Research

on Toxic Algae

Pfiesteria-Like Organisms

- Occupational Risks of Crabbing
- Neurobehavioral Effects of Exposure in Rats
- Consumer Health Risks of Exposed Seafood
- Effects of Nutrients on Zoospore Stage

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• An Exploratory Study of Potential Human Health Effects of Deteriorating Water Quality Among North Carolina Crabbers

David Griffith, Kristen Borré, Aaron Schecter and Vernon Kelley

East Carolina University

• Neurobehavioral Effects of Dinoflagellate Toxins in Rats

Edward D. Levin

Duke University

• Consumer Health Risks Due to Incidental Exposure of Fish to *Pfiesteria piscicida*

P.D. McClellan-Green, Duke University Nicholas School of the Environment Marine Laboratory

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• Environmental Control of *Pfiesteria piscicida* Outbreaks: The Role of Anthropogenic Nutrient Loading

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University of North Carolina at Chapel Hill Institute of Marine Sciences

Resources for these studies were provided by state public health agencies when they were housed within the Department of Environment, Health and Natural Resources.

Occupational Risks of Crabbing:

A Report with Special Emphasis on the Public Health Threat of *Pfiesteria piscicida*

An Exploratory Study of Potential Human Health Effects of Deteriorating Water Quality Among North Carolina Crabbers

Final Report of the Project

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I. EXECUTIVE SUMMARY

I. a. Background and Study Rationale

During the summer of 1995, massive fish kills in North Carolina estuaries, combined with several anecdotal accounts of individuals afflicted with a variety of symptoms attributed to contact with the toxic dinoflagellate known as *Pfiesteria piscicida,* encouraged the state of North Carolina to commission several studies of different aspects of water quality. These studies come at a time that toxic algae blooms and other incidents that suggest deteriorating conditions of water quality translate directly into deteriorating conditions of work for fishers, marine workers, and other coastal residents who interact with the marine environment regularly.

Several anecdotal accounts have linked *Pfiesteria* to ill health effects among individuals in laboratory contexts and among individuals directly exposed to fish kills, particularly marine construction workers and others working in the Neuse River. In the laboratory cases and a few others, these accounts have been backed up by clinical examination and tests, particularly of cognitive skills, which have shown severe impairment among those exposed to laboratory samples and mild and reversible impairment among individuals exposed in the wild. To date, however, conclusive diagnoses have been difficult to document in the absence of a specific toxin. In the summer of 1997, a complex of dinoflagellates similar to *Pfiesteria piscicida* was found in association with a fish kill in the Pocomoke River in Maryland. For the first time, individuals who were known to have been in contact with the fish kill were tested and found to suffer cognitive impairment.

Given the potential threats to human health of an organism that seems so highly varied, resistant, and adaptive, and the severe health problems experienced by those working with the

organism in the lab and in areas affected by fish kills attributed to *Pfiesteria,* a team of East Carolina University researchers undertook a survey of the health of crabbers, using a sampling design that allows comparisons among two groups of crabbers (those working in and those working outside of *Pfiesteria*-affected waters) and a third group of non-fishing individuals. Such comparisons are strengthened with detailed data on the specific areas where crabbers set their crab pots and where fish kills, algae blooms, and other water quality problems have been known to occur.

This study thus presents the results of research into the health of crabbers and coastal residents in North Carolina. It addresses the question of whether or not crabbers fishing in regions affected by fish kills, as well as toxic dinoflagellates (e.g *Pfiesteria piscicida),* suffer worse health than either crabbers fishing in areas with fewer fish kills or non-fishing residents of coastal communities along the Pamlico and Neuse Rivers and the Pamlico Sound.

I. b. Data

In 1996, a team of researchers interviewed a target population of 253 crabbers who work in the Neuse and Pamlico Rivers and southern sections of the Pamlico Sound, here called the Pamlico crabbers, and compared their responses to questions about their recent health to two control populations: 1) 115 crabbers who work in the Albemarle and Currituck Sounds, the Alligator River, and northern sections of the Pamlico Sound, here called the Albemarle crabbers; and 2) 125 non-fishing residents of the communities of the crabbers, here called the Community Controls. More detailed analysis of the cases resulted in reassigning one of the Albemarle Crabbers to the Pamlico group because this particular crabber set his pots in the Pungo River during the height of the summer crabbing season. In addition, two Pamlico Crabbers were

dropped from the analysis when it was determined that they set no traps during the summer of 1996.

The team also collected detailed data on crabbing territories for 295 of the 368 crabbers interviewed; the research team compared these data to maps on fish kills provided by the state of North Carolina, various scientists in North Carolina, and the National Marine Fisheries Service HEED Project, paying particular attention to those areas where toxic dinoflagellates, now known as *Pfiesteria*-like species, have been found in conjunction with fish kills. These territorial data allow closer analysis of the incidence of illness among crabbers who have fished in areas known to have hosted fish kills which are highly correlated with *Pfiesteria*-like dinoflagellates, as well as areas that have suffered repeated fish kills without specific dinoflagellates having been identified. Members of the research team accompanied crabbers on their vessels to determine the extent of crabbers' exposure to the water during the course of a typical work day. Finally, several interviews were conducted with health providers, coastal residents, individuals who believed to have been afflicted with various sicknesses due to their contact with the water, and others interested in and informed about the potential health threats associated with *Pfiesteria*-like organisms.

