential for growth, they are probably no more misleading than the "heterotrophic potential" that is often quoted in the literature.

Another general technique to estimate the overall condition of the bacterial population is the energy charge (Chapman and Atkinson 1977; Karl and Holm-Hansen 1977). This is the ratio of the energy-charged nucleotide-phosphate bonds to the total nucleotides. Healthy, active bacteria have a score approaching 1.0, while senescent organisms score around 0.6. The ADP and AMP are first converted enzymatically to ATP, and then assessed by the luciferin-luciferase reaction, so

careful manipulation is required, especially with small quantities of material. The method has all the advantages and disadvantages of the ATP biomass evaluations. The major advantages are that the resulting values relate directly to the natural situation and that from the ATP figure an estimate of the biomass can be obtained without further work. The major problem lies in interpretation, since algae and flagellates will also contribute to the nucelotide concentrations.

The overall mineralization rate is another general method in use for a long time and familiar as the basis of the classical biochemical-oxygen-demand assessment of pollution. While very useful for this purpose, it is difficult to interpret in terms of the ecology of the sea, if only because the time scale is long enough for anomalies to develop. Periods as long as 10 days at elevated temperatures of 30°C have been proposed for freshwaters (Sorokin and Kadota 1972). Shorter-term respirometer measurements using plankton concentrates have been suggested (Pomeroy and Johannes 1968), and these are more likely to be closer to the values occurring in nature. However, White and his colleagues (1979) comment that the addition of inhibitors such as chloroform, toluene and trichloroacetic acid "effectively stop biological activity, often with little effect on the chemical oxidations." Perhaps one of the most neglected areas of marine chemical research is that of the abiological oxidation of organic substances. However, as means for detecting very small changes in O_2 tension in sea water are developed, oxygen changes may well become a very important way of recording bacterial activity in the environment.

Another way of measuring the rate of oxidation is to use an indicator that changes color when it is reduced. Such substances are 2,3,5, tetraphenyltetrazolium chloride or 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride,

which are reduced to water-insoluble formasan, which is extracted with solvents such as chloroform and measured spectrophotometrically. The method has been applied to sea water and sediment samples (Iturriaga and Rheinheimer 1975), and soils (Cassida 1977). Skujins (1976), reviewing the technique as applied to soils, points out that the result is dependent upon factors such as population density, pH, substrate concentration, and concentration of the indicator; consequently, standardization of the conditions is important, and the method is more useful for indicating patterns of change than absolute values. The advantage of the method is that a sample can be examined immediately after it is collected.

A more kinetic approach (Wright and Hobbie 1965) entails adding radio-labelled substrates to freshly collected samples of sea water or sediment and counting the radio marker that evolves during a few hours incubation period. By using a series of concentrations within the range of those expected in the environment and by plotting them appropriately, it is possible to show whether they follow Michaelis-Menten kinetics. It is as interesting to know that on occasion they do not as it is to find that at other times they do (Wright and Hobbie 1969;Vaccaro and Jannasch 1967; Williams and Askew 1968). For example, sediment samples enriched with organic material for 24 hours "improved" analytical uptake patterns (Hamilton and Prelan 1970), while samples diluted 1/1000 in sterile sea water showed saturation kinetics (Vaccaro 1969). What kinetics did these bacteria show in the natural environment, and what was the nature of the change in the bacterial community induced by enrichment in one case and dilution in the other?

Besides revealing that the familiar biochemical techniques are not invariably followed, this technique has given rise to the concepts of

heterotrophic potential and turnover time. The former is defined as V_{max} of the Michaelis-Menten plot and indicates the maximum rate at which the substrate would be degraded under conditions applying at the time, providing the concentration of the substrate had reached saturation values. Now the concentration of organic material in the marine environment is generally very low, so that saturation conditions may not be reached. Moreover, conditions such as temperature and the presence of inhibitors are not constant. So it seems likely that this factor is an interesting biochemical artifact rather than an ecological fact and conveys little information of real value.

Similarly, turnover time, T, can be a source of confusion. It may be defined as $\frac{S_t}{dS/dt}$ where S_t is the substrate concentration at time t and dS/dt is the velocity of the reaction. Clearly this ratio has the dimensions of time, and if the rate remained constant and independent of concentration, it would represent the time taken for the concentration S_t to reach zero. Indeed, the approach made by Dietz and his colleagues (1977) assumes a constant velocity. However, the Michaelis-Menten equation requires that at low concentrations of substrate, the reaction velocity is dependent upon the concentration; that is, the kinetics are first order, but as the amount of substrate increases and velocity approaches its maximum, the velocity of the reaction becomes independent of substrate concentration so that zero-order kinetics apply (Cornish-Bowen 1976). Therefore, the constant velocity assumption does not remain valid for all concentrations. Now a measure of the range of substrates over which the reaction has first-order kinetics is given by the K_m, but its value is often not indicated in the literature. Still, since organic concentrations are low in the sea, it is reasonable to assume that reactions are generally first order, such that T is not a constant. There must, however, be a number of

important exceptions, as on detritus and at the interface between two phases such as the air-water interface.

Nevertheless, by using the Wright-Hobbie technique, plotting turnover time, T, against the concentration of added radio substrate, it is possible to calculate the turnover time of the natural substrate without knowing its concentration (Wright and Hobbie 1966). But these calculations assume a constant velocity reaction that may not be true at the concentration of the substrate pertaining to the sea at that time. Hence, any attempt at an ecological interpretation is difficult.

Another approach to activity determinations is to study the rates at which single enzymic reactions take place. Nitrate reductase estimated by following the NADH-dependent formation of nitrite by phytoplankton showed that the enzymic response was related to variations that might be expected to be of ecological significance (Eppley, Packard and MacIsaac 1970). Comparative studies of glutamate dehydrogenase in laboratory grown cultures has also been used and then applied to areas of upwelling (Ahmed, Kenner and Packard 1977). An adaptation of this idea, which promises to be very useful in survey studies because of its high throughput, is illustrated by the work of White's colleagues (White et al. 1979; Morrison et al. 1977). p-Nitrophenylglycosides are incubated with the sample and the reaction is followed in the development of color using a spectrophotometer. This method might well be amenable to automation and hence very useful for survey work.

Conclusions

This paper is neither a report on new data nor a review in the usual sense. Instead, it is an attempt to ask if the data currently appearing on

microbial biomass and activity really provide insights into the ecology of marine microorganisms and to set out a few of the problems, conceptual and practical, that haunt the study of the microbial aspects of marine biology. The microbiological ecologist has inherited the ideas and terminology of the macroecologist and the techniques of the classical bacteriologist and biochemist. There is a danger that the terminology of the macroecology will be misused by the microbial ecologist. The term "community" is one example. And caution is needed when producing data using biochemical methods, often at considerable expense in terms of ship time and resources, because the information thus gained may not increase our knowledge of the way marine microorganisms live in the sea.

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Role of Microorganisms in Environmental Asessments

ARTHUR M. STERN

I have taken the liberty of changing the title of my assignment from "Methods for Monitoring and Regulatory Purposes" to "Role of Microorganisms in Environmental Assessments." In this paper, I shall deal with the following subject areas: (i) the role of environmental assessment in the regulatory process under the Toxic Substances Control Act, (ii) the role of microorganisms in determinations of chemical fate and effects, and (iii) problems and issues related to both of the above.

Role of Environmental Assessments in the Regulatory Process

In considering microbiological methods for environmental assessment, it is not difficult to conceive of evaluating some definable attribute of microorganisms or their activities. In so doing, we are really dealing with techniques

of measurement. However, there are many problems associated with developing methods for making environmental assessments because they require a set of definitions covering a myriad of variables about which little is known in composite.

The term environmental assessment is an all-encompassing one and generally applies to the evaluation of many individual elements. The integration of these individual evaluations provides the basis for making a risk assessment as shown in Figure 1. From this point, the regulatory process involves an integration of risk and benefits analyses, the identification of certain regulatory options, and finally, the taking of regulatory action.

A health hazard assessment results in predictions of adverse human effects if and when critical concentrations of chemical pollutants are released into the environment and is based, among other things, on the analysis of data from *in vivo* and *in vitro* testing. An ecological hazard assessment results in similar predictions for organisms other than man that could be exposed to chemical pollutants. An abiotic hazard assessment involves an analysis of the potential nonbiological effects of released chemical pollutants and includes concern for atmospheric impacts, structural damage, etc. An environmental exposure assessment results in the identification of human and nonhuman populations at risk. Finally, a chemical fate assessment results in predictions with regard to concentrations of chemical pollutants in all environmental media and must consider, among other things, the physical/chemical properties of pollutants, their transport and persistence, volumes released, modes of release and disposal, end uses, distribution, etc. The predicted environmental concentration ranges must be integrated with the minimal concentrations producing adverse health, ecological, and abiotic

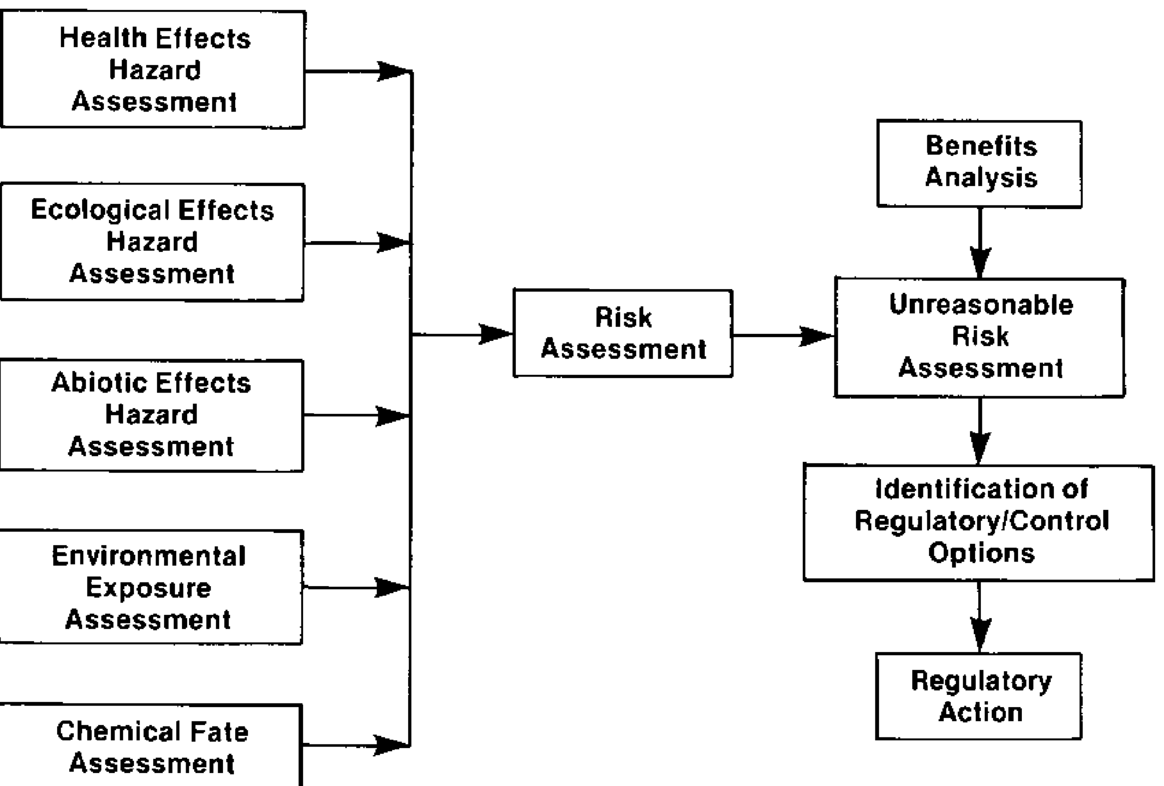

Figure 1. Role of exposure assessments in the regulatory process.

effects to arrive at an assessment of chemical risk.

Microorganisms play a role in both the ecological hazard and chemical fate assessments. In the remainder of this paper, I shall discuss the nature of microbial involvement in these assessments and some of the major problems that must be resolved before we can more fully understand the significance of this involvement.

Role of Microorganisms in Chemical Fate Determinations

Overview. Many organizations both inside and outside government have attempted to devise schemes for evaluating the environmental concentration of chemicals after they have been released. Some of the schemes have considerable merit, but none fully provides answers that are not based on a multitude of assumptions. Furthermore, many of these assessment schemes require informational inputs that are impossible to obtain legally, technically, or both.

Figure 2 is a simplified schematic of the types of inputs that may be critical for conducting a fate assessment. Although time does not permit more than a cursory treatment of this flow plan, there are a number of informational elements I would like to bring to your attention. Since the objective of a fate assessment is the derivation of "steady-state" chemical concentrations in all environmental media (i.e., air, water, soil and sediment), the starting point has to be the identification of point sources (manufacturing and/or processing sites), chemical uses, and if possible, product distribution. Thus, the assessment process depicted in Figure 2 starts with the analysis of production and end-use data and focuses on a "worst-case" situation

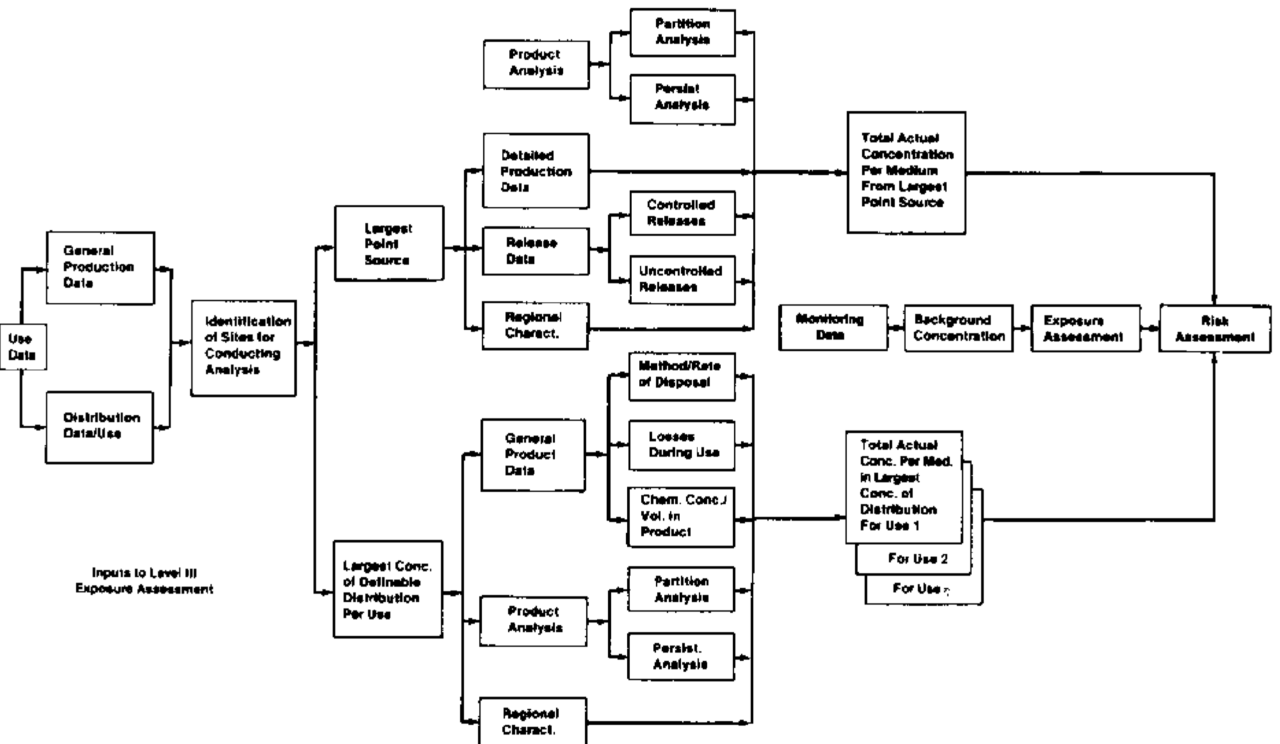

Figure 2. Flow plan for conducting environmental exposure assessments.

involving the largest point source and the highest concentration of definable distribution per end use. For each source (or each of several sources) of environmental release, information is required on the product(s), production, release, and the region(s) in which release occurs. In the next level of detail in this scheme, one can see the role microorganisms play in fate assessments. This level includes partition and persistence analyses. The former involves calculations of the distribution of chemical pollutants into various environmental media and is schematically presented in Figure 3. It can be seen that many processes are involved in the distribution/transport of chemicals into air, water, soil, and sediment.

Chemicals are potentially capable of undergoing degradation in all media, and it is a chemical's resistance or susceptibility to degradation that determines, in large part, its ultimate "steady-state" concentration in a particular medium. The major mechanisms for degrading chemicals are chemical, photochemical, and biological. With the possible exception of air as an environmental sink for chemical accumulation, microorganisms potentially play a major role in the degradation of chemical pollutants in all media. Whether a chemical is released directly into air, soil, or water/sediment or is redistributed into these media after release, it is subject to exposure to a wide spectrum of microbial populations possessing the potential capability for promoting chemical transformations. Evaluating the possibility, extent, and rate of microbially mediated transformations constitutes a very important part of the fate assessment process.

Some problems associated with biodegradation analyses. Most of the information we now have concerning the transformation of organic compounds

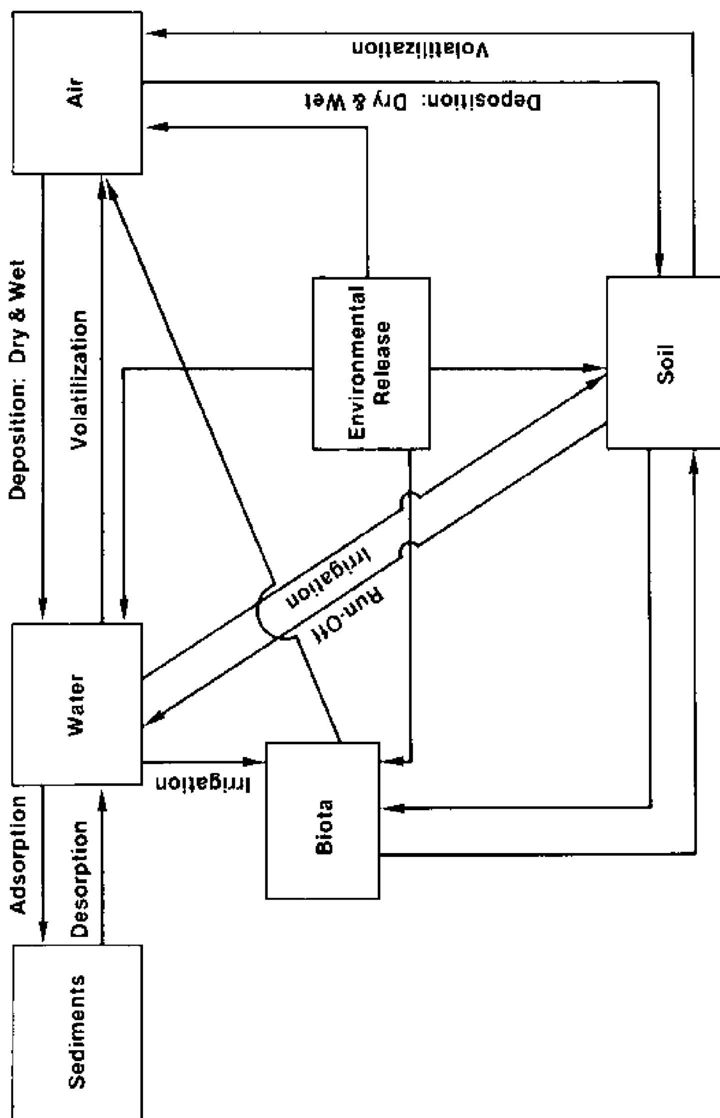

Figure 3. Components of a partition analysis.

has been derived from detailed laboratory studies in which single substrates and single species of microorganisms were used. There has been some work with microcosms in which "natural" population mixtures have been exposed to combinations of chemicals in complex environments. These efforts leave a lot to be desired in terms of replication, data interpretation, and extrapolation to *in situ* conditions, although some recent microcosm work appears to be very promising for preliminary screening purposes. Initial approaches to testing for biodegradation in our organization represent compromises between the two alternatives; i.e., natural mixtures of microorganisms are exposed to single chemical candidates in a laboratory milieu that is controlled and amenable to replication. This is not a particularly ideal compromise, but it appears to be the best approach one can take considering current deficiencies in our knowledge and the extent of our responsibilities under the Toxic Substances Control Act. The Act implies that we must identify methods that could apply to the entire spectrum of chemical compounds already in commerce or that will achieve that status in the future. Ideally, any methodology we provide as guidance or as a requirement should be validated, amendable to standardization, and acceptable to the scientific community. We certainly have not yet met all these goals.

I would now like to discuss other and more specific problems we encounter in considering tests for biodegradation. Temporary solutions of these problems or issues sometimes require decisions based exclusively on personal judgement. Although decisions arrived at on this basis may often appear to be arbitrary, no decision has permanent status and all decisions must be amenable to modification as our knowledge and experience expand. Some of these issues are not restricted to biological mechanisms but apply to chemical and photochemical transformations as

well. The problem of defining "significant degradation" is one that has stimulated a great deal of controversy for many years. Do we gear our definition to a finite exposure time or to a percent loss of parent compound? It may be that an acceptable definition of significant degradation cannot be a general one but, rather, will be a definition that is compound specific and dependent upon both the mechanism of degradation and the concentration of chemical released into the environment.

Another problem concerns the design of experiments to assess degradation. The whole purpose of testing is to extrapolate laboratory results to predictions of what occurs in nature. Obviously, it is impossible to duplicate nature in the laboratory, so the question is, "How close can we come to meeting this goal within the confines of what we already know and what is economically feasible and possible to do with available human resources?"

Another difficulty associated with extrapolation to the "real world" involves the relationship of results from the testing of a single mechanism of chemical alterating in the laboratory to what happens in the environment where pollutants are subjected to multiple degradation mechanisms. Are we justified in assuming that the rate and extent of degradation from all mechanisms are additive? I believe that examples can be found in which degradation by one route can preclude the possibility of degradation by another route.

Finally, there is the general problem of what to do about stable degradation products.

This involves their identification and the decision as to which degradation products are important or significant. Degradation products must be addressed because there are several examples of situations in which they have proved to be more toxic than the parent compounds from which they were derived. However, the designation of importance to any one or several of them in the absence of data concerning their toxicological effect can be erroneous if based solely on their relative concentrations in degradation mixtures. Since separation and identification of components of a mixture may be an arduous and expensive procedure, it may be possible in some instances to test degradation mixtures for biological activity before undertaking a detailed chemical analysis. However, this choice would have to be left to the manufacturer or his agent and would, in large part, depend upon the particular compound under consideration. Needless to say, there are a number of problems associated with this approach, the most serious of which is the possibility that some potentially hazardous components in a mixture may be present at concentrations below their toxicity thresholds.

I would like to turn to some problems specifically associated with biodegradation testing. The areas of particular concern are the choice of inocula, media, and substrate concentration. Of these, the choice of inocula is probably the most difficult to make. First, the decision regarding the use of pure or mixed cultures poses a problem. While pure culture testing is easy to handle and results are relatively easy to interpret, it precludes the impact of interspecies interactions such as synergism, commensalism, symbiosis, and antagonism that occur in the real world. On the other hand, there is some question about whether these relationships can be duplicated in the laboratory even if natural mixtures are used as inocula.

Furthermore, if we were to use natural mixtures of microorganisms as inocula, we would still have the problem of deciding what sources to use and how to handle them prior to initiating testing. Keeping in mind that we have to devise testing protocols general enough to be applied for use anywhere in the country (i.e., not specific for one type of chemical manufactured, distributed, and used in a well-defined geographical area), it would seem logical to select a "typical" source containing a "typical" distribution of microorganisms. Since this is as impossible to do as to define a "typical" soil, we must explore other alternatives. One possibility is to allow the use of any source and to require: (i) a complete characterization (physical, chemical, and biological) of that source and (ii) the use of structurally related reference compounds whose degradation characteristics are known as comparators.

Another problem concerns the size or concentration of inocula in biodegradation testing. Concentrations of pure culture inocula can be readily manipulated, but this is not true of natural mixtures. The latter can be adjusted with regard to total biomass, but the maintenance of relative concentrations of microbial components is difficult and depends upon pretesting treatment which could easily result in significant divergence of population behavior from that which could be expected *in situ*. Of equal importance is the fact that rates of degradation are related to numbers and types of microorganisms in inocula, and any attempt to accelerate the process by increasing inoculum concentration could lead to false conclusions. This problem is intimately associated with selecting criteria for or defining degradation.

The use of adapted or nonadapted inocula poses another problem. If the chemical of concern

is released into the environment as a result of an accident or spill and thereby represents a single instance of environmental insult, a case can be made for the use of nonadapted inocula. However, most of our attention has to focus on those pollutants which are or can potentially be released into the environment on a continuous basis and, as a consequence, provide the microorganisms with which they come into contact sufficient opportunity to adapt to their presence. One solution to this problem is to test adapted and nonadapted inocula in parallel.

Finally, there has been considerable controversy about the importance of anaerobes in biodegradation. It is difficult to minimize their importance when one looks at such environmental media as sediments and eutrophic waters where dissolved oxygen levels can be very low. Therefore, I believe that some testing for anaerobic degradation of chemical pollutants must be incorporated into any battery of tests designed for evaluating biodegradation.

Another group of problems we must resolve in order to identify suitable protocols for biological transformation is associated with the selection of test media. Should we conduct tests in laboratory culture media or in "natural media" (i.e., river, lake, ocean waters, etc.)? Some of the arguments for and against either alternative are similar to those presented for the use of pure or natural inocula. If we use laboratory media, how can we possibly extrapolate results to the natural environment? Again, one possible solution appears to be the use of a natural medium that is characterized as fully as possible and the use of reference compounds that would be run in parallel with the compound of interest.

An additional problem in the area of chemical fate assessments is the selection of

substrate or candidate chemical concentration. It would be ideal to utilize that concentration of chemical which is likely to appear in the environment. However, calculations of environmental concentrations are dependent upon, and would therefore have to follow, chemical transformation testing. The concentration of chemical substrate is important since responses can run the gamut from stimulation to inhibition when the concentrations of some chemicals are varied. Furthermore, there is evidence that, because of dependence upon concentration gradients or other reasons, some compounds will not undergo transformation until a critical environmental concentration is reached.

Role of Microorganisms in Ecological Effect Determinations

Microorganisms play another role in the determination of chemical risk. In Figure 1 we saw that the impact of a chemical pollutant on organisms other than humans is an important factor in the evaluation of risk. As we all know, microorganisms are ubiquitous in nature and play a major role in maintaining most, if not all, ecosystems. Therefore, any pollutant that can adversely affect natural populations of microorganisms can potentially disrupt those ecosystems. For example, microbes are largely responsible for the decomposition of cellulose and protein wastes and, thereby, assure the continuous availability of vital nutrients to other life forms such as plants. It is not difficult to visualize how a massive interruption of this process could set off a chain of events that could lead to major dislocations of plants, animals, and, eventually, humans. Consequently, some provision must be made in the assessment of the ecological impact of chemical pollutants for

determining whether or not they are potentially capable of adversely affecting key groups of microorganisms.

Included in a list of tests which has been compiled to provide guidance for the manufacturers of new compounds are procedures which are designed to determine whether chemicals will adversely affect certain types of microorganisms. Common to all tests for ecological effects is the problem of selecting the species of organisms to use. Ideally, the test species chosen should be representative of other forms of similar biota in a particular ecosystem with regard to their sensitivity and response to chemical challenge. However, in the case of microbial tests we are also interested in species known to play critical roles in biogeochemical cycles for which reasonably good tests are available. I do not intend to go into detail with regard to the tests themselves except to say that the issues this type of testing raises are similar to those already discussed for persistence testing. Thus, selection of species, extrapolation from laboratory to natural environments, size of inocula, substrate concentrations, etc., have to be considered carefully.

Summation

I have discussed the role of microorganisms in the process of developing a scheme for evaluating the fate and ecological effects of chemicals after they are released into the environment. Although we are not locked into any particular approach, we have a fairly good idea what types of information have to be considered in developing one. I have attempted to identify some of the problems that we recognize in this regard. I do not believe there is any doubt that many of the problems associated with the determination

of chemical transformation and ecological effects on microorganisms are complex and require considerable research and development before they can be resolved. The issues already discussed, and many I have not had the time to present, should provide the basis for a substantive dialogue for some time to come.

^{14}C Heterotrophic Studies: A Comparison of Four Ways of Calculating Kinetic Parameters

CAROL D. LITCHFIELD

During the past few years the concept of applying microbial uptake and respiration rates to environmental analyses has gained general acceptance. Since its first introduction by Parsons and Strickland (1962), heterotrophic potential measurements have seen many improvements in technique, such as including in the final calculations the respired $^{14}CO_2$ (Hobbie and Crawford 1969; Kadota, Hata and Miyoshi 1966) or using tritiated substrates (Azam and Holm-Hansen 1973).

Inherent in all of the measurements is a recognition that one is making rate measurements and, therefore, that there are certain technical constraints imposed on the analysis. There have been numerous reviews of the assumptions involved in the "saturation kinetic" model (Litchfield 1976; Thompson and Hamilton 1974; Wright 1973; Wright 1974), and one need only recall here that the linear plot of the transformation of the data as unit time divided by percent utilization (t/f) versus the added substrate concentration (s)

should result in the straight line shown in Figure 1. To obtain these types of plots, therefore, one must use multiple concentrations of the test substrate, and unless one knows the system very well, it is necessary to use multiple incubation periods as well. These strictures assure working under conditions where, among other things, the transport systems are saturated and changes in the available substrate concentration are not limiting, nor are there changes in the microflora (Williams and Askew 1968). In other words, all of the requirements for Michaelis-Menton-type kinetics used in enzymology (Mahler and Cordes 1966) or growth rate studies (Monod 1942) must be fulfilled.

In 1966, Wright and Hobbie noted that there were cases in which the test system would not become saturated at some finite substrate concentration, and therefore first-order kinetic calculations should be used (Figure 2) (Wright and Hobbie 1966). In fact, when data plotted according to the Hanes plot above result in a line parallel to the X axis, one can usually replot the data according to the first-order model and thereby obtain information on the rates of decomposition that would otherwise be lost. Such calculations result in turnover times (T_d) and the uptake rate (v_d) if the *in situ* substrate concentration is known (Wright and Hobbie 1966). Therefore, unlike the "saturation kinetic model," the first-order model assumes a direct proportionality between uptake or respiration and the substrate concentration.

Because of the time-consuming nature and intensive personnel and equipment requirements for analysis according to the saturation kinetic model, it has been suggested that either a single incubation time period coupled with multiple substrate concentrations (Griffiths et al. 1977) or a single substrate-single time period could

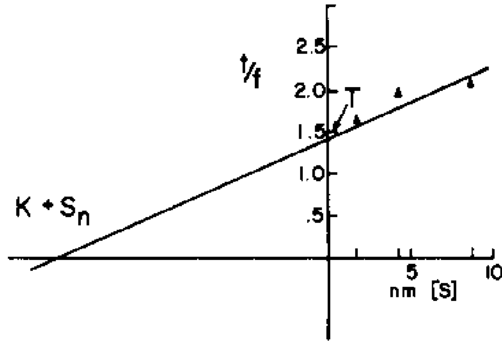

Figure 1. Typical plot of the data from a ^{14}C mineralization study which has been transformed to the Hanes plot describing the saturation kinetic model (Hobbie and Crawford 1969).

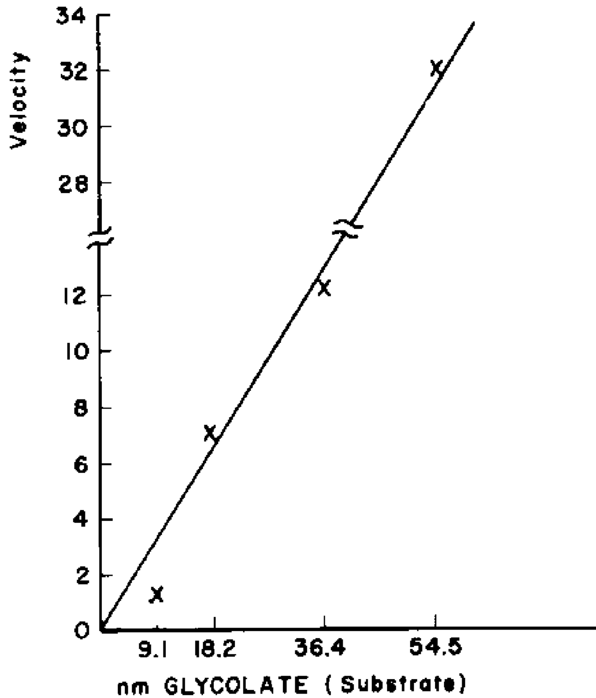

Figure 2. Typical first-order plot of data which do not fit the saturation kinetic model transformations shown in Figure 1 (Wright and Hobbie 1966).

be used (Kadota, Hata and Miyoshi 1966; Williams 1973; Williams and Askew 1968). The former approach would reduce considerably the amount of work, but the latter approach would certainly enable one to use many more substrates in the same amount of time, or to test more stations. Both procedures would, therefore, cost less per sample in terms of personnel, time, and expenses.

Therefore, at least four different approaches have been suggested for estimating microbial heterotrophic activity in aquatic environments: (i) multiple substrate concentrations tested at several different time periods to determine which condition will provide the best model for saturation kinetic calculations; (ii) a single substrate concentration tested over multiple time periods and using the v versus t to calculate turnover times and uptake rate; (iii) multiple substrate concentrations at a single time period, assuming first-order reaction rates; and (iv) a single substrate concentration used for a single time period.

During the past few years we have been testing the heterotrophic potential and mineralization capacity of the microbial flora in the New York Bight. In all cases we have used two ^{14}C-labeled substrates, urea and glycolic acid, for which we can measure quantitatively the *in situ* concentrations (Nakas and Litchfield 1976; Shah and Wright 1974; Wright and Shah 1975; K. Edenborn and C. D. Litchfield, unpublished data). The water column and sediment experiments were designed to permit the calculation of the kinetic parameters based on the saturation model, i.e., multiple substrate concentrations and multiple incubation periods. Both heterotrophic mineralization and heterotrophic uptake were calculated for the water samples by use of standard techniques (Wright and Hobbie 1966), whereas only the heterotrophic mineralization rates were determined

for the sediments by use of procedures described elsewhere (Litchfield et al. 1979).

Subsequently, it was decided to examine the uptake data with a view to comparing the different methods of calculating turnover times and/or uptake rates. Data from one water column station and one sediment station were selected and are shown in Table 1. The initial calculations were made according to the saturation model (Wright 1973) and then were recalculated by the other three suggested procedures (Griffiths et al. 1977; Kadota, Hata and Miyoshi 1966; Williams and Askew 1968; Wright 1973).

Only data for mineralization, i.e., $^{14}CO_2$ respired, for urea as the substrate are given. From Table 1 it is evident that the uptake rates *(v)* calculated by the four recommended methods result in an extremely wide range of values. For the sediments, the range is from 4.09 to 23.42 nmol/h; the turnover times range from 2.26 to 12.95/hr. Both of the kinetic parameters have an approximately 6-fold range. When the water column data are examined, the uptake rates vary from 92.26 to 1.13 nmol/h. In this case, the saturation kinetic values for uptake rates were higher, and more closely approximated similar values calculated for the first-order model. Both the single substrate-single time and single substrate-multiple time techniques gave substantially lower uptake rates (from 20 to 100 times). As would be expected, the effects on turnover times were reversed: shorter turnover times were calculated for the saturation and first-order models, and approximately 10-fold longer turnover times were calculated for the other two procedures.

Previous studies with similar sediments (Litchfield et al. 1979) had shown that the coefficient of variation was significantly less when turnover times were calculated from replicate

Table 1. Comparisons of different methods for calculating kinetic parameters[a].

Sample	Incubation time (h)	Saturation kinetic system					First-order kinetic system		Multiple time-single subst concn[b]		Added sub-subst concn (nm)	Single time-single subst concn	
		T_t	V_{max}	K_t+S_n	v_t	r	T_d	v_d	T	v		T	v
Sediment[c] (R-20)	0.167	2.30	0.88	-2.04	23.12	0.76	8.22	6.44	3.8	13.93	1.95	4.45	11.9
							-		9.5	5.57	3.90	11.48	4.61
									NS	NS	7.81	8.80	6.12
	0.50	2.26	0.77	-1.75	23.42	0.87	8.90	5.95				5.89	8.99
												10.18	5.20
												10.60	4.99
	1.00	3.78	0.58	-2.18	14.00	0.71	12.95	4.09					
Water (211-surface)	0.167	0.31	11.2	-3.52	92.26	1.0	0.98	29.2	5.2	5.5	1.95	4.81	5.95
									NS	NS	4.88	10.18	2.81
									NS	NS	7.81	14.40	1.99
	0.50	0.65	5.72	-3.72	44.00	1.0	1.37	20.9				7.66	3.73
												14.49	1.97
												18.97	1.51
	1.00	1.05	5.36	-5.61	27.24	1.0	1.86	15.4				10.04	2.85
												20.42	1.40
												25.34	1.13

[a] All values calculated by the methods of Wright and Hobbie (1966). T = turnover time in hours; V_{max} = nanomoles of substrate/hour; v = uptake rate in nanomoles/hour; r = correlation coefficient; $K_t + S_n$ = transport constant + *in situ* substrate concentration.

[b] Because of different basis for calculation, the single substrate concentration used is listed for the multiple time periods which correspond to those listed for the other three systems. NS = not saturating; therefore, calculation not possible.

[c] Only $^{14}CO_2$ respired has been used in these calculations.

samples than were the corresponding maximal uptake rates. A rule of thumb developed from those sediment studies indicated that a greater than fourfold range in turnover times was necessary before a statistically significant difference was obtained (Litchfield et al. 1979). Similar replicate studies have not been reported for replicate water column analyses. However, Griffiths et al. (1977) did note that the correlations between the saturation kinetic method and the single substrate concentration-multiple time period assay were better for the sediments than for the water column, where presumably they also found 5 to 50 times difference (Table 1) (Griffiths et al. 1977).

In summary then, application of the ^{14}C heterotrophic technique to natural samples still requires extreme attention to detail and care in execution. The turnover times seem less susceptible to manipulations during calculation, and the uptake rate is extremely sensitive to the methodology employed. The ideal method would still seem to be to attempt to use saturation kinetics and to go to the shorter methods only as a last resort. Certainly, there seems little justification for the single substrate concentration-single time period analyses. This point was made previously by Gocke (1977) and is reemphasized by the data reported here. Above all, we must remember that heterotrophic potential/mineralization data are not absolute; there is too large an inherent error, which even the best statistical procedures cannot remove. However, when coupled with other data and certainly when performed during seasonal studies, this technique can be a most valuable tool for estimating changes in the microbial response to environmental pressure.

Acknowledgments

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Microbiological Testing Under the Toxic Substances Control Act

WILLIAM E. GLEDHILL

Federal regulations, such as the Toxic Substances Control Act (TSCA) (Environmental Protection Agency 1979), will have major impact on the future research in the chemical industry. The TSCA requires extensive testing of new chemicals prior to manufacture and of certain existing chemicals for which sufficient data are lacking. Among the numerous test requirements under the TSCA are two categories of microbiological tests. The first, under ecological effects testing, requires measurement of the toxicity of chemicals to metabolic processes of several ecologically important microorganisms. The second, under persistence testing, requires establishment of the biodegradability of chemicals. The purpose of this discussion is to describe briefly proposed testing in these two areas, to present some key concerns about the tests, and to indicate areas where future research is required.

Microbial Effects

Short-term shake-flask or bottle tests are

proposed under the TSCA to assess toxicity of chemicals to microorganisms involved in the carbon, nitrogen, and sulfur cycles. Different concentrations of the test chemical are used to establish levels inhibitory to (i) cellulose decomposition by *Trichoderma longibranchiatum*, (ii) ammonia production from urea by soil or sediment microorganisms, (iii) sulfate reduction by *Desulfovibrio desulfuricans*, and (iv) growth of four species of algae.

In the absence of inhibition of these processes, at concentrations of the chemical which might be expected in the environment, no further testing is necessary. Adverse test results indicate a need for further testing using mixed populations from natural sources and examining other functions such as nitrogen fixation, dehydrogenase activity, invertase activity, nitrification, and pectin or protein degradation.

The rationale for and the appropriateness of these tests is subject to question. Because of adaptive capabilities, cell structure, and the protection afforded by the environment, microorganisms, in general, are less susceptible to the toxicity of chemicals than higher organisms. There are, indeed, problems associated with both the assessment of chemicals effects on microbial activity and the interpretation of test results (Grossbard 1973). Microbial processes, however, are essential in key element cycles. Microorganisms are responsible for all of the nitrogen fixed from the atmosphere, approximately one-third of all primary production of fixed carbon and regenerated oxygen, ammonia conversion to nitrogen, oxidation of sulfides, and conversion of organics to carbon dioxide and methane (Kelly 1978). Therefore, some testing for chemical toxicity to this class of organisms would seem appropriate.

Are the most appropriate test organisms

proposed? Rather than representatives of major biogeochemical cycles, would it be better to select organisms for testing on the basis of taxonomy? It is conceivable that certain chemicals would have no effect on the proposed test organisms, but could have significant effects on other species. Conversely, a compound could inhibit all test organisms with the proposed methodology, but under actual environmental conditions have insignificant effects. Selecting a variety of representative environmentally important species of bacteria, fungi, yeasts, algae, and protozoans on the basis of taxonomic considerations and ease of laboratory growth could simplify the testing requirements and increase the utility of test results. Microorganisms could be cultured in test tubes, and the concentration of test chemical inhibiting microbial growth could readily be established. Toxicity results could be expressed in terms of the EC_{50}, the concentration of test chemical which inhibits growth by 50%. Since microbial toxicology is a new field, it is only through continued evaluation of various test organisms that the most appropriate aspects could be selected.

Another concern with the proposed toxicity tests is how the results can trigger meaningful regulation. Adverse test results can only indicate the need for further testing. Further testing under the TSCA is an open-ended process. Limits to testing must be set. With certain chemicals it may become necessary to determine how toxicity to pure laboratory cultures relates to toxicity under actual environmental conditions. This aspect will be discussed in more detail below.

Microbial Degradation

Biodegradation procedures are almost as numerous as the number of chemicals on which

biodegradation has been studied (Howard et al. 1975). Very little interlaboratory validation of methods has occurred. Those tests which have been subjected to validation have only assessed primary biodegradation, i.e., the disappearance of parent material. Standardized and validated methods to measure ultimate biodegradation, the complete mineralization of a chemical to inorganic components, are not available.

Biodegradation test protocols under the TSCA are unique in that they center on ultimate biodegradation, a step in the right direction. Tests are designed to measure (i) the extent of dissolved organic carbon disappearance in a synthetic medium and in an activated sludge system, (ii) carbon dioxide evolution or oxygen uptake in closed bottle tests, and (iii) gas production in an anaerobic digestor. For the time being, these tests will be of general utility; however, they have several drawbacks which will hinder future regulatory needs. The indirect methods used to assess biodegradation require addition of relatively high levels of test chemicals to the medium. Such levels would not be encountered in the environment and, with certain chemicals, would be toxic to microorganisms. Biodegradation of these chemicals would not occur in the test systems, whereas they may be readily degradable at environmental levels. The shake-flask and activated sludge methods are of limited utility. They rely on dissolved organic carbon measurements which do not permit assessment of the biodegradability of chemicals that have low aqueous solubility or adsorb to microbial biomass. Furthermore, all of the laboratory tests proposed under the TSCA may be meaningless when extrapolation to the natural environment is made. Biodegradation characteristics of chemicals at environmental concentrations may be entirely different from those at concentrations normally evaluated in laboratory systems (Wszolek and Alexander 1979).

Areas for Future Research

Proposed TSCA test methodology has drawn attention to the fact that a significant amount of research is required in the areas of both microbial toxicology and degradation. As indicated previously, laboratory methodology is needed that simulates more closely microbiological events occurring in the natural environment. In the future, the use of laboratory microcosms to examine the fate, persistence, and toxicity of chemicals may satisfy this need. From the viewpoint of microbial ecology it would be useful to be able to go to the natural environment to determine whether chemicals are having adverse effects on specific microbial processes. Are there key "indicator microorganisms" which can be used to detect subtle long-term effects of chemical pollution? Goals such as these are, perhaps, unattainable; however, the pragmatism of federal regulation necessitates the devotion of substantial effort to this area.

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Polyphasic Study of the Microbial Ecology of Bacteria-Phytoplankton Interactions

MICHELINE A. G. BIANCHI

The microbial ecology of eutrophication has been under study in my laboratory for several years. The research project involves the use of a system I have formed called the ECOTRON.

Plankton populations, in continuous primary production, are monitored in a large outdoor tank containing approximately 40 m^3 of seawater. The seawater is pumped into the tank from a lagoon (Figure 1). The water flow is continuous, permitting renewal of the water in the tank and control of the population density, and preventing accumulation of metabolic products. Fertilization of the tank is accomplished by addition of nutrients, i.e., phosphate, nitrate, and silicate, to the continuously flowing seawater entering the tank.

Enrichment induces an increase in the natural plankton population, a phenomenon familiar to planktologists. Data on the bacterial populations are collected at established time intervals. A

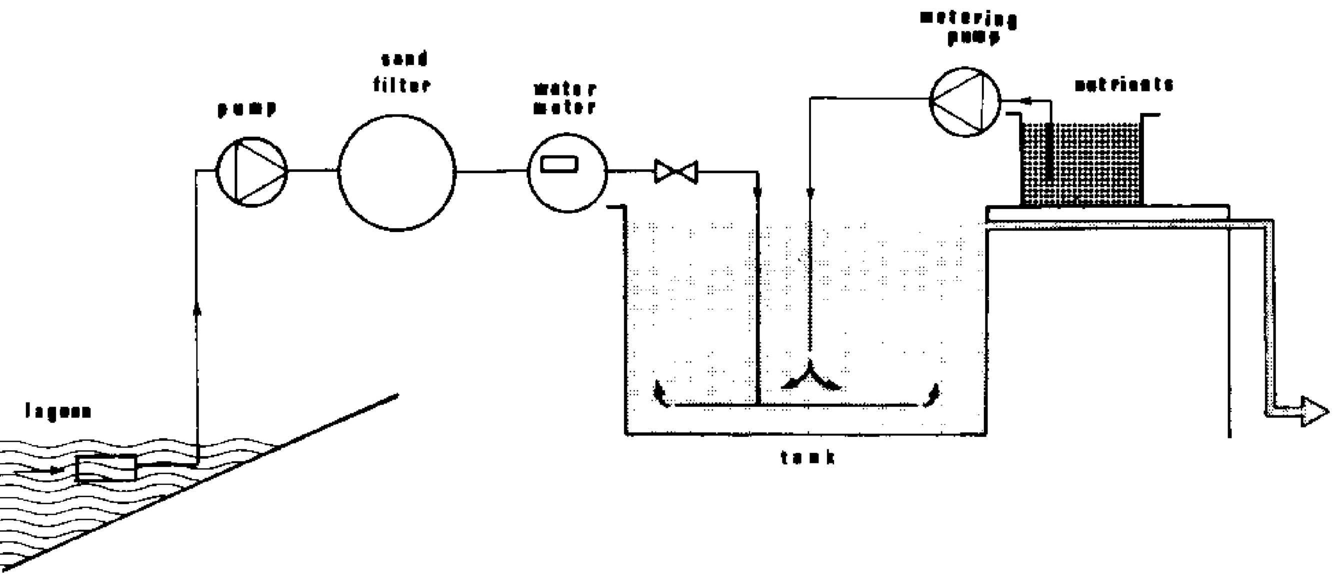

Figure 1. Diagrammatic representation of the ECOTRON employed in this study.

singularly important advantage of the ECOTRON is that it provides an excellent opportunity for multidisciplinary study.

Analysis of the dynamic interaction of phytoplankton and bacterial populations is a major objective of our study. Therefore, bacterial and plankton biomasses, as well as the taxonomic composition of these groups, are measured. Autotrophic and heterotrophic activity are monitored also.

It is recognized that the experimental design of the ECOTRON is simplistic because, in the natural environment, interactions are complex and interwoven. Nevertheless, interactions of algae and bacteria can be examined and conclusions can be drawn that will be useful in understanding these interactions. It should be emphasized that phytoplanktologists, biochemists, and bacteriologists work very closely on this project, carrying out sampling and collecting data on the same schedule.

In Figure 2, the measurements taken on the phytoplankton and bacterial populations are summarized. The experimental procedure employed in the ECOTRON experiments is as follows. Biomass (both plankton and bacterial populations) measurements are made. The dynamics of the bacterial and phytoplankton populations, measured by use of diversity indices, are then recorded. Pure cultures of bacteria are isolated. The 2216 medium of Oppenheimer and Zobell (1952) is used because it is commonly employed by marine microbiologists and, therefore, permits comparison of results of our experiments with those of other investigators. With a special grid, 30 strains are selected from the count plates prepared for each sample. Each of the 30 strains is characterized by means of 90 morphological, biochemical, and nutritional tests. The results of these tests are coded, and the

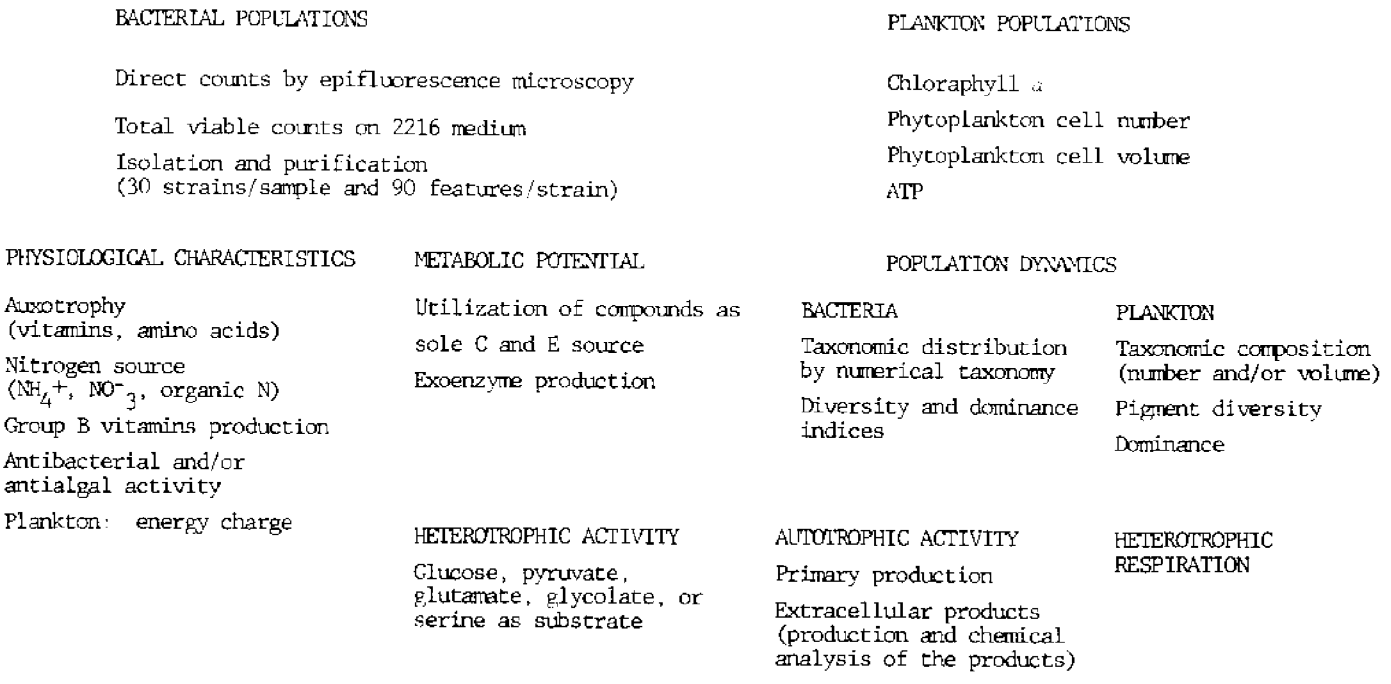

Figure 2. Flow chart of the sampling schedule and analytic procedures employed.

taxonomic data are analyzed by numerical taxonomy methods.

Diversity and dominance indices are calculated so that the bacterial and phytoplankton communities can be compared seasonally and according to treatment of the tank. For bacteria, because of the difficulty in identifying species, two types of bacterial profiles are established. From the clusters obtained by numerical taxonomy, ecological, i.e., metabolic, profiles are calculated. Taxonomic profiles are also developed, for which identification and classification are done only to the generic level, which is sufficient for the purpose of the ECOTRON studies. Phytoplanton and bacterial populations are enumerated in the same samples.

In addition to the above, the physiology and metabolic potential of the populations, bacterial and phytoplankton, are measured. Auxotrophy, nitrogen source, vitamin B production, and production of antibacterial and/or antialgal metabolites are measured, as well as energy charge of the plankton populations. Autotrophic and heterotrophic activity are also measured (see Figure 2).

Figure 3 provides results obtained during an experiment conducted from 27 April to 17 May 1977. At the beginning of the experiment, when oligotrophic seawater was present in the tank, plankton and bacterial populations were diverse. The bacterial populations were able to utilize low-molecular-weight compounds. Of the taxonomic groups, the pseudomonads were predominant, and these bacteria were able to utilize a variety of organic compounds as sole carbon and energy source.

With addition of nutrients, an increase in plankton biomass occurred. The diversity index decreased, while the plankton dominance increased. Interestingly, the diversity of the bacterial population increased. Bacterial diversity was

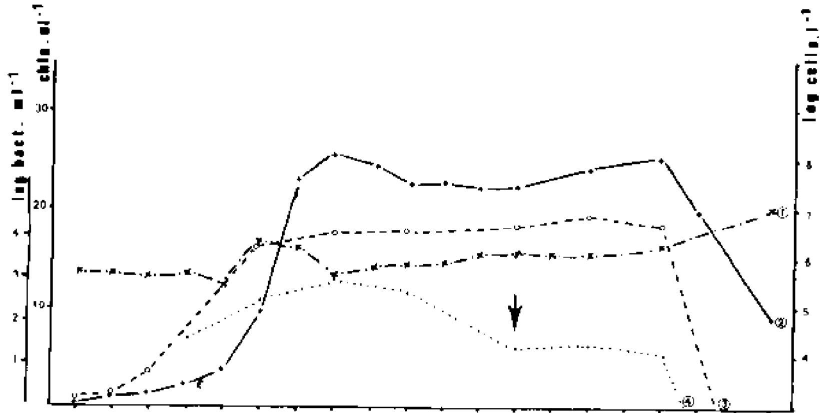

Figure 3. Changes in bacterial and phytoplankton populations, including structure and activity (spring 1977).

observed to be high when the energy charge of the plankton population was high. Thus, it appears that whenever the phytoplankton population was "active," the bacterial population diversity increased, and when a major component, e.g., *Chaetoceros* spp., of the plankton population decreased, a lower energy charge for the plankton occurred and the diversity index of the bacteria was reduced. Concomitantly, there was increased breakdown of high-molecular-weight compounds by bacteria, i.e., predominantly by the vibrio group of bacteria.

At the end of the 20-day experiment, the phytoplankton population returned to a community structure similar to that present at the beginning of the experiment in April. The diversity index of the bacterial population returned to a high level, the same as the start of the experiment.

Figure 4 gives the results of an experiment conducted during the fall season of the same year, 24 October to 24 November 1977. During phytoplankton growth, intense heterotrophic activity was found to be directly related to excretion of extracellular products. The two maxima for excretion of extracellular products corresponded very closely to the two maxima observed for glucose assimilation. Total viable counts of the bacteria also increased at this time.

When primary production decreased, heterotrophic activity was no longer found to be parallel to the production of extracellular metabolites. Again, the bacteria demonstrated a complete population compositional change at the end of the experiment, with a low diversity index recorded, and an increase in the vibrio group of bacteria noted, as was observed in the spring months.

In conclusion, by using a variety of methods suitable for monitoring microbial populations, it

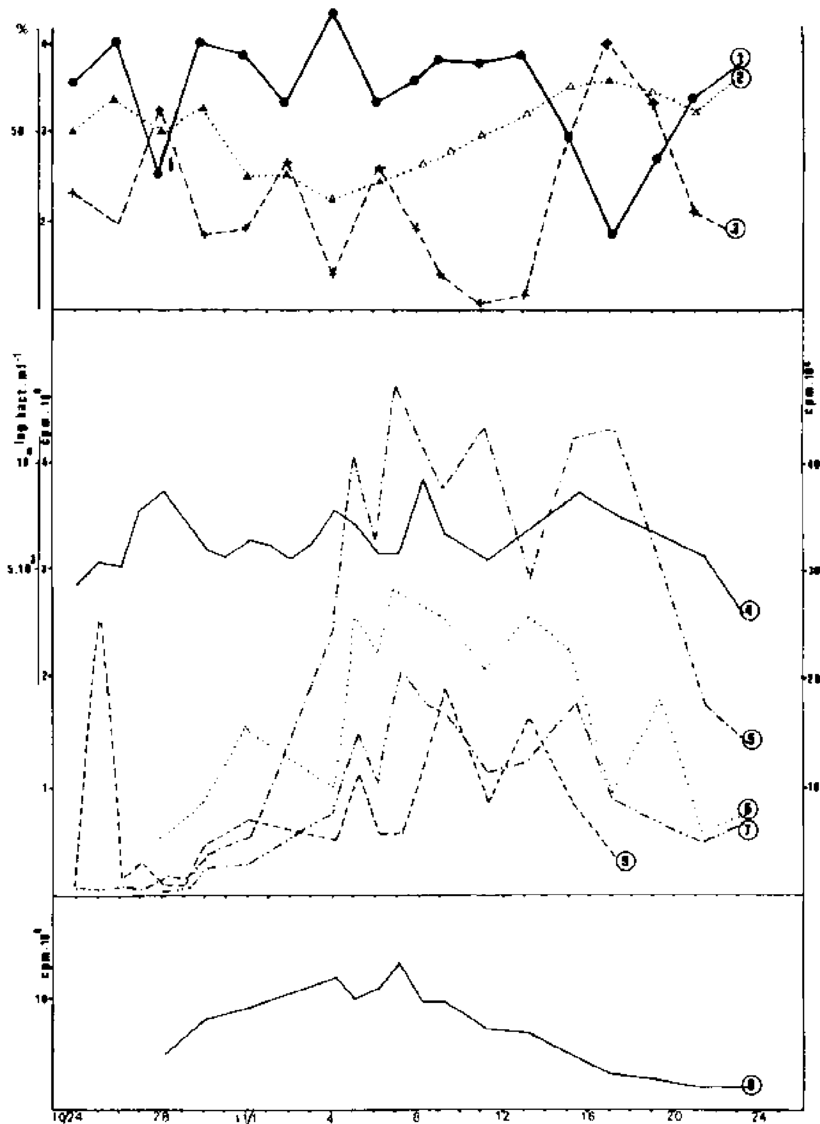

Figure 4. Changes in bacterial and phytoplankton populations, including structure and activity (autumn 1977).

can be shown that bacteria demonstrate fluctuations in population biomass, activity, and generic composition corresponding to similar population characteristics for the plankton. It is concluded that diversity indices are very useful and that a polyphasic approach, both for taxonomy and ecology, is needed if meaningful results are to be obtained for understanding microbial interactions.

Acknowledgments

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Discussion

MICROBIOLOGICAL METHODS FOR ENVIRONMENTAL ASSESSMENT

ALEXANDER: In the microbial ecology of soil, a question was once raised that I think is appropriate to raise again here. How many of your methods, we are asked in soil microbiology, are really of interest to anybody else but the soil microbiologist? Are you giving any information that is useful to higher organism ecology? Does anyone care but us?

A lot of the interest in aquatic microbial ecology comes from the enactment of the Toxic Substance Control Act. This has led to a flurry of activity in government, in a number of industries that will be subject to regulation and in some university and private laboratories designed to examine methods for assessing stress effects on microbial communities and microbial processes. It has been satisfying to see governmental, industrial and academic laboratories moving so quickly into what was before a complete void. I am not sure the situation has been clarified, but at least there is activity.

I would also like to point to two differences

between the more academic research approach to testing methods and the approach Dr. Stern just presented. If we in the Universities do not get an answer today, we can always wait for the next cruise, or wait for the next spring. If this year's data are not too good, we can do the experiment again next year. The problem in regulatory agencies is they cannot wait until next year, because the answer was due yesterday. The second difference is that if we make a mistake, we can recant in a future issue of the journal. If one makes a mistake in this area, by contrast, it may cost millions of dollars or lead to some serious consequences.

FLOODGATE: If I may say so Marty, we are very much aware in the United Kingdom and in the universities of the problems and pressures that beset regulatory people. We know the difficulties that they have, but we just sort of sympathize and pat them on the back and hope that they will not get too worried. But one of the things that bothers me about what we have been hearing in the last few minutes is the idea of a representative organism. If somebody asked me to provide them with a representative bacteria, I wouldn't know which one to give them. I can't find or chose for you a representative American because I just don't know enough Americans to pick out a representative one. I could possibly, using my sense of humor, pick out a representative New Yorker, but picking a representative implies knowing the spectrum. Unless we know the whole spectrum, we can't pick out a representative organism. Secondly, I am bothered by the idea of the "real world." Is there one real world or are there many real worlds? Somebody was telling me yesterday that they had driven 800 miles from the top of Florida to the south of Florida. Now if I drove 800 miles south from the north of Scotland, the last two hundred miles I would be swimming in the Atlantic. America is a very big country and there isn't one real world here, there

are dozens of them. I am not going to teach you geography, but you think from the tropics to Alaska. There isn't just one real world which a regulatory body can pick out, but hundreds of them.

ATLAS: Some were concerned last night when Jim Tiedje said he didn't want to see microbiologists performing more effects work. I am more concerned with the types of effects work called for in some of the EPA regulations. I have been involved in some pesticide testing for industry to meet new registration for EPA regs and I am particularly concerned with some of the sorts of experiments that EPA has been requiring in this respect, particularly with reference to pure culture work. I don't think that the pure culture work and the effects on pure cultures that have been proposed and carried out as part of registration procedures have any relevance. You can show toxicity to pure cultures, but if you look though an environment like soil or sediment with its binding and other effects, its other organisms that may degrade and detoxify or transform chemicals, you simply won't see those same toxic effects. When you finish that sort of testing and you show a toxic effect, so what? The chemical may be toxic to that particular organism in the test tube, but you know *a priori* that the results have no relevance to the sorts of concerns that you must have in the environment. I am much more prone to favor testing in mixed communities, in sediment, water or soil having mixed microbial communities that allow for interaction. I may have a simplistic approach to some of the pesticide work in soils. If you know the general geographic area, the soil conditions and the concentrations to be applied to that soil, then you have some basis for choosing representative soils. The problem is much more complex if you look at widespread utilization. The concern in Great Britain and Europe with regulations for such compounds extends to testing not two or three organisms but let's say ten soils, ten different types

of soils with a pesticide added to determine the sorts of effects. Also, I guess I am much more influenced by the sorts of results that examine effects on processes in natural communities, whether they be cellulose degradation or litter turnover in a community, or nitrification, denitrification or nitrogen fixation in a community. But even there I think I get into some problems and I know industry gets into problems interpreting impacts. If in a test system you see a 5 or 10% change in a rate of some critical process--a process you are willing to say is critical like nitrogen fixation--what does that mean you should do about regulating that particular compound? What does it mean in terms of the statistical variability that normally occurs in such a situation? It's simple if your substance wipes out all activity. You know you have a 100% kill or have lost all activity and you don't have a problem; but when you see the sorts of effects that are 10% effects, you are left with the same question you started with: What should I do about it?

BARTHA: I want to add something to what Dr. Atlas said. Some of the pure culture tests were suggested by EPA in order to explore essentially the interactions of pesticides. What effect will a new pesticide have on the degradation of a previously used pesticide? I agree that this concern is a valid one, but it does open up a big can of worms. After all, how many combinations of substances can you test? The EPA's approach to this problem is "Let's test the effects of the new pesticide on the growth of pure culture which is known to degrade a pesticide." I believe this is a very indirect and probably not a very valid approach. If something like this absolutely has to be done, I think the viable way to do it would be to pick out a few model pesticides, an organophosphate, a carbamate, etc., radiolabel them, put some in a test system with a new test compound and see how $^{14}CO_2$ evolution from this compound is

influenced by the new compound. This would be at least a direct test and a valid one. But how many substances to test? If you play this game, that becomes a big question, especially as the subject is broadened by the Toxic Substances Act from pesticides to including all sorts of other compounds. In practice we may have to abandon this cross-testing all together.

Another remark I would like to make is in response to Dr. Gledhill and Dr. Tiedje. Is there really a substance, besides an antibiotic, that hits microorganisms harder than higher organisms? Well, I agree with them that in the majority of cases the higher organisms are more sensitive. However, if this were a general truth, we wouldn't have any fungicides. Another example that has practical use is the inhibition of nitrification by extremely small concentrations of nitraphrin, also called N-serve. Fractions of a part per million have a very dramatic effect on nitrification, which is actually used for keeping the ammonium fertilizer in the soil and preventing it from being oxidized and the resulting nitrate leached out. These examples illustrate that extremely small concentrations of nonantibiotic substances can have dramatic effects on the biochemical cycling processes.

ALEXANDER: I would like to ask Dr. Stern if there are any cases in which an antimicrobial synthetic chemical was introduced into waters or soils and the fact that it was antimicrobial led to a cancellation of its registration? That is, has this or any country banned a chemical because it has been shown to be antimicrobial? The answer is no, I presume. To me this is a pressing question: Are we now spedning time testing the effects of chemicals on microorganisms only to find later that no one is going to listen to us? Why do all the work and why require industry to to all that testing?

STERN: I think I was a little hasty in saying no, Marty. I think that there is plenty of evidence that antimicrobials do disrupt waste treatment plants and this impacts greatly on environmental loadings. At the moment, I'm not sure that I can provide another example.

WOLIN: I have a difficult time understanding the complete acceptance of the philosophy that if something is biodegradable or might possibly be biodegradable, then our strategy is to let the environment take care of it--rather than trying to focus on the possibility of a technology for biodegradation of potentially biodegradable point source chemicals prior to their entry into the environment. I don't know of any natural microbial biochemical process that has been made commercially significant without eventually taking it from the natural environment and using modern technology to take advantage of that natural process. Any kind of microbial activity in the food-processing industry was derived from some natural process in the past, but I don't think we could enjoy wines today if people had to go around crushing grapes with their feet and letting them ferment in a vat in the back yard. Even antibiotic and amino acid manufacture couldn't actually happen if they were not laboratory-adapted processes brought to pilot plant and production by controlled bioengineering technology. When people talk about economics, I think that focusing on controlled technology may give greater diversity in biodegrading products, because otherwise we are always calling for organisms to do something in a very specific outdoor situation. If biodegradation was carried out in a fermentation tank, there are inexhaustable resources in the microbial world that we might be able to call upon to carry out a biodegradation process that cannot necessarily be found in a back yard. For example, suppose there was an organism in the soils of Kenya that at 37°C could degrade a

compound, but the organism simply isn't found in Tampa Bay. The chemical production of that compound would not be feasible in this area if potential biodegradation was a problem, whereas if the organism could be found somewhere and put in a tank outside a plant and mineralized at that point, I think that there would be greater potential for using chemicals without getting into environmental problems. In addition, I think depending on natural biodegradation destroys the whole opportunity to use modern genetic engineering to handle the technology of biodegradation, because genetic engineering for biodegradation becomes a viable game only if there is a mechanism for using that organism in a controlled scenario. I can't see anybody going out and seeding the world with some organism that is genetically engineered to carry out degradative processes.

STERN: When we talk about regulatory options we are really including control options that go beyond problems associated with biodegradation. This addresses the concern Mike Wolin expressed regarding anticipating environmental pollution and instituting control measures prior to chemical release. I believe that the basic thrust of TXCA is preventative, rather than curative.

ALEXANDER: One has to bear in mind that enormous tonnages of chemicals are released at nonpoint sources. The magnificent control technology in a sewage treatment plant may still have little impact on toxic chemicals in water. So attention must be paid to both nonpoint and point sources. Moreover, sewage is such an important aquatic ecosystem that we should not leave it to the engineers.

Our organizer, Dr. Colwell, wishes to make a point.

is rising to the surface. We should return to our discussion of biomass. It is very interesting that we have come to the same end-point, moving from the applied, i.e., regulatory viewpoint and from our attempt to assess the ecology of the environment at the microbial level. If you change the title of your main graph, Dr. Stern, from problems associated with biodegradation testing to problems associated with biomass estimation, the same uncertainties are involved. What I conclude is that we must gather reliable information about the microbial ecology of the natural system. Trying to apply what we now know before we've properly characterized natural populations leads us to a dilemma. Many participants here are very interested in getting back to discussing methods of estimating biomass.

A question for both George Floodgate and Art Stern is what method should be used to assess microbial activity? Should a melange of methods be used or do we accept the inadequacies of two or three methods and recommend that they be developed further?

FLOODGATE: I think that the important thing here is, if possible, to use a variety of methods, because the assumptions in each of the methods are different. If you take the ATP technique, it is assumed that the bacteria have a certain amount of ATP which is fixed. That's not true, but it's an assumption that you have to make. If you use another technique, for example the electron transport technique, or let's say the LPS technique, then you are making the assumption there is a certain amount of lipopolysacharride associated with each bug. If the two agree, if you get the same sort of trends, then it suggests the results are valid because you are making two different assumptions but are getting the same result. And therefore, presumably, the results you are getting are going in the right sort of direction. On the other hand, you don't want to stand still and say that

any of these methods are in any way perfect. The ATP technique, for all its faults (it has plenty, we know), has developed quite a lot over the last few years and may well develop again in the near future. So I think the true advice I've got to give to Ricki is, use both. Let's by all means use a battery of tests if we possibly can, if we have the time and money and the staff to do it. And the more tests we use the better. If we do get things going together, that's good. If they go against each other, that too is good because it means there is a complication in the system which we haven't taken care of and we've got a reason for looking and finding out what that complication is. But let's also, at the same time, go ahead and try to improve all the techniques we've got and see if we can get more efficient methods. Particularly, we should get better fudge factors. Microecology is moving very, very quickly and what we are doing now will in twenty years time look awfully primitive.

STERN: One of the wisest things put into the Toxic Substance Control Act was a requirement for a review of protocols annually. This means that even the regulatory people realize that the state of the art in a lot of these areas we have been talking about is pretty poorly developed, pretty primitive, and that technology does move on and we had better take advantage of it as it gets here. So nothing we have come up with yet is locked in cement. All of it is amenable to modifications and additions. And as our technology grows, certainly we ought to take advantage of it and make use of it. At the present time we have to go with state-of-the-art technology, so we are stuck with pretty primitive testing, testing which sometimes defies extrapolation to the environment or environments that we are dealing with. But nevertheless, it's the best we've got. We are willing and anxious to incorporate other methods that allow us to make more valid predictions as to what occurs when chemicals are released than we can make now, but for

the moment we must use what we have.

COSTERTON: I think we get two problems with techniques, one is the technical problems with the methods themselves and the other is when we try to hammer the results of a technique into an ecological preconception we get preoccupied. When we are trying to make ATP fit bacterial biomass, we wrench it around and do all kinds of strange things. A lot of these techniques, if we just used them with a measure of horse sense, could give us some very, very interesting results. For starters, take epifluorescence in a river--don't even do the counts all that carefully, but look at the circulating water, the moving water, look at the sediments and look at the submerged surfaces. You find a very, very important answer. Most of the bacteria are not moving with the water at all. They are in the sediments and on the surfaces, so you can do microbial ecology until you are blue in the face on the circulating water and you have the wrong answer. You are working with the wrong population. Or, when we work with a sessile mass that we know contains little wormy, creepy crawlies and algae as well as bacteria, should we just say sessile ATP, and use that as a parameter without hammering out bacterial biomass? There are a great many examples where can could apply horse sense in measuring activities, too. Do we have to fee a system glycolate or feed it glucose to extrapolate to overall heterotrophic activity? Couldn't we just say that the rate of turnover of this compound by this population is very interesting, very high and indicative of some very intriguing things? So my plea would be to use ecological terms in their pigeon holes. Instead, we beat them around trying to get answers where they are not terribly capable of giving us answers. Why don't we just look at these techniques with a measure of horse sense and use the results for what they actually mean, rather than trying to force them?

Session V

MICROBIAL INDICATORS OF ENVIRONMENTAL QUALITY

What Do Water Quality Indicators Indicate?

VICTOR J. CABELLI

The routine examination of aquatic environments for individual microbial species or groups of "similar" microorganisms as indices of water quality first started at about the turn of the century. Specifically, the use of "fecal" indicators was applied to index the potential for human disease consequent to direct or indirect fecal contamination of aquatic environments used as sources of drinking water, food, or recreation. This objective remains as the justification for most of the microbial monitoring programs. However, routine examinations of aquatic environments for certain microorganisms can be and are now performed for other reasons, although on a much more limited scale. These include examinations for certain aquatic microorganisms (i) that can and have caused human disease among recreationists, (ii) that play essential roles in aquatic ecosystems (i.e., in mineralization, cycling of nitrogen and sulfur, etc.) and whose activities are adversely affected by chemical pollution, (iii) that degrade or transform chemical pollutants, and (iv) that cause faunal disease and may respond to nutrient

loading. There is even one coliform species, *Klebsiella*, whose presence in aquatic environments at disproportionately high densities (relative to *Escherichia coli*) is suggestive of pollution with polysaccharide-rich industrial wastes (i.e., pulp and paper mill effluents, textile finishing wastes, etc).

An affecting or affected microorganism itself is measured in some instances; in others, an indicator of its presence is measured. For almost all of these applications, and even for specific uses (e.g., drinking water as opposed to recreational water),measurements should be made for specific microorganisms, or at least for a closely related group of microorganisms, and the results of the examination should be interpreted only with regard to the specific use or type of pollution. Furthermore, certain conceptual and practical considerations limit both the use of indicators and the interpretation of data obtained thereby. This paper will deal with these considerations in an attempt to answer the question: What do water quality indicators indicate? It will, however, be directed primarily at those indicator systems associated with human health effects.

Fecal-specific Indicators

At the turn of the century, three specific organisms were suggested as possible indicators of human fecal pollution and the consequent health effects of using water contaminated with such wastes. They are known today as *Escherichia coli*, *Streptococcus fecaelis*, and *Clostridium perfringens* (Prescott, Winslow and McCrady 1945). These were selected because they were consistently recovered from feces and fecally-polluted waters. But for each of these, a broader group of organisms is now enumerated, to include not only the species in question but other biotypes as well, whose

sources are not consistently and exclusively fecal--total or fecal coliforms instead of *E. coli*, fecal streptococci instead of *S. fecaelis*, and spore-forming, sulphite-reducing anaerobes instead of *Clostridium perfringens*. In each case, no scientific justification exists for the change other than that facile methods for enumerating the specific organisms in question were not available. It might be argued that there was a gain in sensitivity; however, this is not worth the loss in specificity, as there is no compensation for specificity, but sensitivity can also be accomplished by examining larger volumes of water. The justification of not having facile methods for enumerating specific organisms no longer exists. There are accurate, facile methods for the enumeration of two of the specific indicators--*E. coli* (Dufour, Stickland and Cabelli 1975) and *Clostridium perfringens* (Bisson and Cabelli 1979)--and a method for enterococci (Levin, Fischer and Cabelli 1975), a more fecal-specific component of the fecal streptococcus group.

Coliform indicators require further discussion, not only because they are the most commonly used indicators, but also because of the reasons given for retaining the total and fecal coliform systems.

The total coliform population, as commonly enumerated, includes four genera in the family Enterobacteriacae: *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*. It may also include other organisms, notably lactose-positive members of the genus *Aeromonas*. Aeromonads are not normally found in feces, although they can be routinely recovered from domestic sewage and its receiving waters in rather large numbers (Miescier 1977; Rippey and Cabelli 1980). That there can be an impact from the inclusion of *Aeromonas* in the coliform group is shown in Table I (Lupo et al. 1977). Although aeromonads can be easily and readily differentiated from coliforms by the oxidase test, the distinction

is frequently ignored, possibly because of the widespread use of total coliform most-probable-number and membrane filter procedures (Standard Methods for the examination of Water and Wastewater 1976) in which the differentiation is made with difficulty. If this nonspecific indicator system is retained, the inaccurate definition should be modified and methods developed that are compatible with making the necessary distinctions.

The total-coliform system is being discarded for many applications, presumably because *Citrobacter* and *Enterobacter* species are recognized as not fecal specific. However, the total coliforms are being replaced by the so-called "fecal coliforms," a group which includes thermotolerant *Klebsiella* biotypes as well as *E. coli* (Dufour 1976). There never was any evidence that the adjective "fecal" was properly applied. In fact, it has been known for some time that there are substantial extra-fecal sources of *Klebsiella* (Table 2), even for the thermotolerant biotypes (Dufour and Cabelli 1976). In addition, *Klebsiella* is infrequently present in human feces, and then generally only as a minor portion of the coliform population (Table 3). A number of reasons have been given to justify the use of this fecal coliform system instead of *E. coli*. It has been argued that much of the historical data on water quality is presented in terms of fecal coliforms, that the existing standards for recreational and shellfish waters are stated as fecal coliform densities, and that *Klebsiella* should be enumerated as a fecal indicator because it is an opportunistic pathogen. First of all, much of the historical data are given as total coliform, not fecal coliform levels. Secondly, the little epidemiological or even experiential data in support of existing recreational or shellfish standards were developed in terms of total coliforms and extrapolated to fecal coliforms. Thirdly, *Klebsiella* is an opportunistic pathogen of the respiratory and genitourinary systems and not the gastrointestinal tract.

Table 1. Effect of oxidase-modified completed test on the confirmed and completed MPN estimates.

MPN Procedure	Geometric mean/100 ml R.I.[a]	N.Y.[b]
Confirmed	1995	415
Completed	1449	362
Modified completed	1021	252

[a] 26 samples (Rhode Island)

[b] 38 samples (New York)

[c] MPN tubes containing only lactose-positive isolates; oxidase-positive isolates eliminated

Table 2. Source of *Klebsiella* in the receiving waters for a textile finishing plant effluent.

Location	Cells/100 milliliters	
	Total coliforms	*Klebsiella*
Above outfall	300	130
At outfall	2.3×10^8	1.3×10^{8}[a]
Below outfall	3.7×10^6	1.7×10^{6}[a]

[a] About 50% of *Klebsiella* isolates were thermotolerant (Dufour and Cabelli 1976).

Table 3. Distribution of coliform biotypes in human feces[a]

Study year	No. Samples	No. Colonies Tested	Percent of isolates which were *E. coli*,	*Klebsiella*,	Enterobacter or Citrobacter
1975	13	438	99.99	0.01	
1976	15	285	89.0	5.0	6
Totals	28	723	96.8	1.5	1.7

[a] After Dufour (1976).

Finally, there are no data showing that *Klebsiella* infections have been obtained via the waterborne route, much less that they occur at environmental fecal coliform densities of less than 200/100 ml or 14/100 ml, the present U.S. Environmental Protection Agency guidelines for recreational and shellfish-growing waters respectively (U.S. Environmental Protection Agency 1976).

Conceptual considerations aside, there are empirical reasons, at least for recreational waters, why *E. coli* is better as a water quality indicator than fecal coliforms or total coliforms and why enterococci are superior to all three coliform systems. There is every reason to assume that the same would be true of shellfish-growing waters. These come from the results of a three year epidemiological-microbiological study conducted at some New York City beaches (Cabelli et al. 1974; Cabelli et al. 1976; Cabelli et al. 1979). It can be seen from Figure 1 that the mean density of *E. coli* in the bathing water correlated better with the rate of swimming-associated gastrointestinal symptoms than did the mean density of fecal coliforms, or total coliforms, but that the best correlation by far was obtained with enterococci.

Universal Versus Multiple Indicators

In all probability, if there were no historical precedent for using coliforms as indicators, they would not be used as the indicators for these three applications: drinking water, swimming pool water and shellfish-growing-area waters. Total coliforms, so-called fecal coliforms, and even *E. coli* do not meet the primary requirement for the first two applications, nor a very important requirement for the third. These requirements are resistance to the cidal effects of chlorine disinfection (Scarpino 1974) and survival in shellfish

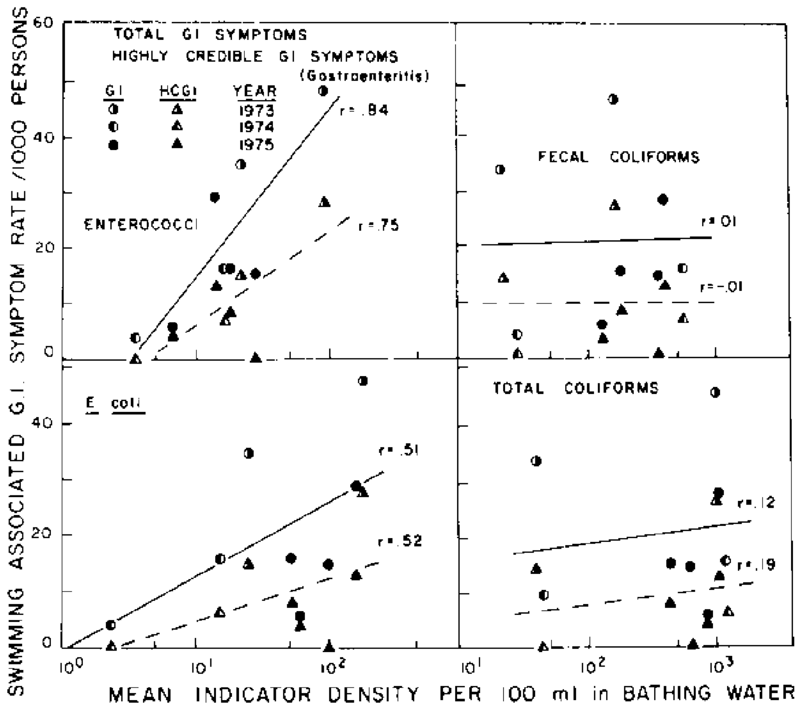

Figure 1. Regression lines and correlation coefficients (r) for swimming-associated rates (swimmer minus nonswimmer) of gastrointestinal (GI) symptoms against the mean indicator density in the bathing water. Highly credible GI symptoms (HCGI) are equated with gastroenteritis, as defined in the text. The data shown are from three years of the New York City study; total and fecal coliforms were discontinued thereafter. The four indicators shown (of a number examined) were selected because total and fecal coliforms are the "classical" indicators, *E. coli* is the most specific and best correlated of the coliform indicators, and enterococci provide the best association to swimming-associated illness.

comparable to that of the pathogens of primary interest, notably hepatitis A virus and possibly some viral agents of gastroenteritis. If there is a need for a microbial indicator system in the first two applications, it is for one that indexes the efficacy of disinfection. Indicating fecal contamination is of limited value because disinfection must be assumed in swimming pools and in many surface sources of raw drinking water. The occurrence of outbreaks of shellfish-associated hepatitis during the winter in temperate zones (Cabelli and Heffernan 1971) coupled with the absence of coliforms in shellfish harvested from grossly polluted waters during this time (Rindge et al. 1965) would imply that hepatitis A virus can persist long after the coliforms disappear from the shellfish or the growing waters subject to intermittent fecal pollution. This is one explanation for the Louisianna hepatitis outbreak (Portnoy, Mackowiak and Karaway 1975) in which cases of the disease were associated with the consumption of shellfish taken from a "clean" area. The area had been temporarily closed because of the influx of massive quantities of fresh, polluted waters following extensive rainfalls, but the closure had been suspended, based upon indicator densities in the water, about two months prior to the harvesting of the shellfish. The coliform densities in the shellfish were not abnormally high.

Even in those instances where it is possible, measuring pathogens is not the solution to the problem, because there is no predictive value in doing so. Nor is the answer to develop more rapid coliform assays, because microbiological methods cannot match the potential for rapidity or automation of chemical measurements of disinfectant levels. The problem is not methodological but conceptual, and there is a need for a more environmentally "resistant" indicator for these specific applications. Several possibilities will be discussed later in this paper.

Indicator Comparisons

Among the microorganisms present in feces, sewage, surface waters, and raw drinking water, there are indicator systems which could better satisfy the requirements for drinking, swimming pool and shellfish-growing waters. Some other systems are being considered; in fact, some recent data suggest that two other fecal indicators, *C. perfringens* spores and enterococci, could better meet these requirements (Bisson and Cabelli 1980). One reason coliforms have not been abandoned completely is that there is a conceptual flaw in the evaluation of alternative indicator systems. In general, the candidate indicator's densities are compared to those of the coliforms, but this is a no-win comparison. If there is a correlation between the two, coliforms are retained; if there is no correlation, the candidate indicator is rejected. One solution would be to examine the levels of all the potential indicator systems against the final arbiter, the incidence of illness among the water users, preferably in the course of prospective epidemiological studies. However, such studies are costly and difficult to design and execute. A poor alternative to the epidemiological approach is to compare the candidate indicator's densities in the water to those of the epidemiological significant pathogens.

Limitations of Fecal Indicators

Periodically it is stated that fecal indicators, notably coliforms, are defective as health effects, water quality indicators, principally because pathogens are recovered at low indicator densities. In fact, the fecal indicator concept has certain limitations (Cabelli 1978). Fecal indicators were never meant to index the risk of disease from pathogenic, aquatic microorganisms such as *Pseudomonas aeruginosa* or *Vibrio parahaemolyticus*, which multiply in the

aquatic environment. Furthermore, for obvious reasons fecal indicator densities are rather meaningless when applied to small fecal sources, and a presumed indicator-pathogen relationship may become invalid during an epidemic of a given enteric disease in the population whose fecal wastes reach the water. The limitation on the size of the source is probably the most overlooked of the three. The application of fecal indicators under these circumstances is improper, as is criticizing a specific fecal indicator because it does not work under such circumstances. The solutions to these problems are to measure the "aquatic pathogen" itself in the first instance, to conduct a sanitary survey in the second, and to maintain surveillance of enteric disease in the discharging population in the third.

Measuring the enteric pathogens themselves, as has been suggested by some, is not the solution to the inadequacies of fecal indicators because as noted earlier, the results of such measurements have no predictive value. That is, the absence of an enteric pathogen in the water at any given point in time is no guarantee that it will not be present shortly thereafter. Therefore, the frequency of pathogen isolation, in contradistinction to the numbers of infective units present, is hardly a basis for assessing risk. Moreover, the two groups of pathogens most frequently measured, salmonellae and enteroviruses, are poor choices for the reasons that follow.

Enteric Pathogens as Indicators

No well-documented cases of salmonellosis in the United States, much less typhoid or paratyphoid fever, have been clearly shown to be associated with shellfish consumption since 1959 (Verber 1972); only two small, questionable cases have been associated with swimming since 1921 (McCabe 1977). The change is probably the result of two factors: the decrease

in the numbers of active cases and carriers in the population and hence of the pathogen in the sewage being discharged, and the more widespread treatment and disinfection of sewage effluents. An additional factor is the virulence of the organism.

The oral infectious dose for salmonellae, as determined from human volunteer experiments, is very large (the ID_{50} has been determined to be 10^6 to 10^7 cells) (Hornick et al. 1970) and, relative to the ID_{50}, the densities even at a "barely acceptable" beach are very low (Cabelli et al. 1974). Thus, the probability of disease via swimming or even shellfish consumption is very small. Such would not be the case, however, if the salmonellae were present in multicelled particles rather than as cells randomly distributed in the water. This explains the absence of recent cases of salmonellosis, since these multicelled particles would most likely be found in bottom sediments near a sewage outfall, and shellfish harvesting or swimming in the vicinity of an outfall is now generally prohibited (Shellfish Sanitation Program 1965). Furthermore, a major function of sewage treatment is to reduce suspended solids, especially the large particles that could contain multiple numbers of salmonellae. The information available certainly does not provide a rationale for recreational water or shellfish-growing-area standards based upon the enumeration of salmonellae, much less for routine monitoring of aquatic environments for the presence of organisms.

The rationale for enumerating enteroviruses is equally tenuous. There have been only a very few outbreaks of disease, including those via drinking water, in which the waterborne routes were shown to have been operative in the transmission of enteroviral disease (McCabe 1977; Cabelli 1978; Levin 1978). Thus, the potential for such disease via the waterborne route is presumed, not demonstrated. The presumption derives from the sporadic recovery of the agents from environmental waters

(Berg and Metcalf 1978), and then only in very low densities, coupled with the much-repeated statement that a single polio virion at the right time and in the right place will infect man. The reference paper for this statement documents the infectious dose of a vaccine strain of poliovirus fed to new-borns by gavage (Plotkin and Katz 1967)--hardly the basis for risk analysis in real world situations. These findings do not warrant standards based upon the numbers of these agents in environmental waters, expensive monitoring programs for their presence, or conclusions of risk drawn only from the presence of these agents in environmental waters. It is of interest that Moore's (1959) conclusions on the improbability of recreational waterborne disease were derived from epidemiological information on poliomyelitis and typhoid or paratyphoid fevers.

Coliphages as Indicators

Earlier some conceptual reasons were given why the densities of enteroviruses, much less their presence in environmental waters, should not be used as the basis for establishing health-effects, water-quality criteria. There is another reason: the unavailability of accurate, precise, facile and inexpensive methods for their concentration and quantification. Methodological limitations are most often cited as the reason for not enumerating these and other enteric pathogens in the aquatic environment and this has led to a search for a good simulant for these epidemiologically unimportant enteric pathogens. The coliphages, which could be plaqued on *E. coli* B, were considered and generally rejected because one component of the population enumerated on *E. coli* B as the host. All the T-phages were shown to be more sensitive to the cidal effects of chlorine than most of the enteroviruses, notably poliovirus (Scarpino 1974). When the male-specific coliphages, f-2 and MS-2, were discovered and characterized, it was thought that the solution

was at hand because these colphages resemble the poliovirus in that they are octahedral, single-stranded RNA phages. Furthermore, it was thought that they could serve as water quality indicators as well as viral simulants. But there is a distinction between the two applications. Both simulants and indicators can be used to study the fate and transport of the pathogen from source to target. However, indicators should be consistently and exclusively present in the feces of warm-blooded animals, notably man. The f-2 phage is undoubtedly a good simulant for the poliovirus. Its greater resistance than *E. coli* to chlorine is well-documented (Scarpino 1974) and there is evidence that it survives better in the aquatic environment (Kott and Buras). However, as noted earlier, there is no evidence for the significant transmission of poliomyelitis via waterborne routes. Furthermore, as can be seen from Table 4, none of the coliphages measureable on *E. coli* K-12 Hfr meet the very important requirement for a fecal indicator--consistent presence in human feces in at least moderate densities (Lupo, Dufour and Cabelli 1977). They could be considered sewage indicators, but certainly not fecal indicators. Finally, their survival characteristics relative to those of hepatitis A virus or the rotaviruses and parvo-like viruses are unknown.

There are coliphages in sewage and its receiving waters that are markedly more resistant to the cidal effects of chlorine, heat, and residence in the aquatic environment than f-2. Their presence in sewage was suggested by the comparison of *E. coli* and coliphage densities in post-chlorinated effluents (Table 5) (Lupo, Dufour and Cabelli 1977). By the stepwise increase in the free, residual chlorine level in sewage from several sewage treatment plant effluents, they were found to comprise about 30% of the coliphage population plaqueable on *E. coli* K-12 Hfr. When tested in "pure culture," they were resistant to rather high levels of free, residual chlorine (Figure 2; McBride 1979). Characterization

Table 4. Recovery of coliphage from feces as determined from ability to form plaques on K-12 Hfr.

Species	No. Samples	Coliphage recovered at density 2- 200 PFU/gm		200 PGU/mg	
		No.	%	No.	%
Man	58	12	19		
Man	92			0	>1.1
Cat	7	0	>14	1	14.
Chicken	3	0	>33	2	67.
Cow	10	2	20	5	50.
Dog	8	1	13	2	25.
Goat	8	0	>13	0	>13.
Horse	4	1	25	1	25.
Pig	9	0	>11	0	>11.
Sheep	8	1	13	2	25.
Rabbit	5	0	>20	0	>20.

Table 5. Densities of coliphage and coliforms in post-chlorinated effluents.

Sewage Treatment Plant	Coliphage[1] PFU/100 ml	Coliforms[2] PFU/100 ml
A	>67	>1
B	>67	>1
B	133	>1
C	150	>1
D	2800	>1
E	5000	>1
F	5500	>1
G	6000	>1
F	6000	>1
H	8000	>1
I	14000	>1
J	17000	>1
J	19000	>1
C	1000	4
E	16000	56
J	3000	200
J	6000	200
E	800000	200

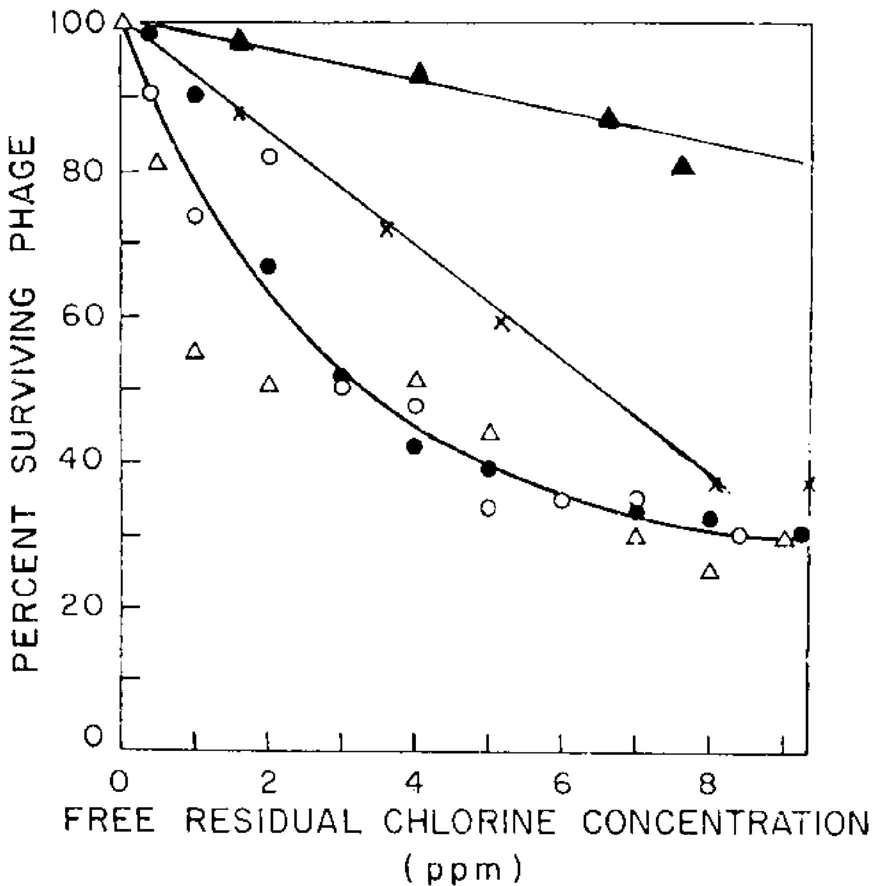

Figure 2. Survival of coliphage exposed to increasing concentrations of free residual chlorine. The curve is defined by adding hypochlorite to secondary stage sewage effluent to given concentrations of 0, ● and Δ, holding the samples for 20 min., and plaquing them on *E. coli* Hfr. Although heterogeneous initially, the population became more homogenous with increased chlorine treatment. By 6 ppm, it was composed almost entirely of male-specific filamentous DNA phages that reacted to titer with anti-Fd sera. The line defined by the Δs is for an isolate (N-31) having the above characteristics; that defined by the Xs is for the male-specific octahedral, RNA phage f-2 (after McBride).

by electron microscopy, serology, nucleic acid composition, and male specificity showed them to be identical or similar to the filamentous, male-specific, single-stranded DNA coliphage. They show promise as viral simulants, at least for certain applications.

Human Versus Lower Animal Fecal Indicators

Existing guidelines and standards for drinking water, recreational waters, and shellfish-growing areas are stated in terms of total coliform or fecal densities. Neither these systems nor *E. coli* distinguishes between human and lower fecal sources of pollution; yet there is little doubt that human fecal wastes are inherently more hazardous than those of lower animals. The consequence is that some resources impacted by nonpoint sources or stormwater run-off are closed to the public because of assumed health risks for which there is no evidence one way or the other. Some states have recognized this and have caveats in their standards which allow such resources to be used. There are two requirements to resolve this question: the first is epidemiological studies to actually assess the relative health risks of human as opposed to lower animal fecal pollution, and the second is an indicator that is specific for human fecal wastes. The results of an epidemiological study conducted at a Lake Pontchartrain beach, as part of the USEPA program to develop recreational water quality criteria, suggest that the health risks associated with stormwater run-off are less than those with wastewater discharges (Cabelli 1980).

The system currently used for making this distinction, the fecal coliform/fecal streptococcus (FC/FS) ratio (Geldreich and Kenner 1969), is inadequate, inaccurate, and frequently misleading. In effect, a ratio is obtained from the densities of two heterogeneous populations, neither of which

is entirely fecal specific and both of which have different survival characteristics in the aquatic environment. Furthermore, ratios are difficult to deal with, especially on a quantitative basis and rarely is the source of fecal pollution in a given area purely animal or purely human. Finally, the distinctions are often clouded, because a number of lower animal species, like humans, have high FC/FS ratios (Table 6) (Wheater, Mara and Draqui 1979; Thomas and Levin 1978) and because *S. bovis* can be recovered from humans (Wheater, Mara and Draqui 1979). Even the proponents of its use point out that FC/FS ratios can only be interpreted at their extremes, that is at ratios greater than 4 and less than 0.6 (Geldreich and Kenner 1969). At best, the ratios are of value only in the immediate vicinity of a "pure" source of pollution. With such restrictions, visual observation or a sanitary survey probably provide information as meaningful. It is time this imprecise, inaccurate, and misleading parameter is laid to rest and a search for a human fecal-specific, environmentally resistant indicator is started.

Bifidobacteria are probably the most human-specific fecal indicators which have been considered. However, their poor survival characteristics through sewage treatment, disinfection and transport in receiving waters (Resnick and Levin 1977) preclude their general use. Nevertheless, the recovery of bifids indicates an immediate, unchlorinated source of human (and to a lesser extent porcine) fecal wastes.

Criteria and Guidelines

The existing microbiological guidelines and standards for recreational, drinking, and shellfish-growing waters are supported by little more than a historical precedent for their use. Very limited and somewhat equivocal epidemiological data are

available to support guidelines for the first two (Stevenson 1953; Peterson and Hines 1960), but none exist to support standards for shellfish-growing waters. Some calculations were made to support the existing shellfish and drinking water standards (see McCabe 1977); however, they were based on the erroneous assumption that the human ID_{50} for *Salmonella typhosa* was one (Hornick et al. 1970). Aside from the choice of the indicator, the specific numerical limits and the epidemiological base which supports them, there is yet another problem: none of the three were derived consistent with risk analysis. There are two consequences of this defect. The first is that individuals who set the guidelines, those who promulgate standards from the guidelines, and those who are compelled to use the guidelines have a limited option--to accept or reject some limiting value for a given indicator density (sometimes referred to as a "magic number"). The second is that, where a guideline or standard is based upon a "detectable" risk (for drinking water and recreational waters), it has to be made more restrictive as better detection procedures are developed. Moreover, with time the flimsy basis for the derivation of such values is often forgotten and the values themselves become sacred.

The fecal coliform standard for recreational waters (U.S. Environmental Protection Agency 1976) is a case in point. As noted earlier, it has gained wide acceptance and apparently the shortcomings in its derivation (National Technical Advisory Committee 1968) from the results of the Stevenson studies (1953) have been forgotten. Detectable health effects were associated with total coliform (TC) densities of about 2000/100 ml in prospective epidemiolgical studies conducted at two freshwater sites, but not associated with the one saltwater location. The 2000 TC value was extrapolated to 400 fecal coliforms from TC-FC relationships observed sometime later, on the same stretch of the Ohio River where one of the two freshwater studies was conducted. The value was

reduced by one-half to a log mean of 2000/100 ml in order to prevent the effects observed, and the 400/100 ml value was retained as the limit not to be exceeded more than 10% of the time. What is often forgotten about these studies is that (i) of necessity, a weak experimental design was used, (ii) the statistical analysis was questionable, (iii) the fecal coliform values were extrapolated from total coliform densities and thus have some of the weaknesses of the TC indicator, (iv) there were no health effects observed in the study conducted at the saltwater beaches, and (v) in the Lake Michigan study the total coliform value was associated with all symptoms, but in the Ohio River study with gastrointestinal symptons.

Most important of all, these guidelines and standards are based upon inferred or detectable health risks, but as noted earlier, the need is for guidelines and standards derived from criteria arrived at by deliberate decisions as to acceptable risk. In fact, one such criterion is now available from the results of an epidemiological-microbiological program conducted by the USEPA (Cabelli 1980). The criterion is a quantifiable relationship between swimming-associated gastroenteritis to the mean enterococcus density in the bathing water.

The nature of the gastroenteritis observed, the age distribution of the cases (Cabelli et al. 1979), and differences in the rates observed among Alexandria residents, Cairo tourists at Alexandria beaches, and participants in the U.S. studies (Cabelli 1980) suggest a viral etiology, specifically the human rotorviruses or parvolike viruses.

Furthermore, a differential (swimmer-minus-nonswimmer) attack rate for gastroenteritis of about 1% was associated with an enterococcus or *E. coli* density of about 10 per 100 ml in bathing water. Because this represents the ingestion of a single enterococcus or *E. coli*, it suggests that

positive findings would be obtained from carefully controlled, prospective epidemiological studies of other potential waterborne routes of transmission.

Human Health Effects and Nutrient Loading

Vibrio parahaemolyticus, Aeromonas hydrophila, and *Pseudomonas aeruginosa* are aquatic microorganisms that can cause disease in man and aquatic fauna. They have been responsible for swimming- or marine food-associated cases of disease (Cabelli 1978; Levin 1978) and there are data indicating a relationship between their densities in water to nutrient loading, including loading from the discharge of sewage effluents. These three organisms are of special interest. If their densities in the water can be associated quantitatively with waterborne disease in man and with man-mobilized nutrient pollution (specifically that from municipal sewage effluents), this association could provide the basis for health effects guidelines against such discharges. These guidelines could be stated in terms of the agents or the nutrients which stimulate their growth.

Toxigenic biotypes of *Vibrio parahaemolyticus* have caused outbreaks of disease with the consumption of marine foods (Barker and Gangarosa 1974). Closely related organisms have been found responsible for wound infections among swimmers in coastal and estuarine waters (Ryan 1976). Mixed findings have been reported regarding the assocation of *V. parahaemolyticus* densities to nutrient loading from municipal sewage treatment plant effluents. The findings from a study conducted in Narragansett Bay, Rhode Island (Watkins and Cabelli 1978) clearly showed such an association, as seen from the correlation to fecal indicator

Table 6. Ratios of *Escherichia coli* to fecal streptococci in fecal specimens from man and animals as determined from the recoveries on FC and KF media.

Samples Source	No.	m-FC/KF Range	Mean
Man	12	3 - 1,864	296
Cattle (inside)	5	2.02 - 0.5	0.2
Cattle (grass)	5	0.1 - 27	10
Sheep	6	0.4 - 79	23
Horse	5	0.01 - 22	5
Pig	10	0.2 - 52	13
Cat	7	0.1 - 35	8
Dog	6	0.6 -102	24
Rabbit	4	0	0
Rat	4	0.02 - 0.2	0.07
Mouse	4	0.04 - 0.06	0.05
Hen	5	0.1 - 3	1
Duck	6	0.3 - 38	7
Pigeon	7	4.0 - 98	97
Seagull	8	0.4 - 60	20

Table 7. Correlation coefficients for *Vibrio parahaemolyticus* density versus physical, chemical and biological parameters.

Parameter[a]	r[b]	Parameter[c]	r
E. coli density	.81	Kjeldahl N	.09
Enterococcus density	.70	NH_3	.11
C. perfringens density	.85	$NO^-_3 + NO^-_2$	.06
Viable heterotrophs	.30	Total phosphorus	.13
Station depth	.09	Orthophosphate	.20
Water temperature	.01	Dissolved organic C	.53
Salinity	.27	Chlorophyll *a*	.12
Dissolved oxygen	.54	Phytoplankton	.22
pH	.12	Net zooplankton	.55
Transparency	.80		

[a] for entire bay

[b] correlation coefficient

[c] for Providence River and Upper Narragansett Bay stations

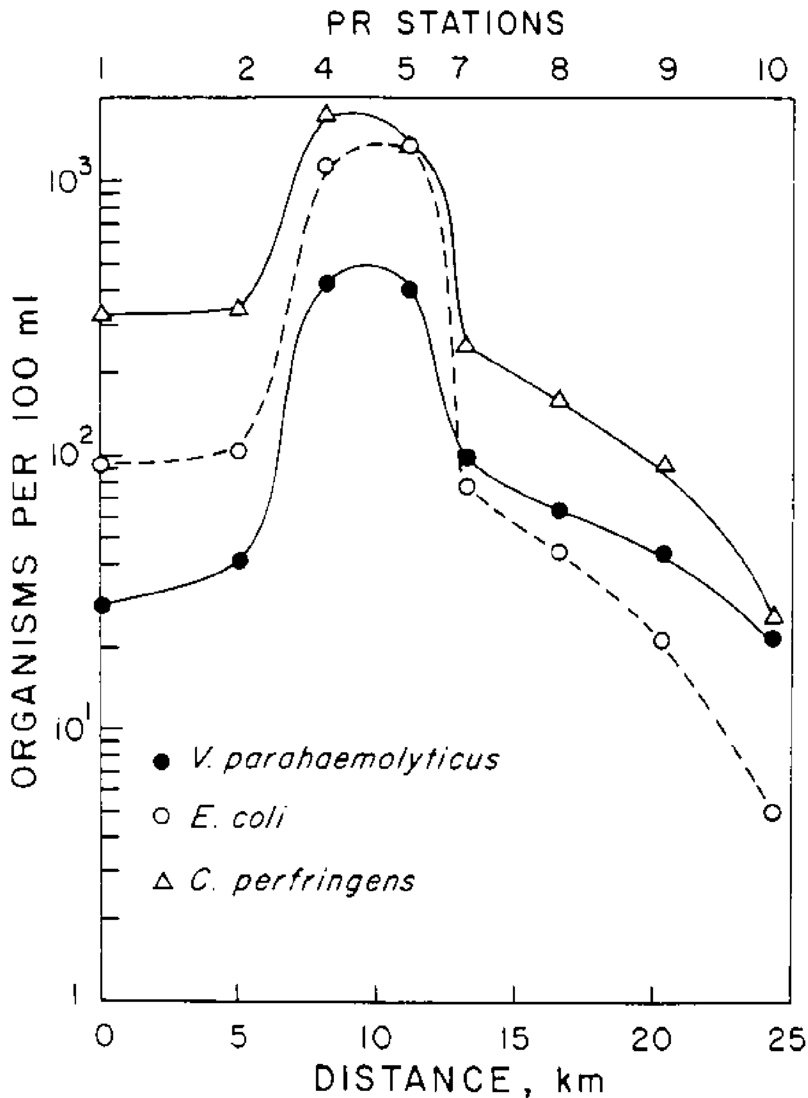

Figure 3. Relationship of *Vibrio parahaemolyticus* densities to those of the fecal indicators *Escherichia coli* and *Clostridium perfringens* "above" and below a known municipal wastewater discharge (PR4) on the Providence River and Upper Narragansett Bay, Rhode Island (after Watkins and Cabelli).

densities (Table 7) and the horizontal distribution of the organisms in the bay relative to the sources of pollution (Figure 3). Furthermore, *V. parahaemolyticus* densities correlated with transparency, dissolved oxygen, and net zooplankton densities, but not with the levels of various nutrients in the water or with phytoplankton densities. The observed association with zooplankton and the seasonal distribution of the organisms confirmed earlier observations (Kaneko and Colwell 1975; Kaneko and Colwell 1973).

Further evidence for an association with zooplankton densities and the absence of a direct relationship to sewage pollution was obtained from laboratory investigations. Of a variety of particulates added to the water, including zooplankton, bottom sediments, silica gel and phytoplankton, only net zooplankton (whether dead or alive) and chitin particles supported the growth of the organism (Figures 4 and 5). Added sewage, even in the presence of the aforementioned particulates, did not stimulate the growth of the organism.

These findings suggest an indirect association of *V. parahaemolyticus* densities to man-mobilized nutrient loading of estuarine and coastal waters, mediated through zooplankton and the organisms in its food chain. However, the genetic, ecologic and epidemiologic relationship of *V. parahaemolyticus* to its toxigenic biotypes remains an enigma yet to be deciphered.

The evidence for an association between *Aeromonas hydrophila* densities in the water and nutrient loading (including loading by man-mobolized wastes) is even more compelling. *A. hydrophila* densities may be useful not only as a basis for developing recreational water-quality criteria but also as an index of trophic state. Like *V. parahaemolyticus*, *A. hydrophila* has a seasonal distribution, being found in the water only during the summer months in temperate zones

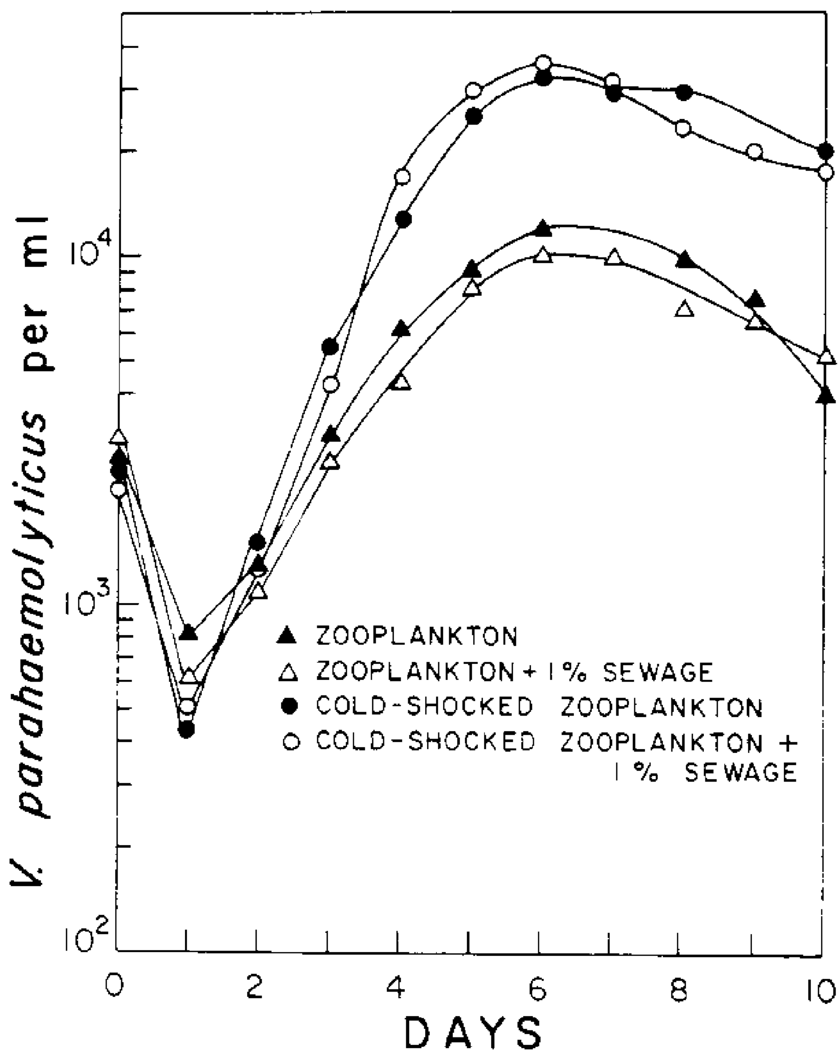

Figure 4. Effect of zooplankton and cold-shocked zooplankton, with and without secondary sewage, on the densities of *Vibrio parahaemolyticus* seeded into filtered estuarine water (salinity 10 ppm) (after Watkins and Cabelli).

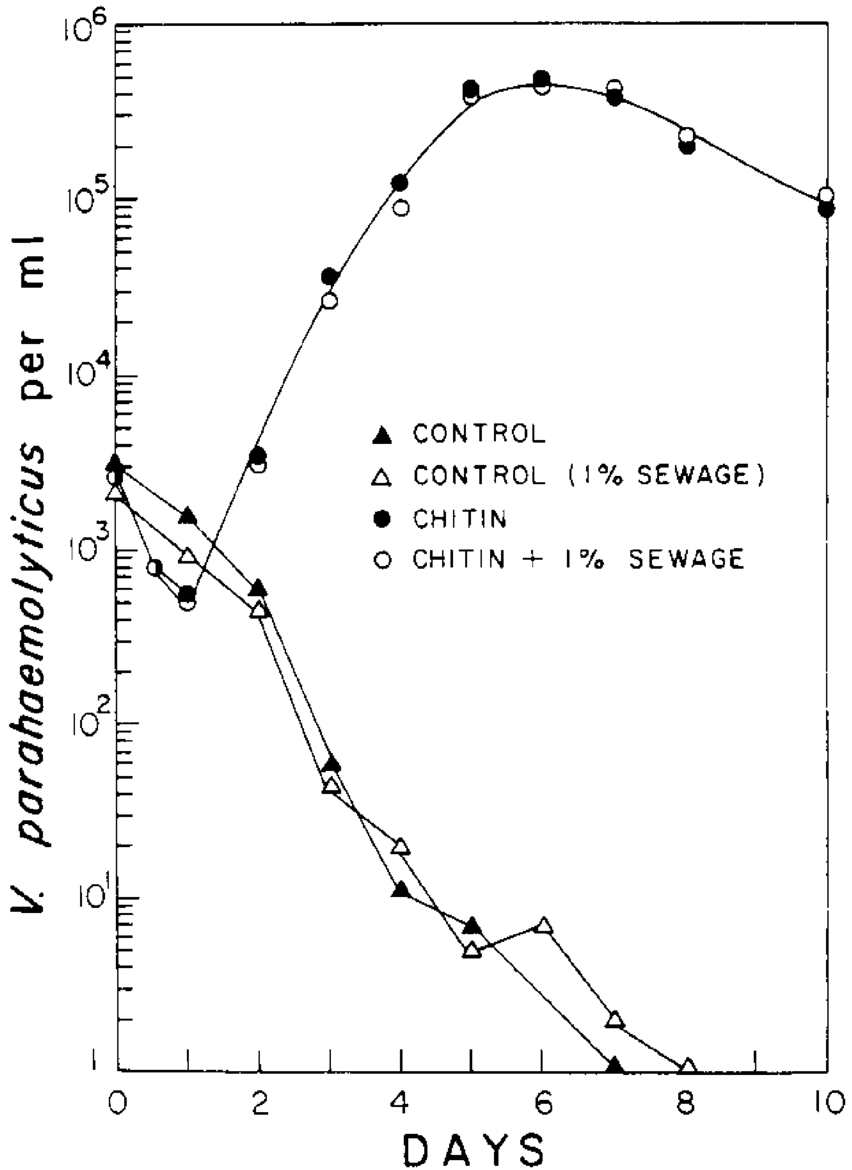

Figure 5. The growth of *Vibrio parahaemolyticus* in filtered estuarine water supplemented with chitin, to which secondary sewage was and was not added (after Watkins and Cabelli).

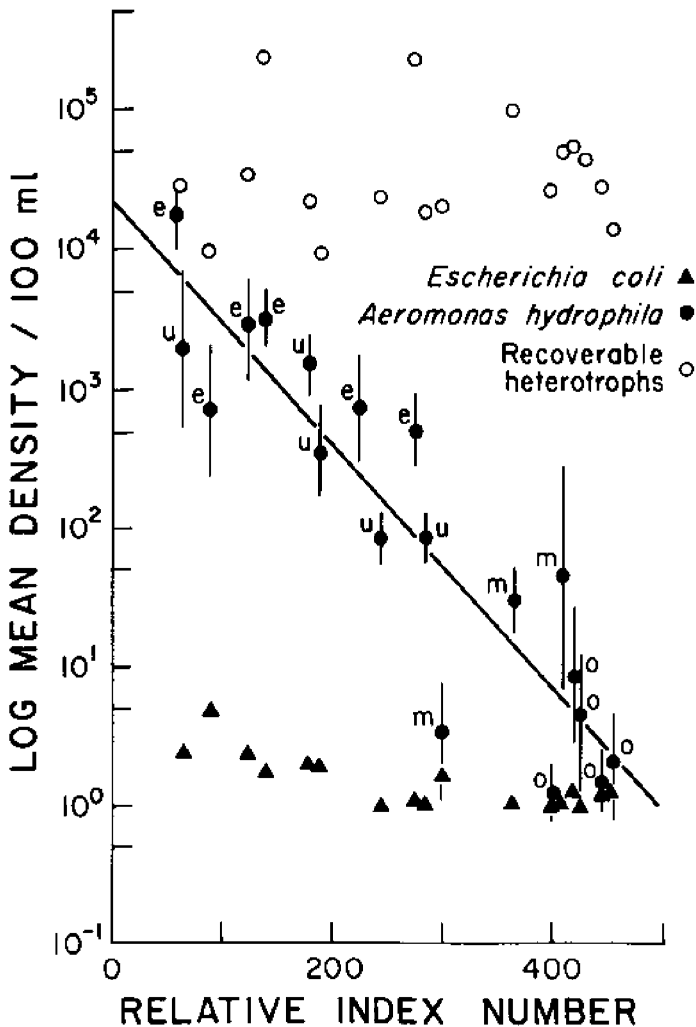

Figure 6. Relationship of *Aeromonas hydrophila*, *Escherichia coli*, and "recoverable" heterotroph densities to the Relative Trophic Index. Geometric mean bacterial densities are from a minimum of eight samples. Vertical lines for the *A. hydrophila* densities are 95% C.L. Index number calculated using the present data and the 19 bodies of water only (r = .91, p .0005 for *A. hydrophila*; r = -.24, p .10 for recoverable heterotrophs relative to RTI).
e - eutrophic; m - mesotrophic; o - oligotrophic, as determined from the National Eutrophication Survey (NES); U - Rhode Island lakes and ponds not included in NES, trophic state undetermined.

(Cabelli et al. 1974; Rippey and Cabelli 1980).

When *A. hydrophila* densities were plotted against the relative trophic index, as calculated by the procedure developed for the National Eutrophication Survey (U.S. Environmental Protection Agency 1972, 1977), a regression line was obtained with a correlation coefficient of 0.91 (Figure 6). No correlation with recoverable heterotroph or *E. coli* densities was obtained. The regression line showed that this measurement is of relatively little value in trophic state determinations, and the lack of correlation with densities showed that the aeromonads being measured did not come from sewage discharges.

Water quality indicators can be used to index a number of conditions related to the health of aquatic ecosystems and to the potential for health effects among individuals using aquatic environments as sources of water, food, and recreation. However, their use in monitoring programs provides very specific information with regard to potential or actual health and ecological effects. Therefore, the indicators to be used should be chosen and the data produced analyzed against very specific questions and with consideration of the limitations of the systems.

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Human Pathogens in the Aquatic Environment

RITA R. COLWELL

The occurrence and distribution of human pathogens in the environment has, until recently, been determined by application of the indicator organism concept. Presence of selected organisms, such as *Escherichia coli*, has been interpreted as indicative of the potential hazard of enteric pathogens, including *Salmonella* and *Shigella* spp. However, many organisms pathogenic for man, viz. *Vibrio parahaemolyticus* and *Vibrio cholerae*, occur naturally in brackish water and estuarine environments (Kaneko and Colwell 1975; Colwell et al. 1977; Kaper et al. 1979). The more common of the waterborne diseases are caused by *Salmonella*, enteropathogenic *E. coli*, and related organisms excreted by man and warm-blooded animals, and the presence of these microorganisms has been shown to pose a human health hazard. Microorganisms potentially pathogenic for man are proving difficult to isolate from the natural environment when they are present in small numbers, especially if methods for isolation and characterization originally designed for use in hospital laboratories

are employed. Many problems associated with the direct isolation of pathogens from the aquatic environment have been recorded (Colwell 1978) and a major difficulty is that methods are used that do not take into account environmental stresses upon microorganisms discharged into the aquatic environment or their adaptation to conditions common to estuarine and brackish waters, such as low temperature, low nutrient concentration, and increased salinity.

An important aspect of the microbial ecology of marine and estuarine waters, about which little is known, is the alteration of community structure of the natural microbial flora induced by introduction of pollutants. Bacterial species in estuaries have been shown to demonstrate seasonal cycles and convincing evidence has been gathered for a seasonal cycle for *Vibrio parahaemolyticus* in waters of the temperate zone (Kaneko and Colwell 1975). Microorganisms associated with the biota, water, and sediment of estuaries and coastal waters achieve an ecological equilibrium. When sewage, industrial wastes, or other pollutants are introduced into the aquatic system, alterations in species composition of the microflora will occur. That species selection will take place has been documented by studies employing selective and enrichment techniques. Dominance of *Aeromonas* and *Klebsiella* spp. in sewage effluents provides an example of such selection. Changes at the macrobiological level are readily discerned when fish kills, clam mortalities, oyster mortalities, marsh grass disease, noxious odors or a general decline in the aesthetic qualities of receiving waters occur. Such effects may be irrevocable, or, at the least, not readily remedied without heroic effort. Conversely, changes in microbial populations occur rapidly, if not immediately upon introduction of allochthonous materials to the aquatic system and, if promptly detected, can be reversed. Thus, microbial

parameters may offer a fine-tuning mechanism for rapid detection of changes occurring in the estuarine and marine ecosystems.

Recent work has shown that the microbial flora of areas receiving petroleum, pesticide or heavy metal discharges will contain a significant proportion of petroleum-degrading, pesticide-metabolizing, and/or heavy metal-mobilizing bacteria, depending on the pollutant influx (Colwell and Walker 1977). Directly stated, when oil is present, petroleum-degrading bacteria, yeasts, and fungi increase in number and when mercury accumulates in the environment, as in a polluted harbor receiving industrial waste, the number of mercury resistant bacteria will also increase (Nelson and Colwell 1975). Thus, a microbial response can be detected, even at the molecular genetic level (Olson et al. 1979).

In some areas of "pollution microbiology" new methods developed by microbial ecologists can be applied with very good success. Coliform "die off" in estuaries and coastal waters, for example, may ultimately prove to be a phenomenon of the improper use of methods for recovery of these bacteria; i.e., plate count or viable count methods employed for obtaining direct counts of viable bacteria, when applied to the natural system, may show that the die-off is, in fact, a result of stress, with the bacterial cells remaining viable but not able to be cultured by standard methods of plate counting. Other problems with the indicator organism concept have been reviewed elsewhere (Colwell 1978).

Whether human pathogens in estuaries and coastal waters pose a health hazard is a question long debated among environmental microbiologists. Recently, unequivocal documentation of infection of a diver was published, with the source of the pathogen being traced to the polluted water in

which he was diving (Joseph et al. 1979).

Many of the heavy metal-resistant bacteria isolated from polluted water have been found to be antibiotic resistant (Allen et al. 1977). Whether populations of antibiotic-resistant bacteria in estuarine and coastal waters receiving heavy metal-enriched wastes is a serious human health hazard is, of course, yet to be proven. Species selection and alteration of the natural microbial community structure provide useful indices of the "ecological health" of an environment and should be considered by microbial ecologists measuring environmental impacts.

Vibrio spp. occur naturally in the estuarine and marine environment. The distribution of *Vibrio parahaemolyticus* in estuaries and bays has been well documented. This vibrio has been found to be associated with crustaceans (Sizemore et al. 1975). Surprisingly, *Vibrio cholerae* has also been found to be widely distributed in brackish water and estuarine environments (Kaper et al. 1979). *V. cholerae* has been found in brackish water areas of Chesapeake Bay, but a significant relationship between the occurrence of *V. cholerae* simultaneously with *Escherichia coli*, the generally accepted indicator organism, or other fecal indicators has not been observed. In fact, *V. parahaemolyticus* and *V. cholerae* are chitin-digesting organisms and their association with chitin-containing invertebrate animals may be useful in this context. *Vibrio cholerae* and related vibrios can be readily isolated in areas of low salinity, and, in fact, the distribution of *Vibrio* spp. appears to be controlled by a variety of environmental parameters, including salinity, temperature, and nutrient concentration. The vibrios also attach to surfaces and this characteristic may be very important if attachment to surfaces provides improved survival and reproduction for them in the natural environment. The

natural occurrence of *V. cholerae* and related vibrios in the brackish water environment suggests that the presence of these organisms is not an indicator of fecal pollution. Rather, it is important to establish at what levels the vibrios pose a public health risk, i.e., is there a "Vibrio Index" where the numbers of vibrios present per unit sample represent a potential human health hazard? Thus, population size, rather than occurrence, becomes the key factor.

Recently Rondle et al. (1978) presented the hypothesis that the source of some cases of cholera might be effluent discharge from aircrafts, pointing out an apparent relationship between distribution of isolated outbreaks of cholera and major airline routes. This hypothesis appears untenable in view of the ecological data accumulating for *V. cholerae*. Furthermore, since aircraft discharge only washbasin effluent, with sewage held in chemical tanks, *V. cholerae* in wash basin effluents would be subjected to drying and to lethal effects of ultra-violet irradiation. More importantly, flight paths of regular airline services from where cholera is endemic to Europe cover the entire estuarine and brackish water areas of those countries where sporadic cases of cholera occur. In fact, the incidence of cholera carriers on aircraft coupled with the dilution factor of cholera aerosols make it unlikely that this route of infection is a feasible explanation for the spread of cholera. The link between disease and planes carrying cholera-infected passengers is much less probable than that between the natural habitation of *V. cholera* in estuarine and brackish water areas and the occurrence of cholera when sanitation or proper food handling practices are lacking.

The occurrence of *V. cholerae* in Chesapeake Bay and Louisiana in areas free of fecal contamination has been demonstrated. Bashford et al.

(1979) have isolated *V. cholerae* 01 from marshes in England. The association of *Vibrio* spp. with estuarine and marine copepods (Sochard et al. 1979) has been demonstrated and preliminary results of studies in Chesapeake Bay and in the canals near Matlab, Bangladesh, provide evidence for association of *V. cholera* with plankton (manuscript in preparation). The conclusion, then, is that *V. cholera* is, indeed, a component of the autochthonous flora of brackish water, estuaries, and salt marshes of coastal areas of the temperate zone. Work is in progress in our laboratory to determine the basis of the commensal relationship of *V. cholerae* and related vibrios to plankton species, including larvae of crustacea, as well as association of these microorganisms with adult shellfish. It is hypothesized that the enterotoxin may, in fact, play a role in salt tolerance and osmoregulation in crustacea.

In summary, microbial ecology studies will prove to be very valuable in understanding occurrence, distribution, and survival of pathogens in the aquatic environment. Clearly, pathogens in the aquatic environment can no longer be considered allochthonous, but in the case of *V. cholera* and *V. parahaemolyticus* should be recognized as autochthonous. From this perspective, it becomes far more productive to seek to elucidate the role of these organisms within the microbial communities of the aquatic ecosystem than to search for the source of their entry to the environment.

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Gut Critters are Stressed In the Environment, More Stressed By Isolation Procedures

WARREN LITSKY

After listening to Dr. Colwell and Dr. Cabelli concerning the difficulties in enumerating and isolating indicator and other enteric microorganisms, my sympathies go out to them. I too know the frustrations associated with environmental microbiology. Yet in some cases these frustrations are the result of attempting to place a square peg in a round hole and vice versa. By this I mean that perhaps most of our difficulties stem from borrowing techniques developed for clinical microbiology and applying them to the environment directly, or with little modification, but without adequate evaluation. We all know that the technology and methodology for isolating organisms literally sprang up in the clinical laboratory. These isolation procedures usually dealt with a pathological condition that was optimum for the growth of the causative agent, namely, the pathogen. In other words, most if not all of the growth requirements--nutrients, moisture, temperature, time--were optimized and most often encouraged growth of the pathogen in pure culture. As a result, the clinical bacteriologist was mainly concerned with the isolation of a culture from an actively growing, well maintained, extremely large

homogenous population. Thus it is no wonder that the clinical microbiologist was usually successful in his attempts to isolate and/or enumerate these pathogens, be they enteric or whatever.

Now let me switch scenes from the clinical laboratory to the environmental laboratory, which in most cases could be found in the most recessed corners of the basement of City Hall, and usually doubled as the janitor's storage closet. In their attempts to monitor the environment, these grand old men, whose memory I cherish dearly, literally lifted the methods of their clinical counterparts and applied them to the environment. This would have been fine if the environment--water, air, and soil--encouraged and maintained the growth of enteric organisms similar to the animal body. Unfortunately, this is not the case; the enteric population of the environment differs enormously.

Let me try to make my point with the following example. Try to visualize, if you can, a specific salmonella in my gut. For him or her it's easy street; life is beautiful. The environment in which this organism is maintained is bountiful and provides all the necessary requirements for optimum growth--at least, in my gut it is optimum! There is enough food, the correct oxygen tension, no UV lights, no antibiotics, and few if any predators. Eventually, this little critter will begin to multiply, resulting in a case of salmonellosis which inconveniences me at first, but eventually hospitalizes me. A sample is taken...But what kind of sample? A fecal sample in which the salmonella are actively growing and are present in extremely large numbers. The sample is streaked on an unusually toxic media on which up to 99% of the organisms are lost. But this is no major catastrophe because the high numbers of pathogens in the sample and the surviving population will manifest themselves on the recovery medium.

It is in this area that we, the environmental

microbiologists, made our biggest blunder. We took the methodology and techniques of the clinical laboratory without considering that we were faced with the problems inherent in the recovery of stressed organisms--and most of these enteric organisms, be they indicators or pathogens, are stressed in environmental samples. The moment feces are excreted from the body into an aquatic environment, the optimum growth conditions are radically shifted: the nutrients are diluted, the oxygen tension, pH, temperature, etc. are changed for the worst. Should our little friend survive the trip to the sewage plant, and the sewage treatment process, he is subjected to a lethal dose of chlorine. Should, by some miracle, the bacteria survive, it is usually in a very sad physical state, more dead than alive, which we now refer to as stressed. Using our present methods for isolation, these cells, which were hit by every conceivable hostile environmental factor, are usually cultured on the isolation media which we borrowed from our clinical friends, and it is little wonder that we end up with very little or no growth. It sould not surprise us that we fail to isolate enteric organisms from the environment after we hit them with every conceivable inhibitory factor, including disinfection, and then, to add insult to injury, we employ highly toxic media for their isolation.

Finally, I would like to reiterate that it is my belief that most indicator and pathogenic microorganisms, whose normal habitat is man and/or animal, are stressed when they are excreted into the environment. The more quickly we realize this and employ more efficient isolation and enumeration procedures that cater to their stressed condition, the more successful we will be in isolating these microbes from environmental samples. I also make a plea for a reevaluation of isolating procedures employed by the environmental microbiologist, with the hope that more of my colleagues will realize that there is a difference between enteric bacteria in a fecal sample and in a water sample; this difference is stress.

Indicator Bacteria and the Occurrence of Viruses in Marine Waters

CHARLES P. GERBA*

The release of human pathogenic viruses into the marine environment through sewage outfalls and polluted rivers has been an area of concern from the recreational standpoint and as a threat to important shellfish-growing areas. Human pathogenic enteric viruses (i.e., poliovirus, adenovirus, infectious hepatitis virus, etc.) usually occur in domestic sewage and survive in significant numbers even after conventional secondary treatment, including chlorination. They can also survive in seawater and marine sediments for a few days to several weeks (Gerba and Goyal 1978; Smith, Gerba and Melnick 1978) and have been detected in coastal bathing and shellfishing waters (Gerba and Goyal 1978). The eating of raw or inadequately cooked shellfish from polluted water is known to lead to the transmission of hepatitis and gastroenteritis (Gerba and Goyal 1978). Hundreds of cases of shellfish-transmitted hepatitis have been reported, and enteric viruses have been detected in oysters taken from the East and Gulf coasts of the United States (Gerba and Goyal

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1978). Enteroviruses have been detected in oysters taken from shellfish-growing areas that met acceptable bacteriological criteria for shellfish harvesting. Shellfish take in viruses during feeding and accumulate them in their digestive tracts.

Effective control of enteric bacterial disease spread by recreational waters and shellfish has resulted from the establishment of bacteriological standards using total coliform indices as the basis for limiting recreational use and shellfish harvesting. Much controversy has centered around the adequacy of these standards to reflect a viral disease hazard (Berg et al. 1976). This has resulted from the longer survival time and resistance of enteric viruses to disinfectants than indicator bacteria, as well as the apparent low numbers necessary to cause an infection. Enteric viruses have been reported in drinking water, marine water, and shellfish that met acceptable bacteriological standards. Unfortunately, previous data on the occurrence of enteric viruses in marine waters and shellfish are limited and usually not quantitative.

We have recently compared the results of several studies that we conducted along the upper Texas Gulf coast, where a substantial amount of quantitative virological data was collected (Goyal, Gerba and Melnick 1977; Goyal, Gerba and Melnick 1978; Goyal, Gerba and Melnick 1979). These data were compared to bacteriological indicators and other environmental factors on a statistical basis. Variables common to all these studies were analyzed by multivariate regression. Although multivariate analysis indicated that the number of viruses detected in water was related to rainfall, salinity, and total coliforms in the water, the amount of variation in the number of viruses accounted for by these factors was not large enough to make them good predictors.

The only environmental factors for which a clear relationship with virus isolation could be demonstrated were the occurrence of rainfall within 24 hours of sampling, and salinity of water. There was also some indication that a logarithmic correlation existed between the presence of virus and turbidity, which was probably influenced by the rainfall. The increase of virus in water after periods of heavy rainfall could result from disturbance of sediments containing viruses (R. L. LaBelle, C. P. Gerba, S. M. Goyal, J. L. Melnick, I. Cech, and G. F. Bogdan, manuscript in preparation), runoff, or flushing of sewage-laden waterways that empty into the bay. Although the statistical model, which estimates the number of viruses detected in water based on three factors--presence or absence of rain, water salinity, and number of total coliforms in water--fits well with the observed data, the amount of variance described by this model is only about 16%. Thus, a large amount of variance remains unexplained and is likely to be due to other factors not considered here. Such variance could be related in part to errors encountered in the measurement of the parameters studied, i.e., sampling variations from differences in the efficiency of virus detection from one sample to another.

Enteroviruses were detected 43% of the time in recreational waters considered acceptable as judged by coliform standards, and 44% of the time when judged by fecal coliform standards. Enteroviruses were detected 35% of the time in waters that met acceptable standards for shellfish harvesting. Our failure to correlate the occurrence of enteroviruses in marine waters with indicator bacteria and their occurrence with high frequency in water which met current bacteriological standards indicated that these standards do not reflect the occurrence of enteroviruses, and perhaps other human pathogenic viruses, in marine waters.

Without epidemiological data, it is difficult to assess what this discrepancy means in terms of the possible failure of indicator bacteria to represent a viral disease hazard. Epidemiological studies to establish a relationship between viral disease and the presence of viruses in water would be a formidable task, and doubt has been expressed that such studies would yield meaningful results (Berg et al. 1976).

It is felt that current epidemiological methods are not sensitive enough to effectively detect virus disease transmission through water because clinically observable illness occurs only in a small number of people who become infected, and because of the widely varying incubation periods. These facts, and considering the low infective dose of viruses (Westwood and Sattar 1976), has led some to suggest that the presence of enteric viruses in any water is indicative of a potential viral disease hazard (Berg 1971).

The need for standards governing the sanitary quality of marine waters used for recreation has been recognized by public health officials for many years. In response to this need, most states have adopted standards based on federal recommendations for the sanitary quality of waters used for bathing and shellfish harvesting. Total and fecal coliform bacteria are in general use in the United States for judging the acceptability of contact recreational waters and shellfish-harvesting waters. Water is generally not considered acceptable for contact recreation when the total and fecal coliform densities of 1000 and 200 per 100 ml, respectively, are exceeded.

These standards were based on epidemiological studies on Lake Michigan and the Ohio River, where detectable health effects were associated with a total coliform density of 2000/100 ml. This was extrapolated to a fecal coliform density of

400/100 ml. This value was reduced to 200/100 ml on the assumption that the quality of direct contact recreational waters should be better than that which produced a demonstrable health effect (Cabelli 1978).

Such standards have been applied universally to both marine and freshwater bathing areas. More recent epidemiological studies among swimmers at marine bathing beaches have also indicated a relationship between indicator bacteria and gastrointestinal illness (Cabelli 1978). The Environmental Protection Agency is currently conducting a program to develop such water quality criteria for recreational waters (Cabelli, V. What Do Water Quality Indicators Indicate?, American Society for Microbiology Conference on Aquatic Microbial Ecology, Clearwater Beach, Fla., February 1979). The result of these findings thus far is that a swimming-associated gastroenteritis, primarily in children, can be quantitatively associated with the quality of the bathing water as measured by *Escherichia coli* or enterococcus densities. The gastroenteritis typically has a short incubation period, an acute onset, a short period of relatively benign symptoms and no sequelae, although in some individuals the symptoms may be disabling enough for them to remain home, remain in bed, or seek medical advice. The association between illness and the presence of as few as 10 *E. coli* per 100 ml suggests that the agent(s) responsible for the observed illness is highly infectious, is present in sewage in large numbers, and/or survives much longer than *E. coli* in the marine environment. These characteristics, along with the nature of the illness, suggest a viral etiology, probably related to rotavirus or parvo-like viruses.

Because of their ability to concentrate bacteria and viruses from water during feeding, there is a greater potential risk associated with

shellfish consumption than with recreational use of the same waters. The present microbiological standard in the United States for shellfish-harvesting waters requires a median of 70 coliforms per 100 ml, with no more than 10% of the samples exceeding a value of 230 (Portnoy et al. 1975). Enforcement of this standard has resulted in the absence of shellfish-associated typhoid in this country since 1959 (Cabelli 1978). However, outbreaks of shellfish-associated infectious hepatitis and nonspecific gastroenteritis continue to occur (Gerba and Goyal 1978; Portnoy et al 1975). The most noted recent outbreak was of infectious hepatitis associated with the consumption of raw oysters in Texas, Louisiana and Georgia, in which the oysters were harvested from waters meeting national sanitation standards and were certified for oyster harvesting (Portnoy et al. 1975).

Melnick (1976) recommended consideration of a limit of one infectious unit of virus per 10 gallons of recreational water. This standard was exceeded in about one-third of the samples referred to in the present study. Because of the lack of epidemiologic data, such a standard is arbitrary and reflects limitations of current detection methodology for enteric viruses in water, rather than disease risk. Still, it may be conservative in view of factors such as: (i) the efficiency and concentration methodology of enteroviruses averages 50%, and (ii) current concentration and detection methods are optimized for enteroviruses and are not capable of recovering rotaviruses, infectious hepatitis virus, adenoviruses, or the Norwalk agent which may also be present in wastewater discharges. In fact, current methodology is only optimized for detection of less than 40% of the enteric viruses which could be present in sewage-contaminated waters.

The effect of environmental factors controlling enteric viruses in marine water may be

greatly influenced by geophysical parameters (i.e., bottom topography, shoreline contours, water depth, inflow changes, etc.), so that it may be difficult to apply findings of this study to other coastal areas. Clearly, more work is needed on factors controlling the occurrence of viruses in marine waters for the effective management of marine water quality.

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Discussion On Microbial Indicators Of Environmental Quality

DOUGLAS A. WOLFE

I would like to direct my discussion to only two points, and both of these points fall into the area of how to apply research results to environmental assessment and regulation.

The first point concerns the concept of indicator species. The very words imply that we are talking about an assessment tool, a management tool--whether we are concerned with characterizing a real-world situation, such as the ecological health or potential human pathogenicity in Chesapeake Bay, or the selection of appropriate species for testing of microbial toxicity or microbial degradation in response to the Toxic Substances Control Act. In both cases, we are looking for tools which are *simple*, *easy*, *cheap*, and *meaningful*. People are generally successful at applying the first three of these criteria, but the discussions during this meeting have repeatedly been stymied by whether or not particular measurements, methods, processes, or species are meaningful to an understanding of what goes on in the real world.

Unfortunately, the real world is extremely complex: nearly all the speakers yesterday and today either started out or concluded by reminding us that the natural microbial system is very complicated, with all its species interactions, cometabolism, and geochemical cycles (and in these discussions we have avoided those associated parts of the biosphere which are probably of greater concern to the environmental decision maker or manager, i.e., the fish and other macrofauna).

If indicator species are recognized merely as a tool, however, I think we might get over this mental hurdle. For, as a tool-making beast, man has continually had to make compromises--to make do with the best he has at a particular time. It seems rather clear that indicator species are best at indicating their own presence and, therefore, the presence of whatever it is that they do best, whether they are pathogens, nitrifiers, hydrocarbon degraders, or whatever. There can be no single species indicator of all important functions or potential problems that we recognize in natural systems. We simply must accept this and proceed to make arbitrary selections of our tools based on our best understanding today. The tests of the tool should be, "How well does it work for its intended purpose?" and "Is the tool really needed, or can we satisfactorially manage environmental quality based on a different, more comprehensive tool?"

We heard from Dr. Cabelli that, given today's understanding, we would not select coliform counts as an appropriate measure of the quality of drinking water or shellfish-growing areas. Total or "fecal" coliforms do not reflect the fluctuations of other potential pathogens in aquatic environments. The real question, however, should be "Does the *regulation* based upon total or fecal coliform counts provide generally adequate protection against potential pathogenicity in the environment?" Would a regulation based on *Escherichia*

coli be more effective? The tool should not necessarily simulate or reflect the real world; it should only permit us to avoid as cheaply as possible the problems of potential pathogenicity. If our understanding of aquatic microbial ecology has now progressed to a state where we are concerned, for example, with risks of pathogenicity from other sources not correlated in time or space to coliforms, then let us select alternative or additional indicators. That is, we should fix the tool to cover those concerns and not expect the tool to do a job for which it was not intended. The ultimate test of the regulation tool is how well it works to protect the environment or human health, and this test unfortunately takes time. Prior to committing ourselves to any particular management approach, we should carefully evaluate the advantages or disadvantages of proposed alternate or additional indicator species to make sure that they are necessary accessories and not just cumbersome extra knobs that will provide no real additional protection. Our management system must also be flexible, however, so that it is able to test new tools for appropriate periods and then improve or discard them based on the gathered experience.

As Dr. Cabelli pointed out, we frequently tend to use an obsolete tool just because we have used it for so many years, and that brings me to my second point, which is a general concern that we have not developed effective mechanisms for incorporating current ecological theory or understanding into the environmental assessment and regulatory process. Researchers are slowly developing the necessary environmental understanding upon which environmental regulation must be based, and the regulatory agencies are itching to regulate; yet it seems that there is no effective communication link between the two groups.

Dr. Stern mentioned that one of the criteria

which proposed regulations should meet is acceptance by the scientific community, and he stated that the Environmental Protection Agency will soon be circulating draft or preliminary protocols for review and comment. I think that this is a step in the right direction, although I believe that the Environmental Protection Agency and other regulatory agencies should try to involve active research scientists as early as possible in the actual formulation of those draft protocols. Standing advisory committees with rotating and staggered membership from the scientific community may be an effective mechanism for involving scientists on a continuing basis.

An early step in this process should be the identification of realistic management alternatives, i.e., decision options. Each option or potential tool should be evaluated jointly by regulators and scientists in light of the best environmental understanding currently available, and then a particular option should be deliberately selected for its simplicity and economy of implementation and for its expected effectiveness in protecting broad sectors of the environment. Finally, this interaction between regulators and researchers needs to be continuous. Criteria or regulations must be reviewed and updated regularly and efficiently in the light of recently developed understanding.

Our understanding of ecosystem function may never be complete, but that need not prevent us from devising regulations which adequately protect the environment from pollutant impacts. In fact, since we can never be sure *a priori* that the methods will be adequate, the only way ever to be sure is to devise the regulations and give them a try. What we must be sure of is that we have done everything practicable to ensure that all relevant information is considered prior to selection of the regulation, and that adequate mechanisms are devised for their review and updating.

Where is the mechanism by which our perpetual monitoring of coliforms can be reevaluated? Here at this meeting scientists are making suggestions, backed up by data on how to improve the basis of environmental monitoring and regulation, but alas, I fear the scientists are once again talking to themselves. Can the regulators hear? Can they understand and apply the suggestions?

Discussion

MICROBIAL INDICATORS OF ENVIRONMENTAL QUALITY

M. HOLDER-FRANKLIN: Please show the slide. We have found the mysterious handle that everyone is looking for; it is factorial analysis. This method compresses the enormous variance of a large data base and defines bacterial factors derived from a natural population. The bacterial factor analysis is then correlated with the results of a factor analysis on the chemical and physical data. The two analyses are then compared using the correlation coefficients shown on the slide.

The bacterial factors are designated as MX, an abbreviation of the river sampled. The Meduxnekeag is an international river which has a watershed in New Brunswick and Maine. It flows into the St. John with all its goodies and is of some interest to government agencies, hence the positioning by Environment Canada of the Naquadat monitor for physical and chemical information. The physical-chemical factors are coded NQ. We have very good data to show that MX factor 1, which has been interpreted as aerobic versus anaerobic metabolism and nutritional versatility, correlates with

Naquadat (NQ) factor 3 which is oxygen (mg/l. or % saturation). Another high correlation was shown between MX factor 4, salt requirements and salt sensitivity of the bacteria and the NQ factor of specific conductivity. This factor also includes the ions SO_4, Ca, Mg, K, Na, Cl, NH_4, HCO_3. There are a total of 19 physical and chemical parameters measured. The correlations of bacterial factors compared to physical-chemical factors are up to 0.9 at this time, after some improvements in the analysis. MX factor 5, denitrification-nitrate reduction, correlated to some degree with all four NQ factors. The four Naquadat factors are specific conductivity, factor 1; water temperature and nutrients, factor 2; dissolved oxygen and pH, NQ3; carbon dioxide concentration, NQ4. MX5, denitrification, correlated negatively with NQ3, oxygen concentration,and positively with temperature and water nutrient concentration, NQ2. All of the 19 chemical and physical parameters correlated with one or more bacterial factors. Carbon dioxide was a very valuable indicator for us because we hadn't had any measurement of algal activity. We now use chlorophyll *a*.

To reiterate, these two analyses, the bacterial and the physical-chemical, were performed separately and brought together using the Pearson product moment correlation coefficient. All the appropriate statistical tests of the data have been made.

The bacterial factors were obtained from the matrix of 223 physiological and nutritional tests on the bacteria. I would also like to mention that two of the Meduxnekeag factors did not correlate with the environmental parameters. Before concluding, I should point out one unexpected finding. Meduxnekeag factor 3, interpreted as large polymer production, was related to growth at 4°C within that factor. However, we did not observe a correlation between MX factor 3 and any of the Naquadat factors.

The bacterial factor matrix is the test

response of 1600 isolates to 223 physiological and nutritional tests. The bacteria are tested immediately after pure cultures are obtained. The Naquadat parameters come from the water itself. That is a quick summary of the work and type of analyses we are now doing.

PASSMAN: Dr. Holder-Franklin, did you just do regression coefficients on that or did you try to do some principal component analysis and develop some eigenvalue relationships to your principal factors? Exactly what kind of relationships were you showing us?

HOLDER-FRANKLIN: I consider principal component analysis for our purposes a synonym of factorial analysis. We've done regressions, as well as several other kinds of analysis on this data. The best correlations were obtained with a Pearson product moment coefficient relationship between the two sets of factors.

AUDIENCE: I have a couple of comments to make about what Dr. Cabelli mentioned with his first slide concerning the holy writ of using fecal coliforms and total coliforms as indicators of fecal pollution. We recently conducted a workshop to look at a slightly different aspect of the wastewater treatment problem. While compiling a review of the work that had been done, I was somewhat dismayed to see that people chose the very same organisms for indicators of pathogens in municipal sludges. People quantified total fecal coliforms and then were amazed to see that they were actually present in sludges. One of the things we have spent most of our time talking about is this very issue of indicator organisms, and of course there were lots of coliforms in the sludge. Unfortunately it is not quite clear what their presence or disappearance in sludge or in wastewater really means. But we have spent a lot of time deliberating about what types of characteristics one

would like to see in an indicator organism system. One key parameter is, do we want to use organisms that are present in very detectable numbers? As Dr. Litsky mentioned, an organism such as *Salmonella* in a water treatment system is a stressed organism. Under the best conditions, we find maybe a couple hundred per dry weight gram and after treatment the number drops down to undetectable levels, so people conclude that the organism has been destroyed. Well, if we take a look at that material after it has sat around for a while, low and behold, we see *Salmonella* again. What I think we are really looking at is the variation in reproducibility of the data within--plus or minus--the detection limits. So we've got to go to other organisms, not necessarily pathogenic, which in laboratory experiments show the same responses to stress as pathogenic organisms do, in order to quantify them. For example, the bacteria present in much higher concentrations in the matrix and following similar sorts of fates can be used to get a grasp of what's actually happening to more important organisms that are not present in readily detectable quantities.

CABELLI: You are correct. It seems that both you and Dr. Litsky want me to repeat a statement which we have often made and which is evident from my presentation. There is no universal water quality indicator. The choice depends upon a number of factors--the nature of the untoward event you are attempting to predict or prevent, the water use, prior treatment of the water, etc. These, in turn, determine the salient characteristics of the indicator. For drinking water, chlorine resistance is of major importance; for recreational waters, a predictable relationship to the illnesses involved or their etiological agents is important. The major problem with using a pathogen such as *Salmonella* as a water quality indicator is, as I said earlier, not one of methodology, but rather one of concept and predictability.

LITSKY: It is my opinion that we have recently entered into a new era which will soon be creating innumerable problems. Please allow me a minute to discuss this in order to get it on the record. Under PL 84-660, the Federal Water Pollution Control Act (July 9, 1976), we have spent billions of dollars for wastewater treatment. Our efforts to upgrade treated effluents the past decade has been so successful that tertiary treatment has now become a common term. In fact, these efforts were so successful in solving the effluent problem that we have exponentially increased our sludge production, thus creating another dilemma. In the meantime, EPA in all its wisdom mandated that "thou shalt not use the ocean for sludge disposal by 1982." This puts states such as New York and New Jersey in quite a bind since they have employed ocean dumping as a means of sludge disposal for quite some time.

For the past few years the Office of Water Research and Technology of the Department of Interior and EPA have funded research to demonstrate the feasibility of adding renovated wastewater and sludge to forests and agricultural lands. In fact, the 1977 Clean Water Act PL-217 encourages land applications by providing a 10% subsidy for land treatment processes. Additional incentives for considering land treatment are the requirements that grant proposals for waste treatment facilities funds include land applications in their evaluation on treatment alternatives. In short, the federal agencies are encouraging the addition of sewage sludge to forests and agricultural lands so that megalopolises such as New York-New Jersey will not be innundated by this stuff.

While there have been a few research and demonstration grants to show that this means of disposal is possible, I contend that a great deal more data regarding heavy metal toxicity and plant takeup are necessary. Furthermore, adequate disinfection of sludge must be demonstrated in order to protect our water supply in terms of adjacent

rivers and streams, reservoirs and surface supplies. I contend that we have not adequately researched these parameters in terms of protecting the health and welfare of the public.

Three years ago, Robert Ward and I initiated a limited study to determine the longevity of *Salmonella* in agricultural plots to which municipal sludge was added. Granting that the summer of 1977 was extremely wet, our analysis of the soil-sludge mixture from our test plots was cause of grave concern. We found the presence of *Salmonella* for eight months after the application of dried municipal sludge to our plots. By this short summary I wish to alert this group to the explosive situation that we may be getting into if we continue to encourage the disposal of sludge on forests and agricultural land before we have the answers regarding heavy metal toxicity, heavy metal uptake by plants in the food chain, and adequate sludge disinfection. I also would implore my ecologist friends to consider the effect of land disposal of sludge on the natural aquatic biota via runoff, percolation and the like. Are we creating another monster?

CABELLI: Sludge is another matter altogether. In sludge there is the potential for salmonellosis in spite of the agent's high ID_{50}, because of the presence of particles containing multiple numbers of salmonellae.

HAZEN: This is addressed to Dr. Cabelli and Dr. Colwell. We recently isolated *Aeromomas hydrophila* from 135 out of 147 lakes, rivers and estuaries in 26 states and Puerto Rico. We found higher densities in pristine alpine lakes in the Grand Tetons than Dr. Cabelli found in sewage effluent. Very high and very low numbers of *A. hydrophila* were evident in bayous in Louisiana. In addition we also found quite high numbers in Yellowstone National Park in thermal springs that were without any fecal contamination. These springs

were without any fecal contamination because the source water was superheated (93°C) and the effluent immediately ran down a steep slope. The highest densities of *A. hydrophila* were at *A. hydrophila's* thermal optima, midway down these slopes. We also found that *Aeromonas* densities were higher in lotic habitats than they were in lentic habitats, where densities occur in estuaries. Estuaries, however, were also the most variable. In addition to this survey we have also conducted detailed studies of 23 sites in Albemarle Sound (just south of the Chesapeake Bay) and in 15 rivers and 10 lakes in North and South Carolina over the last four years. These sites were sampled at 1 meter intervals in depth, in triplicate, and our current data matrix contains over 3,000 samples of *A. hydrophila* and 8 to 23 other simultaneouly collected water quality measurements.

When we analyzed the current matrix, using factorial analysis of variance and multiple correlation analysis, we found no correlation between *Aeromonas* and fecal coliforms, total count, total organic carbon, dissolved organic carbon, particulate organic carbon, dissolved oxygen, nitrate or chlorophyll. We did find very significant correlations between *Aeromonas* and conductivity, redox potential, phosphates and temperature. From all of this, it doesn't appear that *A. hydrophila* is a very good indicator of trophic level and/or sewage. I should add, however, that we have found in our chemotaxis studies that *A. hydrophila* is attracted to organics. Specifically, it is attracted to carbohydrates and amino acids differentially and most attracted to fish mucous.

CABELLI: Obviously, you have a set of data that is different from ours, at least in some regards. We obtained an excellent correlation to the relative trophic index and a reasonable correlation to total phosphate, chlorophyll and water transparency. We did do one thing which you apparently did not do. When we found the strong correlation to

temperature, we altered the experimental design and the analysis of the data to eliminate this factor; that is, we conducted our studies in the late spring, summer, and early fall and eliminated from the analysis *A. hydrophila* densities from water samples at less than 15°C and from shoreline samples. A number of years ago, we reported finding rather high densities of *A. hydrophila* in estuarine waters. However, the densities diminished rapidly with increasing distance from known wastewater discharges. This finding was understandable, since we found very high densities of the organisms in the discharges themselves. Moreover, with our improved methods, we now find *A. hydrophila* densities in sewage equal to those for total coliforms.

COLWELL: I'd like to comment. Your work offers a good example of what can be done if the ecology of a situation is understood. You are able to interpret your data in a very useful way. *Aeromonas* is widely distributed in freshwater. Thus, it is not really an indicator of pollution with domestic sewage. *Aeromonas* spp. respond to increased nutrient concentration and elevated temperature. During sewage treatment, a selection for *Aeromonas* and *Klebsiella* spp. occurs. Coliforms, for the most part, tend to be reduced in number or put into "stasis"; which situation applies isn't quite clear. Downstream regrowth occurs, a phenomenon reported in the literature. However, *Aeromonas* spp. are an indicator of disturbance at the microbial level. The species can be interpreted to be, I suppose, an indicator of pollution influx. Near an effluent, counts of *Aeromonas* rise, particularly when the temperature is high (>25°C). Understanding that *Aeromonas* spp. are widely distributed in freshwater environments, as *Vibrio* seem to be in estuarine and marine environments, helps in hypothesizing what occurs when perturbations exist. Clearly, a reduction in species diversity is one obvious possibility. *Aeromonas* happens to be an opportunistic group which becomes

dominant in the situation you describe.

The second point I would make is that methods used to identify *Aeromonas* spp. are not totally satisfactory, particularly some of the methods used for rapid isolation and identification. The quest for *Aeromonas hydrophila*, or related species, may result in missing other *Aeromonas* spp. because the media used are not absolutely reliable.

CABELLI: Rita, I agree with most of your comments. However, let me clarify three points. *Klebsiella* is a part of the total coliform population, and a portion of the klebsiellas meet the definition of fecal coliforms. Secondly, as you point out, understanding the ecology of the situation is important. The interpretation of finding *A. hydrophila* in estuarine and coastal waters is much different than that of finding it in freshwater, especially in the absence of *E. coli*. Thirdly, I can only say that we obtain excellent results with our method provided we use it as designed, for freshwaters and sewage effluents. This is a very special organism Rita has given you. There are probably a dozen cases of wound infections with *Aeromonas*.

HAZEN: About 50 reported; there were three or four fatalities in the last year.

CABELLI: The point is very simple. *Aeromonas hydrophila* is a human pathogen which also is associated with wounds which become infected in the course of recreational activities in freshwaters. Furthermore, it is an aquatic organism whose densities in the water reflect its trophic state. The association of these two observations presents the potential for a limitation on nutrient loading of freshwaters based upon health effects.

FLIERMANS: Concerning *Aeromonas hydrophila*, we have prepared fluorescent antibodies against three different serological strains of *Aeromonas*

hydrophila. We have found the technique very useful in aquatic habitats and *A. hydrophila* is particularly conducive to these kinds of studies. The technique may have been discussed at the first session,which I unfortunately missed. Regardless, these strains of *A. hydrophila* all have the same biochemical properties, but are serologically different. There appears to be a strain associated with the water, one associated with red-sore lesions on largemouth bass and a third strain associated with the intestinal fauna of alligators. We have serologically compared over 300 isolates of *A. hydrophila* obtained by Terry Hazen during his travels as well as by others within the laboratory. These comparisons have been based on quantitative fluorescence microscopy of the fluorescent antibody reaction using a photometer. The statistics are quite interesting in that one can demonstrate three distinct sera groups. These groups have been arbitrarily picked and there are other sera groups which we are not observing at all. I suggest the obvious, in that no technique is a panacea, but the fluorescent antibody technique is a very useful technique that has not been discussed here very much and is quite useful in observing specific organisms in aquatic systems.

TIEDJE: I think maybe we've covered *Salmonella* and *Aeromonas*. There are some other topics that were discussed by the discussants and speakers. Any questions on those?

MCGILLIVARY: I'm with NOAA out of the Ocean Chemistry Lab in Miami. I just wanted to mention to Dr. Cabelli that while we were off the New York Bight looking at natural organic detritus and sewage contamination in sediments about three years ago, while we were looking at hydroxy fatty acids, we came across some unusual peaks on mass spec. These turned out to be sterols, one of which was coprostanol. You are familiar with the work? Alright, the fecal sterol coprostanol has been

suggested by the EPA in this country and by the similar agency in Canada as a sewage indicator as soon as the techniques get developed, which we are not doing. We have done some more work on it--there will be another publication out very shortly in the coming year that demonstrates very effectively that the work originally done by Tabak and Bunch, looking in freshwaters for coprostanol, works equally well in the marine environment. Being a fecal steroid, it is specific as a conversion product of cholesterol only in mammalian guts. It is very stable in geological time, lasting well within the period of concern according to several studies. I just thought I would mention that technique in this context. It is potentially very usable as an indicator of fecal pollution.

CABELLI: We agree as to its potential. However, data are needed to support its use. At the very least it might be employed as a conservative tracer in die-off and transport studies.

NOGRADY: I wish to comment on the interaction of marine bacteria with viruses. In this respect I have to mention the studies of Dr. Magnusson in Sweden. She proved that the Baltic's water has virucidal activity. In her experimental system of viruses and tissue culture, when she replaced the real seawater with artificial seawater, there was no virucidal activity, but apparent cytopathogenic effect. Also it was evident that natural seawater contains some substances with virucidal activity. During the Canadian Medical Expedition to Easter Island (1964/65), I collected seawater samples around Easter Island, and inspired by the results of Dr. Magnusson, I sent her a few samples. It was interesting to learn that the latter samples also had virucidal activity. Magnusson pointed out that the virucidal substance is produced in the Baltic Sea by *Vibrio marinus*. In addition to her studies I found that a Japanese researcher working at the University of Hawaii proved that the

seawater around the Hawaiian Islands also has virucidal activity. It is interesting to note that the Baltic Sea is cold and the weather of the North or South Pacific Ocean is rather warm; we can question the origin of the virucidal activity of seawaters in the latter two regions. It seems that the virucidal activity of seawater is a widely distributed phenomenon and we can wonder how far it is operational in different oceanic environments.

GERBA: I have taken a look at that. Actually, if you look through the body of literature on the influence of marine microbes on virus survival or the role of microorganisms on virus survival in marine environments, there are conflicting results, particularly among investigators in the U.S. who previously were unable to find involvement of marine microorganisms influencing the survival of animal viruses. If you look closely at the body of literature though, I think you will find that most of the investigators in the U.S. who have looked at the problem were dealing with estuarine waters; other investigators in Sweden and Israel have dealt with waters of a more oceanic nature. I suspect that true ocean waters have more virucidal activity related to marine microorganisms than true estuarine water. At least the work that we did when I was a graduate student seemed to indicate that there was a difference in the role of marine microorganisms and viruses in estuarine waters compared to waters of a more oceanic nature. But I believe that's an area that should receive more attention in the future. It has a lot of possibilities.

DUGAN: I've a couple of slides that I thought you might be interested in relative to some bacterial indicators. They are straightforward show-and-tell slides, so it won't take long. We've been looking at some river waters that run through Columbus and we also looked at some near-shore

waters in Lake Erie. It turns out that one of the standard methods for fecal coliform analysis is a membrance filter technique using MFC medium. Lactose is the fermentable carbohydrate in this medium and it contains two indicator dyes: analin blue and a resazuric acid. What we are finding is a number of bacteria which decolorize the dyes and have the capacity to outgrow *E. coli* at either 44.5°C or 37°C. In the upper left-hand corner of this photograph is a membrane filter which contains some of these decolorizing bacteria, and the next slide shows a similar membrane filter which we pulled off the nutrient pad. This is observed when the filters are incubated on a pad or on agar, and you can see that the decolorization goes right through. The next slide shows some of the decolorization patterns. Those two showed a variegated effect but the next slide shows that on occasion the entire slide decolorizes because these bacteria do outgrow the *E. coli*, which negates the whole test in our view. We have isolated five different gram-negative rods, all of which react in various ways. Some have the capacity to decolorize the resazuric acid (which is not really essential in this medium), whereas other isolates have a capacity to break down the analin blue and completely decolorize the plate so that variations in color from red to clear are observed. We are starting to question the whole standard methods technique for identification of fecal coliforms by this method.

CABELLI: I feel compelled to respond to Chuck's comment because of his emphasis on the term "animal viruses." There are no good epidemiological data showing that those enteroviruses, which can be propagated in tissue culture and for which environmental waters have been examined extensively, have been responsible for outbreaks or cases of recreational or shellfish-borne disease. There is ample evidence, however, for waterborne infectious hepatitis and nonspecific gastroenteritis. These have

been associated by the use of the term "animal viruses," to the exclusion of the bacteriophage. Yet there is no conceptual or practical basis for the association. The consequence of this has been a planned distortion of the importance of the "culturable" enteroviruses in the eyes of the public, the courts and even certain state regulatory agencies. Of equal importance, huge sums of money have been spent monitoring environmental waters for these viruses with virtually no output in useable data. Coliphage would have been a much cheaper and more satisfactory alternative. The tragedy is that the credibility of environmental virology is seriously questioned--this at a time when we finally have a handle through epidemiological studies on what will probably be at least some of the important viral agents of waterborne disease.

GERBA: Those are some very good points. What we have is a basis for developing a rationale by developing the methodology to detect their presence. As I said, I don't think developing the methodology for detection say of the rotaviruses and other groups, which are probably related to the epidemiological evidence you just showed, is insurmountable. What I would like to point out, though, is that you may be missing a lot of the cases of other viral diseases, i.e., the diarrhea you observed is probably the easiest symptom to detect, but you may really be missing a lot of the other virus-related diseases. A lot of the evidence referring to animal viruses in water is really based totally on studies with the enteroviruses. And you do have a point that the argument for the importance of waterborne transmission of enteroviruses is going to run out. As we have discussed the last few days, the real emphasis has to be on other viral groups in the future and the development of methodology for their detection in waters. Also, your points are well-taken, especially with respect to the rotavirus,which we have examined more recently. They appear much more

stable in marine waters than do the enteroviruses, which may be relevant to some of the data you have shown here. No, your case with the enteroviruses is well-taken, since the water route could be more important in the transmission of enteric viruses other than the enteroviruses.

Conference Summary

CONCEPTS AND TRENDS

Aquatic Microbial Ecology: Concepts and Trends

THOMAS ROSSWALL

This conference summary discusses the need for basic and applied research in aquatic microbial ecology. In particular it discusses methodology, organisms versus processes, general ecological theory, scale in space and time, microorganisms in relation to other organisms, environmental monitoring, the conventional wisdom, and interaction between microbiologists and "the others."

In summarizing this stimulating symposium, I cannot, as a soil microbiologist with limited experience in aquatic microbiology, and should not formulate a set of research priorities. But I can outline areas of problems that I believe merit consideration. S. A. Waksman once stated that the medium is not so important; if one understands the microbiological processes involved, it is often of secondary importance if one looks at water, soil or sausage (D. Pramer, pers. comm.). The abiotic factors affecting an environment vary in magnitude, but the factors themselves remain the same. Likewise, the biological processes are

generally the same, only their relative rates and importance differ from one medium to another. Let this explanation therefore excuse my attempt to summarize some of the points discussed during the meeting.

Integration is a necessary part of modern science. Not only is it important in space and time, but integration is equally important among disciplines. It has been asserted that one reason for the comparatively small number of aquatic microbiologists is the need for a multidisciplinary approach to aquatic microbiology (Zobell 1973). In recent years we have been witnessing ever-increasing specialization in all fields of science. Today however, there is a tendency to develop expertise in directions that cut across the traditional scientific disciplines. Much of this has resulted from the growing awareness of environmental problems, for which solutions must be sought in a holistic manner. Multidisciplinary research teams are nowadays set up to solve specific problems. The new specialist need not be a pure microbiologist, zoologist or chemist, but one who can grasp the interactions among the parts of the system under study.

The microbiologist must always use various approaches in his efforts to answer questions on the structure and function of microbial communities and their responses to environmental stress. In view of the inherent difficulties with almost all microbiological methods, one must realize that very few if any of these methods will produce the "absolute truth." Using different approaches to solve any one problem requires consistency in interpreting the uncertain results obtained by the use of these methods. Frequently, the need for this consistency will enable us to draw more far-reaching conclusions than those we might otherwise have dared to form by examining the results of the different methods separately. Moreover, the employment of appropriate statistical techniques is imperative in

ecological studies, although this does not seem to be realized by many microbiologists starting from biochemistry and pure microbiology.

The meeting has been extremely stimulating, but in some ways also frustrating. The reason for the frustration may be that we have tried to accomplish two things simultaneously: Firstly, scientists interested in aquatic microbial ecology assembled here for the purpose of discussing new concepts and assessing recent developments. We have now reached a point where we have a fascinating view of the current frontiers of this particular scientific field. This exercise may be regarded as the scientific community's introspection--a look at what it is doing. For the most part scientists find this type of activity not only very stimulating, but essential to the advancement of science.

Secondly, we tried to assess the importance of present knowledge in relation to applied needs, especially with regard to the necessity for regulatory action. The very interesting presentations on this topic have successfully made us aware of the formidable problems facing, for example, EPA. I hope we now realize the important need for rapid action. A new approach will have to be adopted if we wish to present our beliefs to the agencies concerned, but the immediate problem is in convincing ourselves before we can attempt to convey the message to others.

Although I do not wish to sound too negative, my belief is that our best course at present is to entrust a few specialists with the task of suggesting tests for immediate use. We should not just sit back and hope that better methods will become available, but should actively try to ensure their evolution. A concerted effort

should be made toward an integrated development of test methods for use in assessing biodegradability and microbial toxicity. If we do not act now, we will just have to come back later and start all over again.

With regard to important new trends and the development of concepts in aquatic microbial ecology, many examples have been provided during the excellent presentations at this meeting. Some items that I feel require careful consideration follow.

Methodological Concepts

In 1972, a meeting on methods in microbial ecology was arranged (Rosswall 1973). At the same time the IBP methodological handbooks (e.g., Sorokin and Kadota 1972) were published. The ATP measurements have been followed by the suggestion that energy charge is a more appropriate measure, especially in assessing the effect of stress (Ivanovici and Wiebe 1978). Radioautographic techniques have been developed for determining the proportion of actively metabolizing bacteria in samples studied by epifluorescent microscopy for determination of numbers and biomass of aquatic bacteria (Meyer-Reil 1978). The same aim has also been achieved by using a tetrazolium salt and counting the number of cells with intracellular formazan (Zimmerman et al. 1978). By use of the two methods, 2 to 56 and 5 to 36% of the total numbers of bacteria were found to metabolize ^{3}H-glucose and respire, respectively. A method has also been developed for the use of fluorescein diacetate staining for determining the proportion of actively metabolizing fungal cells (Soderstrom 1977), and this method is being adapted for use

with bacteria (B. Lundgren unpublished).

With regard to the methodological approach, the development by Robert Koch of the plate count procedure introduced the "plate count era" into microbiology, an era which has still not declined in aquatic and environmental microbiology. The development of direct count techniques amply showed the discrepancy between data obtained by the two methods, but neither method told us anything about the processes carried out in the environment by microorganisms. With the development of ecosystem research, especially as part of the IBP, microbiologists were forced to develop methods that could determine process rates, and integrated measurements, such as respiration, dehydrogenase activity or ATP content, were used in addition to more specific measurements like the acetylene-reduction method for nitrogen-fixation determinations or cellulose-decomposition experiments. After the pendulum swung between the two extremes of plate/direct count data and process studies, it stopped somewhere in the middle. Attempts are now being made to relate observed microorganisms to processes. Only by gaining a thorough understanding of the physiology and ecology of the microorganisms will it be possible to understand, evaluate and quantify their role in ecosystems.

Another methodological development concerns the system under study. In view of the complexity of natural systems and the artificial nature of the conventional culture flask in the laboratory, attempts have been made to develop systems of intermediate complexity, so-called microcosms. Such artificial ecosystems have, for example, been developed to test the effect of pollutants (Draggan 1976; Draggan and Van Voris 1979;Stern 1980). These microcosms should also prove to be of great advantage in developing the linking of process and observed organism studies.

In the development of these microcosm experiments, and in fact in all aquatic microbial ecology studies, one should always remember that most aquatic environments are cold and oligotrophic. A notion to get away from is that microorganisms are ideal subjects for study because of their inherent fast growth rate, with generation times of less than one hour. Another incorrect notion is that microorganisms, especially bacteria, occur singly or in microarrangements, such as chains or microcolonies of a single strain. Unless stressed, microorganisms always occur together in communities or even in consortia (Hirsch 1980).

Organisms Versus Processes

As already mentioned, a large number of the organisms observed under the microscope cannot be cultivated on conventional media. This is probably due to the fact that their natural habitat is oligotrophic, and most laboratory media have excessively high concentrations of energy sources and nutrients. Special media need to be developed if the oligocarbophilic and oligonitrophilic bacteria are to be isolated. It is also important, however, to ask such questions as: Why can we not cultivate many of these organisms? What importance do they have in the system? These questions are fascinating and the microorganisms intriguing. We must address ourselves to the question of the function of the nonculturable microorganisms, and we hope *in situ* methods for assessing this aspect will be developed. These organisms are so fascinating to look at, the danger exists that we will never get around to asking functional questions.

It was stated during the meeting that "junk media only give you junk organisms." But maybe these junk organisms are the ones in which we should really be interested. This is not like simulation modelling where the rule "garbage in, rubbish out"

holds. The organisms that grow on junk media are the saprophytic bacteria that take part in the breakdown of organic matter and in nutrient cycling. These are the organisms that are generally responsible for the self-purification of water bodies and that act as the working factories of the fascinating ecosystem of a sewage plant. Our knowledge of nitrogen mineralization by these junk bacteria and of the competition between bacteria and phytoplankton, e.g., for ammonium nitrogen, is scanty; what is, for example, the role of the C/N ration in determining net mineralization rates for nitrogen?

Considerable interest has been shown also in the biogeochemical nitrogen cycle in aquatic systems. There is, however, a danger in considering nutrients one at a time. Important interactions take place among elements, and the concept of single limiting factors often does not apply. Increased nitrate levels in water bodies might, for example, lead to increased decomposition of organic matter under anaerobic conditions, nitrate respiration thus liberating phosphorus from the decomposed organic matter into the aquatic environment. Increased sulphate levels may also result in the release of phosphate, as has been shown for Lake Mendota (Brock 1980).

It is important to consider the interaction of different physiological groups of microorganisms, which in microniches may form semiclosed systems with complex patterns of energy flow and nutrient cycling (Slater 1980). These microniches generally develop in close proximity to surfaces that are efficient in concentrating organisms, energy sources and nutrients (Shilo 1980). Such assemblages can never be understood by investigating pure cultures in the laboratory under artificial conditions, and generally they cannot be understood from process rate determinations in the field either. A combined approach is needed using physiological knowledge, microcosm or chemostat

experiments and microscopic observations of microbial assemblages in nature. Perhaps sophisticated techniques will soon be available for linking direct observations with process studies using autoradiography and other methods.

General Ecological Theory

Semantic problems often arise when a group of botanists, zoologists and microbiologists try to discuss general ecological theory. Botanists and zoologists always start by defining the species present and then proceed to draw certain conclusions on the role of these species in the ecosystem. The name of the individual isolate of an aquatic bacterium--if any name can be given to it--in only a few instances gives us any indication of its role in the ecosystem. This has caused the very slow development of the concept of applying general ecological theory to microbial ecology. Some even believe that this is beneficial and that microbiologists should develop their own theory, or, in the words of A. D. McLaren, "...Microbial ecology sounds like fun ... I trust that you will avoid borrowing too many 'rules' from macroecology" (Lynch and Pool 1979). To me it seems that, on the contrary, we have not sufficiently discussed concepts like food-web efficiencies, niche separation and population diversity. Such discussions may be important in order to initiate a fruitful dialogue between microbiologists and other ecologists.

What are the real growth rates of bacteria in aquatic habitats, and what are their assimilatory efficiencies? It is important to integrate estimates over a long period of time to determine whether the results are realistic or not. By way of example, Heal and MacLean (1975) gave a general assimilatory efficiency for microorganisms of 40%, while Nagel de Boois (1976), investigating the

growth of fungi on leaf litter, found the efficiency to be only 10%. If these data are put into an ecosystem context such as the tundra mire with which I have been working, these figures are shown to be totally unrealistic, and growth efficiencies of 70-80% have been found necessary to balance the estimated microbial-growth rates and the energy flow through the mire (Svensson and Rosswall 1979). This only gives a very vague idea of what the real value is, but integration should be made of the observations in an ecosystem context in order to evaluate their plausibility. Estimates of growth rates and assimilatory efficiencies under energy- and nutrient-limited conditions at realistic temperatures are sorely needed.

The concept of maintenance energy is important, but realistic data are scarce. Bibiuk and Paul (1970) used literature data on specific maintenance requirements in trying to estimate growth rates of bacteria in grassland soil and, knowing the annual energy input to the decomposer system, concluded that the bacteria could only reproduce a few times a year. Even if soil fungi were omitted from the calculations, this seems unrealistic when compared with bacterial growth rates observed by other methods (Clarholm and Rosswall in press). If the concept of maintenance energy is to be used, determinations of specific maintenance must be conducted under natural conditions.

Surprisingly, great interest in diversity concepts has been shown during this meeting, and various speakers have mentioned the use of the Shannon index in describing bacterial populations from waters (Bianchi 1980; Staley 1980; see also Kaneko et al. 1978). It is possible that physiological diversity is a more meaningful way to discuss the diversity concept in a microbiological context. A vast amount of data on physiological and biochemical characteristics has been collected over the past ten years in projects concerned with

numerical taxonomy of strains isolated from natural habitats. This data base is an untapped well of information and much more use can be made of it, as it offers unique possibilities for describing natural populations of microorganisms in functional terms. Concepts such as physiological diversity in populations may constitute one important component in analysing the effect of stress on microbial populations (Rosswall 1979). The information collected in numerical taxonomic work can thus be used in many ways other than describing OTUs (operational taxonomic units).

From the data material a correlation matrix between test results, rather than between strains, can be calculated. This gives us important information on how physiological characteristics are linked in populations of bacteria from natural environments. It is probable that certain tests are very closely linked, reflecting, for example, enzymes in consecutive steps of metabolic pathways. Strong negative correlations are less likely on physiological and evolutionary grounds (Rosswall and Kvillner 1978). It is probable that increased knowledge of character correlations in freshly isolated populations of microorganisms from natural habitats would give us a better understanding of the microbial associations in nature (Gyllenberg 1965). Cluster analysis can, of course, be made on test results as well as on individual isolates, giving further information on the linkages of physiological capacities in natural populations (Rosswall and Kvillner in manuscript).

Factor analysis defines the position of the individual population in a multidimensional factor space. Its physiological capacity can be interpreted by the position of the point centroid of the population along the first principal factors. Changes in time over the day (Holder-Franklin et al. 1978) or season (Rosswall and Persson 1980) as well as the effect of external factors like pollution

(de Leval et al. 1976) can be quantified. The extension of the population around its point centroid is also a measure of its physiological diversity (Rosswall and Kvillner in manuscript). The functional diversity is also reflected in the proportion of the total variance in the test results accounted for by the first factors. Figure 1 shows that populations of aquatic bacteria from seven Swedish lakes are physiologically more homogeneous in the autumn than in the spring.

Data on functional characteristics of bacterial populations can also be used for discriminant analyses in which it is assumed that the populations are different, and attempts are made to explain this in terms of the test results. By the use of stepwise discriminant analysis it is possible to determine the efficiency of the test to distinguish between the different populations (Rosswall and Persson 1980). This information can then be used to design the most efficient set of tests for use in describing sets of populations from given habitats. The data from the discriminant analysis can be interpreted in functional terms in the same way as can data from factor analysis (Figure 2). Discriminant analysis is, however, often more efficient in distinguishing between different populations than factor analysis. The discriminant analysis further determines predicted group membership as a proportion of actual population membership, and this is also a reflection of the diversity of the population.

Thus, the conclusion of this section is that the vast amounts of information collected for numerical taxonomic purposes offer unique possibilities for ecological interpretations by the use of other numerical methods such as factor, cluster and discriminant analyses.

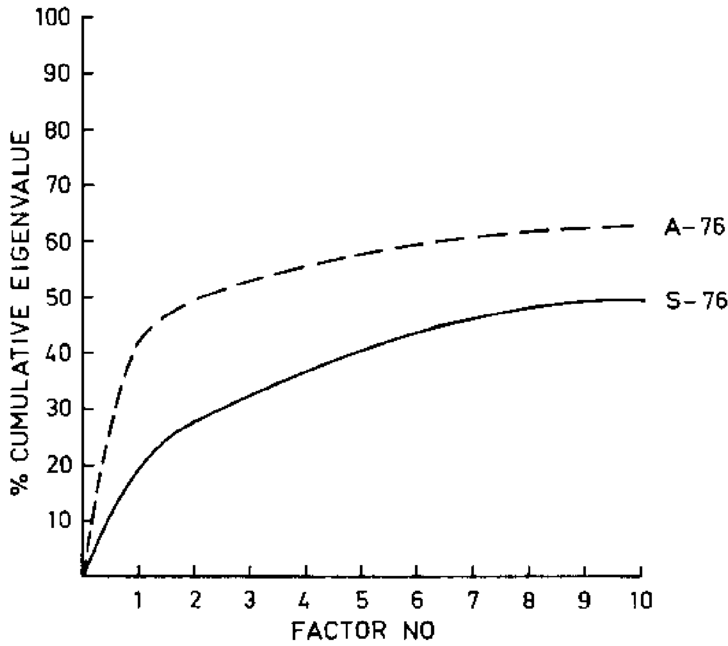

Figure 1. Cumulative percentage of eigenvalues for the first 10 factors of 10 populations of aquatic bacteria sampled from seven lakes. Five populations were sampled in the spring (S-76) and five in the autumn (A-76). The bacteria were subjected to 25 physiological and biochemical tests, and the results were treated by a factor analytical procedure. The relative homogeneity of the autumn populations is indicated by the steep curve (Rosswall and Persson 1980).

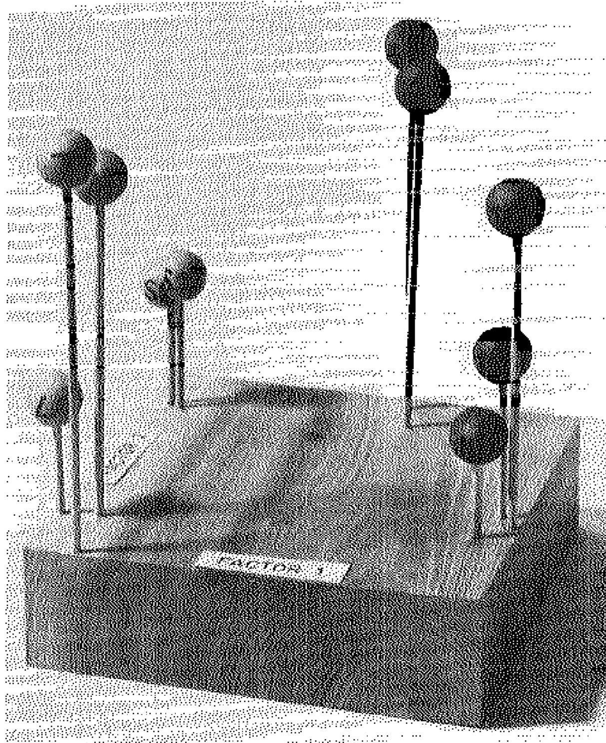

Figure 2. Relative position of the point centroids of 10 bacterial populations (cf., Figure 1) in relation to the first three discriminant functions. Light balls represent spring populations and dark balls autumn populations. The first function (x-axis) discriminates spring from autumn populations; the second function (y-axis) discriminates four populations from one lake (S 1 and S 2) from all other lakes; the third function (z-axis) discriminates spring populations from eutrophic lakes (K and T) from oligotrophic lakes (S 1, S 2, and L). Figure constructed from data in Rosswall and Persson (1980).

Scale in Space and Time

Integration in space is extremely important, for example with regard to inorganic nitrogen metabolism. The use of microcosms is one step towards mimicking nature in the laboratory. It is, however, difficult to extrapolate microcosm information to the mixed-community level in the field. Even if field measurements can be performed, how can this be extrapolated to the ecosystem level? Nitrogen-fixation measurements are fairly simple to perform, but how can the large variability in rates be integrated to the ecosystem level, and is it not impossible to extrapolate this further to produce global fixation rates? (Burris in press.) However, we know that the globe is a closed entity, and by looking at the global cycles we know that we must have consistency in the data base. Extrapolation of community and ecosystem rates of N_2O/N_2 production and nitrogen fixation to the global level will give us an indication of whether the rates are reasonable or not, at least within one order of magnitude (Soderlund and Svensson 1976). With the great spatial heterogeneity observed in nature, this may prove to be very useful.

With regard to time scales, the beauty of the ATP technique is that it measures "something" instantaneously without incubation. Most other techniques for measuring rates call for a certain incubation time. It is necessary to integrate collected data not only in space but also in time. How important are the diurnal variations when attempts are made to calculate yearly rates? One should ask if the result is reasonable after having extrapolated individual measurements to a yearly rate. But remember this--there is no such thing as a typical year.

Microorganisms in Relation to Other Organisms

There is strong competition between bacteria and phytoplankton for available nutrients. This may be somewhat better known than the factors affecting the competition between soil microorganisms and plant roots for available nutrients. It is, however, impossible to consider detritus decomposition, net mineralization, nutrient release and uptake without taking into account the competition for available nutrients. The effect of grazing by protozoans has been studied extensively in the laboratory, but not much is known about the real importance of grazing in natural aquatic habitats. Also, grazing by nematodes might be important not only in terrestrial but also in aquatic habitats (Tietjen 1980).

The interaction of microorganisms and higher organisms can also be viewed from the standpoint that the microorganisms are secondary stress factors. This may be especially important in aquacultures and warrants further attention.

Environmental Monitoring

The importance of, and the problems involved in the quest for microbiological indicator species have been nicely covered in this meeting (Colwell 1980; Cabelli 1980). Indicator species may find certain specific applications, but this is only one of a large number of factors which may be selected when considering microbiological parameters in an environmental monitoring system. Other examples are given in Figure 3.

It is very important to consider test reproducibility, which has previously received too little attention. Certain attempts in this direction have been made in microbial taxonomy (see, e.g., Sneath and Collings 1974).

Miniaturized techniques are also important,

1

Organism

3 Substrate ——————▶ Product 4

Activity

2

1. Biomass
   Taxonomical identification

2. ATP
   Dehydrogenase

3. Litter decomposition
   Cellulose decomposition
   Respiration (O_2)

4. Respiration (CO_2)
   Nitrogen fixation

Figure 3. Different types of microbiological variables useful for a monitoring system (Rosswall 1979).

and methodology can often be adapted from other areas such as clinical microbiology, where there has been a heavy demand for the development of rapid, reproducible and miniaturized techniques (Rosswall 1976). The use of radioisotope-labelled materials will probably be very useful, not only for nutrient- and carbon-uptake measurements but also for testing the biodegradability of a wide variety of compounds, and such techniques lend themselves readily to automation.

Questioning the Conventional Wisdom

During the meeting many speakers stressed the need for questioning the conventional wisdom. Do not always believe in textbooks and in *eminences grises*.

Several examples were mentioned that proved contrary to accepted knowledge, such as anaerobic nitrogen fixation and photosynthesis by cyanobacteria, as exemplified by the shift between oxygenic and anoxygenic photosynthesis on *Oscillatoria limnetica* (Shilo 1980); nitrifying methane oxidizers; denitrifying nitrifiers whereby *Nitrosomonas* spp. use NO_2 as the terminal electron acceptor under anaerobic conditions and reduce it to nitrous oxide (N_2O). The ecological advantages in the last example are manifold (Rosswall 1978) as this may offer a metabolic pathway for the nitrifiers to survive under brief conditions of anaerobiosis, be a detoxification mechanism if nitrite concentrations become too high, and decrease nitrite concentrations in order to minimize competition from *Nitrobacter* spp.

Fudge factors too were examined. Do not uncritically use conversion factors cited in the literature. Such factors often seem to be self-perpetuating once they have entered into the realm of conventional wisdom and are rarely questioned.

Interaction between Microbiologists and "the Others"

The individual scientist has a responsibility to communicate his results and concepts to others. This is normally done through publications in scientific journals, whereby he communicates with his fellow scientists. It is, however, also important that he consider how scientific concepts can be communicated to the general public. This is in no way an easy task, but it is important if we wish society to understand science. In many countries culture and science have been considered things that a developed society ought to have, although there is no real need for either. Only by effective communication with the general public will it be possible to dispel some of the general suspicion with which science and scientists are often viewed.

Communication with so-called decision makers is different. Scientists are increasingly being asked to give advice to legislative bodies or to policy and decision makers. People in these positions cannot use an indecisive signal or amber light. What they require are resolute signals of green or red on which to base their decisions. To sum up in the words of Alexander (1978): "If microbial ecologists ... are not willing to provide the relevant information ..., then society surely has a right to call a halt to the growth of our field and to the extension of our quest for knowledge for its own sake."

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The Regulatory Responsibility

A. W. BOURQUIN

Since I work for a regulatory agency, the Environmental Protection Agency, I should like to approach some of the questions we have discussed from a slightly different point of view. The questions that I face every day sometimes might seem a bit curt to a scientist in academia, but they must be answered constantly by regulatory decision makers. Whenever we are producing data involving monitoring, or data for criteria development, we are asked questions such as: So what? What does it mean? What are you going to give me? Agencies such as the Environmental Protection Agency have a number of regulatory responsibilities, only the most recent being enforcement of the Toxic Substances Control Act. Some hard decisions must be made when funding research, in-house or extramural.

We have been discussing the problem of how to assess environmental stress on ecosystems and the microbiological processes therein. Basically, what we have been discussing are methods--methods

for measuring biomass, heterotrophic activity, biodegradation, and other indicators of environmental quality. What I would like to see discussed now is the question: What can we get out of these methods that can be used by regulatory agencies? I am not sure that we are any closer to agreeing on any particular method or methods as more acceptable than others to the scientific community for assessing any environmental stress. However, I think we have learned a lot about the various methods, including their limitations and their possible benefits. We have addressed various research needs and have just heard a very excellent presentation on this subject. What I would like to add to this is a request that we begin to apply some of this information, particularly in terms of the limitations and benefits of the various methods, to assess the so-called health of a given environment. There are environmental decisions which must be based on the available technology, because we really do not have any time and must make some decisions now. We can modify those decisions later, but decisions are needed now based on the current technology. If people such as those at this conference do not aid in determining which methods should be used with what qualifications, then less qualified people will make those decisions.

In assessing effects of environmental stress on microbial processes, often individual processes are evaluated. As Drs. Tiedje and Alexander both pointed out, no regulatory decision has ever been based on the results of toxicity data from these studies. Dr. Stern, however, felt that we could not afford to omit tests for toxicity that, at a later date, might show a chemical blocking a particular critical microbiological cycle. In our work we usually consider microbial processes and not individual cultures. However, we have to study individual pure cultures to determine what is actually happening to a particular process.

However, we rarely find that one organism is critical to a particular process, so an inhibitory effect on an organism would probably not cause any significant effect in the real world. Nonetheless, I would like to advise some caution in tossing out toxicity testing. Recently, we discovered a significant inhibitory effect of a pesticide on the capacity of an environment to biodegrade other pollutants. Rather than study the effect of the toxicant on a natural cycle, we determined the effect of a toxicant on the biodegradation of another pollutant. These data have made us take a closer look at toxicity testing and the type of information that we may develop in such studies.

I would like to emphasize another point that Dr. Tiedje made: that biodegradation studies should be done under conditions that mimic those of the receiving environment. This was also emphasized by Dr. Rosswall in assessing integrated processes. Although individual laboratory tests using pure cultures and those that assess various degradative processes give us very useful information when making exposure assessments, the fate of a chemical in the environment results from various individual processes acting together; it is an integrative process. Therefore, fate-testing systems for use in predicting exposure concentrations need to be developed to incorporate the complex aspects of the receiving environment. This is particularly true in marine or aquatic environments, and it is for this reason that we have begun using artificial ecosystems. In these systems we think we are more closely mimicking the receiving environment. Bill Gledhill showed you one of these systems that we developed at Gulf Breeze. This type of approach integrates the various degradative processes. We hope that by getting together the various processes in one system we can determine an exposure concentration. We are also integrating our data with those of other people in our laboratory who are

studying toxicity effects. We hope to produce a number at the laboratory level that we can call a hazard assessment for a compound in the marine environment.

Art Stern showed us a list of problems yesterday on degradation testing, and among them was the definition of significant degradation. Dr. Alexander said that we should establish the susceptibility of compounds to mineralization. From our own work in marine systems, we have seen that CO_2 evolution may not always be the best indicator of degradation or detoxification. For example, in our work methyl parathion is detoxified rapidly and we never get more than 10% of the labeled carbon off as carbon dioxide. In the information that Dr. Alexander presented, he made the statement that 7% of CO_2 of the low concentration tested indicated no degradation. We disagree. We think that there is degradation, there is detoxification. These are some things that we need to take a closer look at, particularly in the marine environment. These phenomena may be peculiar to aquatic or marine systems. We really do not know. They may reflect the fact that we are using extremely low concentrations. Marine waters receive pollutants in dilute quantities; therefore, these tests are conducted at low concentrations. As Dr. Tiedje pointed out, we need more information on the biodegradation of low concentrations of substrates. Other organisms that exist at these concentrations may be physiologically different, distinct from those that exist in environments having higher substrate concentrations. This is a question that we cannot answer at this time but a question which we need to address.

I believe that an area requiring a lot of research is biodegradation in the marine environment. We know of at least one compound that is degradable in other environments but seems to be

inhibited in estuarine environments (nitrilotriacetic acid). I am sure that there are others.

Last, as pointed out by Dr. Tiedje, is the concept of predictability. This is probably the bottom line as far as regulatory agencies are concerned. We must be able to predict accurately what will happen to a compound in a given environment. What is the biodegradation potential for a given environment? What is an environment's assimilatory capacity for taking on a pollutant, biodegrading it, assimilating it? I hope these are some points that we can discuss in relation to Dr. Rosswall's excellent presentation.

Discussion

CONCEPTS AND TRENDS

PRAMER: I am sure that our speakers will welcome any questions or comments you may have on what they presented here this morning. In my opinion, this conference has enabled us to hear from those who view microbial ecology as an academic discipline to be pursued for its own sake, and from others who are associated with government agencies responsible for monitoring the environment and for regulatory processes. Members of the latter group, with good reason I think, are calling on the former for help. They are here saying, you are the experts, you are the ones who are more knowledgeable than anyone else. You may not know all you would like to know, but we need you to advise and guide us now. That request--that plea--has been made at various times during this conference and it was repeated here this morning. I think it is exceedingly important that these two positions be recognized as related and interdependent. Those of us privileged enough to spend our time addressing basic questions that primarily are of interest to ourselves--that is, those who deal with microbial ecology as an academic discipline--have some degree

of obligation to respond to questions posed by those who are responsible for safeguarding the public welfare. In previous sessions Dr. Floodgate expressed the academic position and Dr. Stern made clear the need for academics and regulators to communicate. The importance of communications was evident also in Dr. Gledhill's presentation. However, the need was most clearly stated yesterday evening when Dr. Wolfe of NOAA reminded us that man is a tool-using animal and that he, as a member of a regulatory agency, lacked tools for his work. He asked those here who are willing and able to help to respond, and I hope that they will. Tom Rosswall implied that this may not be the best time or place to encourage dialogue between academics and regulators, and he may be correct. Perhaps they should meet on a different occasion and use a different format than that employed here. I will be happy to hear from anyone anxious to speak to this question, or from those present who may wish to further discuss methodology, biomass, or problems associated with estimations of microbial activity.

BARTHA: I would like to make a remark that responds to one of the last things Dr. Bourquin mentioned. In the case of some compounds, $^{14}CO_2$ evolution doesn't seem to be a good indicator of detoxification. As an example, he mentioned the compound methylparathion. Earlier in the conference, a speaker also referred to this by saying that some materials become humified rather than go up in CO_2. Our laboratory has done work on so-called soil-bound residues. I didn't talk about this because we are here at an aquatic conference, but the remark of Dr. Bourquin indicated to me that a very similar problem may occur in the aquatic environment. The residues are not "soil bound" in the sense of being bound by absorptive forces to clay particles, but are actually covalent bonded to humic types of compounds very clearly subject to this type of binding and are aromatic compounds that are free primary amines. Now parathion and methylparathion don't have this character, but on the other hand

they have a nitro group, and in many biological environments this nitro group tends to be reduced to a primary amine. As a result, these substances tend to be integrated into humic compounds and in consequence acquire very long residence times. Dr. Lichtenstein's group showed this with parathion and methylparathion. Our laboratory was involved with anilide-type compounds, where a primary aromatic amine becomes liberated once the compound is hydrolized. A third group of compounds might be subject to this fate and Dr. Bollag recently published a series of papers documenting that phenolic- and chatechol-type intermediates of pesticide degradation are humified, i.e., are polymerized by microbial lactases. Now this type of transformation was shown only *in vitro*, and so far we don't know to what degree this occurs in the natural environment. If this is indeed applicable to the natural environment, of course it would extend tremendously the range of compounds which can become humified in this manner. The studies on the anilide- and parathion-type compounds were done in soil samples, and we know that this is indeed their environmental fate and does not occur only *in vitro*, and so far we don't know to what degree this occurs in the natural environment. If this is indeed applicable to the natural environment, of course it would extend tremendously the range of compounds which can become humified in this manner. The studies on the anilide and parathion-type compounds were done in soil samples, and we know that this is indeed their environmental fate and does not occur only *in vitro*.

BOURQUIN: We are all aware of the binding phenomen in aquatic sediments. My point was that we did have a budget, and evolution of $^{14}CO_2$ did not reflect the extent of degradation in the system. Another point I probably should bring out is that in biodegradation studies you must be aware of what happens to the C-14 label. We do have a certain amount of binding in aquatic sediments. It doesn't seem to relate closely to those studies done in soil studies--all primary amine-type compounds do not

really bind. We have been doing some work with Dimilin, a chitin synthesis inhibitor. In this case, Dimilin hydrolyzes to an aniline compound which is found in the extractable residues. It does bind somewhat, but we can extract it. In the case of methylparathion, it does not degrade in strictly water systems, but must have a system containing both sediment and water. In that case, the compound is first reduced to amino-methylparathion and it is probably this compound or paraminophenol that is actually irreversibly bound. This is another area that needs to be investigated: How permanent are these bound residues?

DALEY: I would like to make a couple of general observations as a Canadian coming down and looking at the American system. I am shocked by the absolute identity between what appears to be preoccupying people here as it relates to the Canadian system. As someone who rides piggyback on applied programs, who does basic research through the back door in applied programs, and who works for the government--an organization that does both the data collection and the regulation--I find it sort of obvious that there is a conflict between scientists wanting to produce information, and regulation. One of the things I find as a research scientist in this milieu is that it's not enough to talk about what you would like to have. Regulatory people, and people who need answers to today's fire fight, require something that they can use, and it becomes a psychological problem depending on your frame of mind. For example, we spent five years developing a chromatographic system that does an elegant job of separating out every single carotenoid and chlorophyll that's ever been seen in a phytoplankton population. And that's absolutely useless to an organization that requires 7,000 chlorophyll analyses in 28 sites in 2 months to try to figure out a particular problem. It forces you to focus back on cost, time frame, and simplicity of method. In a sense, the methodological session yesterday and this morning's comments about regulation

come together. The best way to bring those two groups of people together is to focus on the question: What is the most practical method that can be used now for attacking a particular problem? It doesn't make it any simpler, but it gives a starting point to the argument. To carry on with the chlorophyll analogy, we were given the fact that this was needed in the region, and that there was a standardized methodology required for benthic and pelagic work. All of the people who were using the excuse that the methodologies differed got together and standardized a simple autoanalyzer technique, which from a pure research point of view was simply appalling. But it has sufficient sensitivity and it has sufficient reproducibility to provide useful information for the specific government problem that needs to be answered. Now we have time to go on to other problems because we have resolved that one. It's resolved at a very crude level and it doesn't preclude continuing at some low level to do chromatographic analysis of pigments, but it allows one to segregate, in one's scientific mind, different types of activities. I'd like to emphasize that it takes an organizational technique, in conferences and in the relationship between regulatory agencies and scientists, to get beyond the motherhood issues and past the frustration, or at least to focus the frustration on the real world rather than on the hypothetical.

FLOODGATE: I would like to respond to this idea of the academics versus the EPA people, because we get the same kind of problems. So this is Floodgate's message to the EPA. First of all I think that we must realize that we cannot form a rule or law which is applicable to all places at all times. I think this is one of the things that people who make rules want to do. They search for rules that are universal. In point of fact, I think the first message that any biologist has to give to these people is that you simply cannot do that. You cannot make a rule that is applicable at all places and at all times, even for a very narrow range of chemical

substrates. This is a difficult thing to persuade lawyers is true. Secondly, you can perhaps make a rule which will mitigate against the worst possible disasters--for example, properly controlling the traffic in the English Channel so we get no more AMOCO CADIZ affairs. We still of course can't stop captains from quarreling with each other, which is why we had the TORRY CANYON disaster. No kind of rule will mitigate against the fact that the captain of that ship was quarreling with his first mate and they weren't speaking to each other. In this connection too, in this idea of mitigating against the worst possible disasters, we can use our present techniques. But as we already mentioned several times, these are pretty crude still. So in this state of affairs it seems to me to be more realistic to spend money on monitoring the environment and improving our basic knowledge, rather than spending a great deal of money on carrying out worthless tests. In a sense, it's a question of where are you going to put your available funds. It seems to me that putting the funds and resources into carrying out a lot of meaningless tests, which the industrialists will simply get around either by legal manipulation or possibly by genetic engineering (this was suggested yesterday) is a waste of time and money. It would be far better to spend that money by giving it to Rita Colwell to do some basic ecological research than to simply waste a lot of cash doing tests which really don't mean a darn thing--except that they provide money for lawyers and people of that sort.

PRAMER: Is the position you present being taken by the government of your country?

FLOODGATE: Well, the last time I had dinner at Number 10 Downing Street..... No, Jimmy Callaghan doesn't take me into account these days; he has far too much worry with the unions. I really don't know what the position is because we are like you in a sense, we don't have too much dialogue between the

academics and the other people. But I think we have a slightly better or less formal way of doing it. We tend to have a situation where an industrialist who wants to do something gets on the telephone to people in authority and says please can I dump this in the sea. I think there are two major differences between ourselves and the U.S.A.; firstly, we are a very small country and can be much more in touch with each other. It is much easier to get in touch because we don't have to fly thousands of miles or do anything really dramatic. We just get on a train and are there in an hour. This makes a great deal of difference, in that the industrialist can go and have lunch with the man who is the regulatory man and discuss it over a pint of beer; it makes it a lot easier. And secondly (I discovered this too when I was talking to the Japanese people at the Pensacola meeting), we are all so close to the sea--no part of Britian is more than 60 miles to the sea. When you come to Coventry you will be farther from the sea than any other place in Britain. It's a good sixty miles. This means that because we have the sea all around us and this is a sea with a lot of passing water and a lot of high tides, we can chuck stuff in the sea and dilute it and forget it much more easily than you can. I think that this is a great advantage that we have that you haven't got. The sea is right around us, whereas you have this enormous country where it is much more difficult to get rid of your wastes. The French and the Germans are not quite as lucky as we. We have a residence time in the Irish Sea of one year. This means if a particle gets into the Irish Sea, it is swept out in one year. So in terms, for example, of oil degradation, anything that hasn't happened in a year we can forget, because by then it is out in the Atlantic. But the Baltic has a residence time of 40 years and the Mediterranean has a residence time of something like the same sort of value, so that anything that gets into the Mediterranean or Baltic is going to stick around for a very long, long time. This is one of the reasons why the Baltic and Mediterranean have much greater pollution problems than

the North sea. It's not that the Italians and the French are much worse polluters than the British, it's just that we have a sewage system around our coast that gets rid of it for us.

COLWELL: In a humorous vein, let me assume EPA just got your message and is going to give me its entire budget for basic research. I'd like to ask this august body how it should be spent. Is there any comment about which methods and approaches seem most promising? What areas might best be emphasized?

CHARACKLIS: I am an engineer and I deal with rate processes--chemical and microbiological--in natural environmental systems or engineered reactors. The process characteristics of most interest to the engineer are process rate and stoichiometry. Stoichiometry gives information about the extent of a reaction and the type of products formed. Process rate refers to how fast the processes occur in a given environment. This conference has been useful to me because of the frequent reference to quantitative measurement of process characteristics. However, these quantitative measurements will hopefully be presented with their associated confidence limits if they are to be used in engineering design. One example of process data is biomass yield, a stoichiometric quantity. If x pounds of organic carbon enter a sewage treatment plant, how much biomass is produced? Biomass measurement techniques have been a subject of intense discussion the past several days. Kinetics are usually described with a saturation expression (e.g., Michaelis-Menten or Monod). The region of greatest interest is the low substrate concentration region where the confidence limits are greatest. One of the topics that has come up several times here regarding the kinetics of microcosms is that they are oligotrophic, they are low concentration. In fact, the organisms that are doing most of the work are not even dispersed in the media, but are on surfaces. To a chemical engineer, dilute

systems and surfaces mean heterogeneous reactions, which suggest that diffusion or mass transfer is limiting the rate of the overal process. If that's true, the rates you are measuring may be misleading. The point being here that there are other processes besides the actual microbial reaction occurring, e.g., diffusion, sedimentation. They have all been mentioned here and will definitely affect the kinetics. From an engineering standpoint, material balances are essential in any rate study. The material balances require stoichiometric and kinetic data as input, not just in engineered systems, but also for description and analysis of natural aquatic ecosystems.

Another topic that has come up several times here with regard to the kinetics of microcosms, which are oligotrophic, are low concentration, is that in the organisms that are doing most of the work are not evenly dispersed in the media but rather are on the surfaces. To a chemical engineer, when you start talking about dilute systems, dilute concentrations on surfaces, you are talking about heterogeneous reactions, which immediately will kind of turn on a light in his head. "Diffusion limitations/mass transfer limitations are limiting the rate of the overall process." If that's true, the reaction rates you are measuring may be somewhat irrelevant to the problem. The point here is that there are processes other than the actual reaction occurring--a lot of physical processes, diffusion, sedimentation . . . They have all been mentioned and they will definitely affect the kinetics. So without going into more specifics, I will reiterate that from an engineering standpoint I don't believe in anything except conservation of mass, and therefore material balances are essential in any engineering study, whether it is biological or chemical, and these material balances require input in two areas, stoichiometry and kinetics--not just in engineering systems, but also in natural microcosms.

STEEN: In defense of what Al Bourquin

well as what Dr. Floodgate has said, the point I am going to make is in regard to methodologies. There has been extensive discussion over the last two or three days concerning what is the best methodology for allowing one to make environmental studies that take into account the variation that we know we have in environmental systems. I think it's a matter of being able to accept what is the best methodology or the best test to perform. For microbiologists, the question is: What are we willing to accept as a measure of a particular property that the method gives us? Are we willing to accept the fact that we can measure ATP, or viable counts or energy charge within a 50% range in the environment? We are unwilling, as microbiologists in the laboratory, to accept anything other than maybe 5 or 10%. I am willing to accept that in the environment I am doing pretty well if I can consistently make measurements well within a factor of two.

I think another thing we often overlook when we do microbiological studies and develop methods is the analytical requirement. That's a very high priority when one looks at toxic substances because one, very sensitive methods are required to look at the low concentrations of those compounds and two, because one often overlooks what happens to a compound once it is introduced into a natural environment. Partitioning of compounds in environmental systems is often not considered; volatilization must be considered. By not being aware of other processes we often overestimate microbial participation.

Basically, we have been at fault as microbiologists in being descriptive scientists. We have not been trained as theoreticians and often we have an inner fear of moving over into that area of applying theory or basic principles to analyze microbial systems. There are physical-chemical techniques we can use in our studies with compounds and organisms. There appears to be a very good technique being developed that is related to structure-activity relationships, which the physical chemists have used for years. There are nice correlations between what

we have termed biolysis and alkaline hydrolysis. I think it boils down to getting a quantitative number that we can apply with sound predictability. How to use that number and what reliability to place on numbers is the question.

FERGUSON: I see a general problem--we tend to be beating around the bush of precision versus accuracy. These are two completely different things. The sample mean is the mean of all of our observations and we hope that it is fairly reproducible. We can measure reproducibility very nicely--we call it precision--but accuracy is the relevance of the sample mean to the population mean. In other words, how does the mean of our observations relate to the true mean--the real world? Are our observations relevant? I see a long history of methodologies being used by the microbiologist; however, techniques that work in the clinical laboratory may not work in the natural environment. We have to remember in our presentations to environmentally responsible regulatory agencies that techniques which may have been accurate in the laboratory may not necessarily be accurate in the field.

PRAMER: I think that's a very well made point. It's been said that no one method is adequate, no single procedure can serve any purpose, and I wonder if there is someone here who has thought of a group of procedures that can be recommended collectively. Are there a few methods which, if used together, will give the kind of understanding that is necessary?

STOTZKY: I think that one of the answers was suggested earlier by Tom Rosswall and I wonder whether we have not missed a very obvious point. We use laboratory studies to predict what will occur in the real world--the natural ecosystem--but do we ever really check whether our predictions are accurrate? For example, pesticides have been widely used for some thirty years, and numerous predictions--many of them dire--of their effects on the microbiota

have been made for thirty years, based on laboratory studies. But has anyone looked to see what has actually happened in the field? The bottom line is, are these compounds dangerous in the natural environment, not are they dangerous in the laboratory environment. Why are we not somehow collating the data bases from innumerable lab studies and comparing the predictions made over the years to what is actually occuring now in field studies in Louisiana, Washington, New York and elsewhere? I know that this sounds simplistic, and it is probably not easy to do, but we should think about this. Tom, you have probably given this some thought. How do we go about doing this?

PRAMER: In my own experience, the record of what has been observed in the laboratory in pesticide work and what has been observed in the field is well correlated. To cite one example, we used laboratory studies to obtain an insight into whether or not certain pesticides in soil were undergoing degradation. The results were only partially satisfying because they indicated that degradation was incomplete. That required additional work, also performed in the laboratory, to determine the nature of the undegraded residue. We found there were some products of greater molecular weight that were more complex and more persistent than the compound added initially. When we went to the field, we found that exactly the same thing had occurred, that the added pesticide underwent only partial degradation and there was production of the same large, complex compounds identified in our laboratory studies.

ROSSWALL: I think that unfortunately there hasn't been enough research of the kind you suggest. It is like the social sciences where big social experiments go on all the time which introduce new laws to regulate things--where people can live, what subsistence they get from the state--but there's never any follow up. From the researcher's point of view, what has happened in the past should be

added to any new regulation, and the same thing applies to this field. If we look at the agricultural system, where we have used monoculture with high inputs of fertilizer, high inputs of pesticides for 20 years, then how has this system changed as compared to the rotation system, which maximized biologic nitrogen fixation. Very few studies, if any, have addressed these points. Is there a change in either microbiological functional diversity or Shannon index taxonomic diversity? If so, has this in any way affected the processes, either quantitatively or qualitatively? We have these massive experiments at the ecosystem level that we are not utilizing to the fullest advantage; I'm sure we could find the same for the aquatic system. So I think it is a good idea that we go out and look at the systems where these compounds have been used for a long time, and see what really did happen.

PRAMER: Al, have you had an opportunity yet to look at results in the microcosms and then go to the field and see if you can relate them?

BOURQUIN: Probably some of the better results relating what goes on in microcosms to field work have been acquired in the Kepone studies of the James River and Chesapeake Bay. The studies did not involve biodegradation, but as far as absorption-desorption rates and predicting where the compound is going to end up and in what state it is going to be, the work done in the laboratory microcosms has been used to predict the actual concentrations of Kepone found in the James River of Chesapeake Bay. Other information presented at the Pensacola meeting by Rita Colwell and her colleagues on inhibitory effects they found both in the field and in using pure cultures and mixed cultures in the laboratory showed a similarity to results we found in microcosm studies. We are looking for good field evaluations. We definitely need that kind of information. At the same time, if you look at how water quality criteria are determined today, you find they

are based on good scientific information produced in the laboratory, but which probably has no field validation. It is the best available scientific information, but what it means in the field is still unknown. My point is that we have to use the best information and technology available while we work to improve our methods.

COLWELL: When experiments in the laboratory are designed to take into account as many parameters as possible and as many environmental conditions as can be studied, then the results can be usefully extrapolated to the field. A case in point would be studies done in our laboratory on petroleum degradation. Experiments were done with mixed cultures from environments constantly subject to spills, and from environments which were pristine or at least had minimum impact by oil. These experiments were carried out in the laboratory. From the results observed, a predictive capability was developed. With a rather simple *in situ* sampler, we did experiments in a sub-estuary, the Rhode River of Chesapeake Bay. Results of the field experiments did, indeed, apply to field conditions. Microbial interactions, i.e., population shifts, occurred both in the laboratory and in the field. (These data were published in *Environmental Science and Technology*.) Thus, if one takes into account parameters involved in the environment in model ecosystem experiments in the laboratory, the results can indeed apply to the field. They do correlate with field conditions. If single enzyme reactions--or as you put it, if pennaid shrimp LD50--are measured very precisely and accurately, but without all the other parameters worked into the system, the results when carried to the field have little or no meaning.

A request I'd like to make as a result of discussions in this session is that, when called upon by the American Society for Microbiology to testify before agencies developing or establishing methods for regulation, you participate and take time from your laboratory work to provide expert testimony.

We have a responsibility to ourselves and to the public to undertake these tasks. Participating in hearings, providing wisdom and experience, assisting in the pre-law stages of discussion, before methods or rules become established by government regulation, are very important activities. We should participate and, thereby, help shape or formulate rules and regulations so that they reflect the best knowledge available.

PRAMER: I'll comment on what has been said, knowing that what goes on here may not be characteristic of what is going on in other countries, particularly in Great Britain. What is happening has not been promoted by the American Society for Microbiology, but members of the Society participated in the recombinant DNA debates and were successful in influencing regulatory legislation. As a result of that success, the Society is being asked by members of Congress for additional help on legislation that pertains to food and drugs as well as to the environment, and other areas that have a scientific and technological base. There is increasing pressure on the Society to provide guidance in the development of public policy, and it is my understanding that within the year there will be reorganization of its committees so they will be more able to respond to needs voiced in Washington. There are similar kinds of developments on a state level. In New Jersey, where I live, our local legislators are less well equipped to handle scientific and technical subjects than are our national legislators, but they are faced with many of the same problems. I'm sure you have heard New Jersey called "cancer alley." New Jersey has a high concentration of industry, which is beneficial as well as problematic. Members of the faculty of the state university are being asked by legislators to help with the problem, just as some representatives of federal agencies here are calling on us for assistance. I think the need to respond should be recognized as part of our responsibility today, and it will most certainly be required in the

future. If anything, the need to participate will intensify, and those of us who teach should make our students aware that as professionals they will have a future responsibility to apply their knowledge beyond the laboratory and deal in public policy. Many will undoubtedly be asked, as Rita describes it, to come down to Washington and testify before legislative committees that are seeking scientific and technical guidance.

COSTERTON: We approached this relationship between university researchers and regulatory agencies as if it were a brand new question and of course it has been approached by many different scientific groups and many different government agencies with an awful lot more success than we have had. Let's look very briefly at agriculture. The agricultural product, where productivity and efficiency mean more money for the farmer, is a lot easier to measure than the environmental product. So little increases in efficiency are obvious and hazards are obvious too. One of the things that has happened with the USDA, and the Canadian Agriculture Service as well, is that the interface between the university researchers and the government agencies has been very carefully titrated. Agricultural efficiency can be assessed from better production feedlots and better yields from cereal crops. Understanding that university research is a scattershot type procedure--we aren't integrated enough in the way we are funded or the way we report to put together a coherent statement on how much barley to feed a cow--the government agencies have handled the integration, often in fairly major in-house programs. And here perhaps the regulatory agencies are asking the university researchers to do something that they just aren't suited to do, which is give a rounded picture of an ecosystem. Now, to equate ecosystems to say the Chesapeake Bay, and say the same standards for effluents going into it as for a brackish water in another part of the United States should apply is an outrage. To say that a particular oil-eating river that I work with has to have the same

hydrocarbon effluents as a pristine mountain stream is another outrage. Industries are being forced to clean up a little hydrocarbon slick going into a river that processes tens of thousands of tons of hydrocarbons every year because agencies are legalistic. This then means, if we take the agricultural parallel, that the EPA is obliged to take the scattered information yielded partly by university research, but never integrated, and integrate it so as to draw a picture of the receiving waters in these cases. They should be responsible for telling us how many organisms are in the system and where they are, which I think is the key issue--whether they are in moving water, surfaces or sediments. They are also responsible for telling us how a compound introduced into those systems makes contact with the microbes: does it get bound up in the sediments immediately, does it stay in the running water for a finite length of time, and thirdly (I'm going back to Bill's point, which I think is absolutely vital, that we have to have an engineering concept or process reactor model of a stream), what microbial population does it contact, and at what rate does it change? So we should take this obligation for environmental protection into the agricultural context and, I hasten to add, the medical context, which is very close to this. If you really want to deal with Legionnaire's disease, you don't do it by university research, right? You take the Atlanta group and NIH and they integrate, and they solve the practical problem. So many of the agencies and the university researchers have been sitting back in Alphonse-Gaston types of arrangements, hoping the other fellows will go first and solve all the problems. And if we look at the way other scientific and government groups have interfaced, extremely successfully, it has been the obligation of the government agency to pick up information generated, but not integrated, by the university researchers, to integrate it and make sense of it. Speaking as one involved in several questions about receiving waters right now, the regulations are outrageous, and very soon and very rapidly the public is coming to

realize that these regulations are outrageous. Enormous amounts of money are being wasted and the system isn't even efficient because a great many accidents are still happening because of imperfections in our knowledge of these systems. So drawing that parallel if I could, and if I could be extremely rude, I would cite the university researchers, the ivory tower people who haven't yet integrated their research and are not set up to answer practical problems. The responsibility of the agencies is probably to get really in gear and come in using the keys that university researchers point to, to do the final integration and to give us a final functioning idea of how each receiving water operates.

BOURQUIN: I believe the purpose of this meeting is to attempt to start to integrate information needed by the decision makers in Washington. As well, I think the Pensacola meeting, this meeting, the meeting being planned this summer by industry, are all attempts by the sponsoring government to get the needed input from the scientific community. The regulatory agencies can probably put everything together without this input, but we're going to hear a loud outcry from the scientific community if they do not have an input, and rightfully so. The criteria being developed are proposed guidelines and proposed methods, and I don't see the conflict George pointed out earlier between EPA and academia. I see a cry from the agency saying "help us, let's put it together. Tell us, are these useless tests? What is the best technology now?" Guidelines came out in the Federal Register as proposed tests and we are able to criticize those tests now. So I think the message from regulatory agencies is help us, help us to integrate this information.

ATLAS: I'd like to comment that we seem, in the last number of discussions this morning, to be accepting the fact that there's an integrated agency and it's the universities that are out there and the individual researchers that are not integrated. I

think we have a real problem in integrating different agencies, all of them trying to regulate. EPA is not the only regulatory agency, and even within EPA and other regulatory agencies there are research branches. Now the scientist performing basic or even applied environmental research, the universities, consulting agencies and the like often interface with the research end of these regulatory agencies. The assumption is that if the information is flowing into the research end, it is somehow flowing over into the decision-making end, but I don't think that's true. I think there's a real gap in communication within these agencies among the legal, regulatory, and research ends of the agencies. Many scientists are able to pinpoint research needs, both basic and applied, and they have a mechanism for telling agencies about this; i.e., they send in proposals, they ask for money, they send in a report and they tell about results. These sorts of processes need to be filtered further through the agency and integrated so that they come out in actions. I think there is a gap there that we are just not talking to, and we should be.

TIEDJE: I think part of our problem in the public arena is our diversity. Because we don't speak with a unified voice, the public is confused and it is easy for critics to discount our opinion. This meeting has shown the great diversity of viewpoints. Recently, through our work with nitrogen-13 which requires a cyclotron to produce, I've worked with nuclear physicists and learned how they operate. They come to meetings such as this and they do battle there, but when they leave the meeting, they leave with one voice and that voice is well prepared, well documented--they know what they're going to do and they get what they want. So possibly, if we could reach some concensus and speak with a unified voice, it would be very difficult to disregard or to counteract our position as scientific experts.

PRAMER: Biologists are known for their separation

into many different interest groups: botany, zoology, physiology, ecology, etc. Biology is splintered and fractionated to a point that often defeats its purpose. Biology speaks with many voices, as oppoed to physics and chemistry, and produces cacophony rather than harmony. But let's move from public policy and politics back to a consideration of methodology and methods. Is there someone here with a comment or a question?

NOGRADY: Just to bring up a methodological problem, when we are carrying out some tests in the laboratory on nature and for example, because they were presented, in Professor Shilo's experiments ... we already exchanged some words but I'd like to discuss this problem here. Professor Shilo put some bacteria in a bag, submerged it in water and exposed it to substances which diffused in this bag. I know in other conditions that these kinds of experiments are hampered by the adherence of bacteria. Bacteria are deposited in a short period of time in a very thick layer that virtually stops the diffusion of substances. This makes a long-term experiment difficult to evaluate, or sometimes impossible.

LITCHFIELD: I would like to put into perspective a little of the discussion we have been having on methodological problems. For those of us who are over 30, we have to stop and realize that most of the techniques we have been talking about and the problems with them that we have described are techniques which have arisen within the last ten years. But it's really only been within the last two to three years, or perhaps even this year, that we have had bodies of information that have included multiple testing: ATP to *Limulus* to heterotrophy to plate counts or what have you. When I first started in the field, plate counts were all we had and I think that is one reason why we tend to downgrade them and say they are no good; but the point is that we are finally getting to the point of amassing a body of information. That is, we now have enough information on numerical

taxonomy that we can go to the statisticians, we can use factorial analysis, and cull from all of this information say three tests that are really going to be important in the environment. There are others we won't have to worry about, or there are certain combinations. But we haven't been able to do this until very recently and we should keep that in mind. We also have to select the tools we use for each particular question. There is no universal panacea.

FERGUSON: I got into the business of trying to do field microbiology without a background in bacteriology, and at first I thought that was a tremendous disadvantage, but I was ignorant enough to ask what someone else more informed than I would have thought was a foolish question. I questioned the existing methods that were being applied to work in the field. In about '71, I really started thinking about which methods to use. I went through the microbiological literature for some time and couldn't find what I thought was a good method in the sense of being an integrative method. So I started measuring ATP. Then I bumped into John Hobbie and started doing epiflorescent direct counts of bacteria and also began using radio tracers to study heterotrophy. We also started doing some colony-forming unit determinations, and now I am realizing that I really do have a problem because I don't know enough bacteriology. I'm still attempting to integrate all of these measures of what's going on in the natural environment. I'm finding that the classical methods are not bad, it's that in the past they were badly applied and interpreted. The methods are good, but what are they good for? That's the question.

I was very concerned about short-term versus long-term experiments, trying to look at the response of natural populations to various perturbations. It is very useful to look at the short-term physiological response of a confined population, but what if you want to look at the change of the population in response to some perturbation? You've got a serious

problem, because the bottle itself is a serious perturbation. There was a lot of very good work done in the thirties about what happens when you put water in a bottle. Colony-forming units go up very fast. It was known even then that colony-forming units didn't tell us all of the bacteria that were really in the water. The direct counts were considerably higher, several orders of magnitude. But in the recent literature, I think maybe in the late sixties and early seventies, that wasn't always made apparent. I wonder why, with a technique like acridine orange direct counts, we again have to fight the battle of why direct count procedures using a microscope are better than plate count procedures for estimating total biomass. Let me get back to the bottle effect. Take a rather high nutrient medium and put it in a bottle. It was shown, again in the thirties and forties, that the effect of confinement on this water is not very large because the nutrients are already high. If you take oligotrophic water and put it in a bottle, then things change. What is changing? I took some gulf stream water and put it in three liter bottles and did colony-forming units, acridine orange direct counts and heterotrophic assays of amino acid uptake and turnover and I found that in a period of a day the acridine orange count went up by a factor of about two, that magnitude, and the colony-forming units went up about three orders of magnitude. So where do we stand in terms of trying to run controlled long-term experiments? They'll work in a high nutrient environment, a polluted environment, but we still have problems when we go out into an oligotrophic environment because we really don't yet understand the complete implications of confining a low activity environment. I hope to talk about that some more in the future.

PASSMAN: As a veritable neophite in the factorial analysis line of investigation, I'm encouraged by a lot of discussions on this method and on designing factorial experiments. I've been somewhat

surprised that a couple of essential issues, one just raised by Dr. Litchfield, haven't really been addressed during the discussions of factorial experiments. One of the items is the entire reason for doing factorial design. It's wonderful to be able to analyze 40 to 50 parameters of large numbers of stations and then perhaps describe what the model might be in a given system, but I think the ultimate goal is to develop models based on a semi-standardized type of observation and then to use those models to enable us to take much fewer parameter tests while extending our observations to a much larger field. The other item that Jim Tiedje brought up is that bureaucrats don't want to see reams of papers addressing the impacts of different compounds on organisms. This seems to be one of the problems with which we are faced. Everyone wants to develop unique methods and then go out and perform their own experiments. Accordingly, we have an increasing data base in which the data between one investigator's data base and another's is not directly comparable. Everybody has used slightly different techniques. And when you use a different technique, you can't just go out and say, "Well, my numbers are different from their numbers, therefore my observations do or don't confirm their observations because I have used a different technique." I guess it is part of a microbiologist's egotism to feel that something isn't worth communicating in the scientific literature unless the entire approach is original, so what we are developing is a data base of noncomparable data. We have spent two and a half days talking about developing models to describe impacts of various perturbations on different ecosystems (primarily aquatic ecosystems), but we really haven't gotten down to the central issue of developing a data base. Models must be developed from data acquired using techniques that are directly comparable, at least to a certain point. As we modify techniques, we should compare the modified techniques to the originals--before applying a modification. Someone yesterday talked about this business of establishing the precision of a technique and the accuracy of that

technique before going out and developing a large data base. As has been mentioned by several people, George Floodgate and others, multi-variate analysis is a wonderful tool, but you can't go out into the field and develop models unless you have initial data on the precision and accuracy of the techniques, and if you are using a new technique, unless you go out and take the time to specifically compare any differences in the data that you might get as a result of the change in technique.

COLWELL: Let me offer an amusing metaphor of what I think we are attempting to do. If we were in an UFO, an unidentified flying object, and wished to sample this globe, the Earth, we would fly over a forest, which contains squirrels, birds, and trees, earthworms, etc., and scoop up the whole forest and measure total ATP, total respiration, total counts of living objects, and then try to hypothesize what it is that we have sampled. It would be pretty difficult to separate robin, squirrel, and worm ATP, respiration, etc. The whole lot is stirred together and we try to draw conclusions from a melange. In answer to Randy Ferguson's "dilemma," it's because there are very different microorganisms involved, for example, *Cytophaga, Pseudomonas, Penicillium, Candida*, etc., in the system. Each is contributing to the total system. It comes down to understanding what the functions of each component are. To put all components together, in context, is why we use all of the methods to measure the various activities of the populations. Thus, methods for characterizing microorganisms and assigning activities to given groups of bacteria become extremely important in microbial ecology.

GEESEY: There is one thing that has not been brought out in these discussions and that is the importance of initiating more baseline studies in areas which may not be of great ecological importance at the present time, but are likely to be in the near future. Many of the approaches that have been

considered here for the detection and understanding of ecological disturbance would provide more meaningful information if they were initiated before significant environmental changes took place. I would therefore suggest that we reconsider the importance of basic research in microbial ecology so that we may facilitate improved success in future environmental impact assessment programs.

PASSMAN: As a matter of fact, a few years back the Bureau of Land Management did begin to sponsor quite a number of baseline studies--benchmark studies as they were called. Then in the past year the National Academy of Sciences took a look at what the mission for the benchmark studies was supposed to have been (providing grist for the environmental impact statement mill) and took a look at the types of data that were being generated in these studies (these were very broad multi-diciplinary studies for the most part). I think Alaska and perhaps one or two others are the only ones surviving. NAS determined, basically, that there was too much science being done and not enough production of grist for the EIS mill. Of roughly 14 studies underway, only 3 survived. The problem was people were collecting too much basic information about what was going on in the environment and not doing enough towards answering specific questions that we are hearing now.

PRAMER: There are many problems associated with relating new data to baseline studies and it is very difficult to integrate bits and pieces of information into a meaningful view of a total system. Rita's parable reminded me of a warning issued by Dr. Ernest Gale, one of Great Britain's great microbial biochemists. He wrote that one cannot expect to understand cell structure and function by disrupting cells and separating the fragments for study anymore than one can expect to reconstruct the original design and arrangement of furniture by collecting and examining bits and pieces of wood in a room that has been bombed.

ENGELMANN: I'm with NOAA and I'm the Director of the Outer Continental Shelf Environmental Assessment Program for Alaska, which is co-funded by BLM and NOAA. I've read the academy report and I've been in this Alaskan assessment program for almost four years. It's apparent that there are a number of misconceptions that don't relate to microbiology, but which I can't let stand after that statement. My answer also addresses Ron Atlas I think. At the time the OCSEAP program or the BLM studies programs were initiated I believe, there was a reaction going on to the Alaskan pipeline, to the fact that it had been held up for a couple of years. Something had to be done. Responsible government officials, who were not environmental scientists, had to put a program together, and that they did in a big hurry. They did consult with the scientific community as to what kind of studies ought to be done and, in the Alaskan program in particular, there were over 300 scientists brought together in more than one meeting to determine what ought to be funded. The proposals that came in were reviewed by qualified staff scientists. But the problem in the BLM program was with the decision makers; they didn't really know what they wanted, and it wasn't really their fault either, because the scientific community itself doesn't know how to do an assessment. I guess that is one of the reasons we helped fund this conference.

But not knowing what it is that you want, you can only respond to what the scientists propose. At that time there was a lot of talk about baseline studies. I believe, according to BLM, that the concept of baseline studies originated from the academic community. The concept was to put together a baseline against which future changes could be determined, so that when you had a change in population or a change in process or something, you would know that something had changed and then, somehow, you would change your technology to respond to that. In the case of the NOAA-managed BLM program for Alaska, we never did subscribe to a baseline. I've been very much against it, having been nurtured for over

ten years in the Atomic Energy Commission. I am aware of how fallible baselines are.

We have very much oriented ourselves towards basic science, but really toward processes. What we tried to do was identify those insults that would occur to the environment as a result of oil and gas development, speculate what those impacts might be, and verify those speculations through whatever kind of research we could do. Now initially Alaska was a very poorly known environment. The marine environment was only known in terms of commercial fish and perhaps a few other things in response to some ice work from NSF. We had to begin with what we called reconnaisance studies. That is, we discovered rookeries, hauling grounds, breeding places, and new species. We discovered habitats that were never known to exist, plants that were never known to exist on the floor of the Arctic Ocean, and so on. Well, we have very much tried to stay in the science field. I've been pushing that very much. We've tried to select only those parts of the science that relate to gas and oil development and attempt to be responsible in some way.

Now the Academy report that you spoke about was very critical of baseline studies. It was also critical of the fact that the decision makers didn't know what they wanted and that there was a long distance between the scientists who were doing the work in the field and the decision maker who was supposed to be using the information. It also was critical of the BLM internal organization. Unfortunately, the NOAA program got wrapped up in that by association, even though we only had about two hours of consultation with some young technologist who came out from the Academy. We never did get interviewed.

One of the things we are trying to do in the Alaskan Program is this very thing of integration and I confess that I've learned a lot in three and a half years about how to integrate the information that comes from the scientific community--microbiologists, physical oceanographers, bird people,

mammal people. How do you put that all together in a picture of the lease area so that you can make recommendations that stipulate which tracts to lease and which not to. In doing that we have come to the very firm opinion that we cannot leave Ron Atlas and Randy Ferguson and the people that are actually doing the work out of the integrating activity.

It's also been evident to me that the academic scientists need leadership and an organizing influence that's outside their own scientific discipline. I am a member of the American Meteorological Society and it is very much academic research-oriented, the entire community is. The federal government and program managers such as myself must provide the kind of integrative leadership and opportunities motivating catalytic mechanisms to bring all this together. We also must try to provide the opportunities and the incentives for people to talk about things they haven't talked about or thought about before. I guess this is a full circle which brings me back to Rita and this workshop and why it was sponsored. Thank you for the opportunity.

PRAMER: I think I will say now what I said at the beginning, that all good things have to come to an end and so it is with this conference. And let me thank the organizing committee for providing all of us with this opportunity to meet, to talk, to teach, and to learn.

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