I. c. Findings

The Pamlico Crabbers, Albemarle Crabbers, and Community Controls were extremely similar in terms of their demographic characteristics (e.g. age, marital status), their lengths of residence in coastal North Carolina, and other characteristics that might have an influence on their health, including alcohol, tobacco, and drug use. Their relationships with the medical community are highly uneven, ranging from suspicion of physicians to infrequent use of health care delivery

systems because of lack of insurance or low incomes. According to their own reporting, the sample population is not, in general, unhealthy, reporting low levels of common ailments such as hypertension (15 to 20%) and diabetes (7 to 8%). Physicians interviewed believe that many fishers, often because they lack medical insurance, do not visit health practitioners until they are seriously ill; they also believe that many crabbers practice a kind of "revolving physician" approach to health care, moving from one physician to another for treatments because of difficulty paying medical bills. Physicians interviewed noted, in particular, that fishers suffer from skin problems, ranging from open sores and skin cancers, suggesting that skin disorders comprise an occupational hazard for fishers. Open-ended interviews with fishers support this as well. Eastern North Carolina is underserved by the medical community and many fishers, of independent mind and spirit, view medical practitioners as condescending and invasive and hence resist interacting with the medical community in any fashion.

Comparisons among the three populations, as presented in the following table, show that, in most cases, the two groups of crabbers and the Community Controls report similar levels of illness and injury. This was true of both low and relatively high levels of illness. The data show that all three groups suffered from relatively high incidences (between one quarter and one-third of each population) of allergies and related respiratory ailments. With one exception, the three populations were similar in terms of the incidence of illness within their groups. The one exception to this was that both groups of crabbers report higher incidences of skin disorders than the Community Controls. However, comparing the Pamlico crabbers with the Albemarle crabbers, both groups report nearly identical levels of skin disorders. Close analysis of the specific types of skin disorders found that around one-third of these had been diagnosed as skin

cancers, and those physicians who have experience treating fishermen reported that most skin problems associated with fishing are either skin cancers or are secondary infections caused by failing to treat a puncture wound from a fin or a crab pincer. This suggests that skin disorders are a common occupational hazard among crabbers and cannot be attributed to the presence or absence of *Pfiesteria* or similar dinoflagellates.

Executive Summary Table: Percent who Reported Symptoms: Crabbers and Community Controls Compared

Symptom	Com. Controls	All Crabbers	Pamlico	Albemarle
Personality or Memory Changes	9.6	7.3	7.9	5.3
Problems with nerves, etc.	5.6	7.0	7.5	6.2
Headaches, seizures, etc.	8.8	14.1	12.6	16.8
Respiratory Problems*	24.8	23.7	19.4	35.4
Dermatological problems*	11.2	24.6	25.3	23.0
Digestion, stomach problem	8.0	9.3	9.5	9.7
Heart, circulation problems	20.0	16.1	17.0	15.8
Urination, bladder problems	4.0	7.0	7.1	6.2
Felt unwell, fatigued	6.4	6.5	8.3	3.5
Problems with physical activities	8.0	5.9	7.5	3.5

* = statistically significant

These findings were further confirmed with two additional steps in the analysis: 1) examinations of the relationship between levels of exposure to the water and incidence of illness; and 2) examinations of fish kill data in conjunction with data on crabbers' crabbing territories. First, increased exposure to water (as measured by the numbers of traps watermen pull per day) does not result in increased incidence of illness. Second, among crabbers who set their pots in regions that have been affected by fish kills, far greater percentages of the population reported no ill health affects than those who reported sickness; chi-square analysis shows that, with one exception, there are no statistical differences in levels of illness between those who fish in areas

that have experienced many fish kills and those who fish in areas that have experienced few or no fish kills. The single exception to this was the case of fatigue. Slightly more crabbers reported fatigue in fish-kill areas than in non-fish-kill areas. On closer inspection of the cases of fatigue in fish-kill areas, however, the reasons given for fatigue were either overwork or were related to other illnesses such as heart trouble or diabetes; they were not, therefore, related to water quality conditions.

In addition, the data on crabbing territories show the two crabbing populations to be distributed throughout the Albemarle Pamlico Esutarine System, as well as up and down the Neuse, Pungo, Pamlico, and other rivers that have experienced large fish kills in recent years. In all cases, well individuals outnumber ill individuals, by ratios of between five and nine to one. A ranking of areas in terms of ratios of well to ill crabbers, however, does show that the two areas that are known to have experienced fish kills—the Neuse River and the Pamlico & Pungo Rivers—do have lower well to ill ratios than those that are less prone to experience fish kills. This may be some cause for concern regarding the impacts on human health of these areas as opposed to others.

I. d. Conclusions

Based on this study, there are extremely low health risks associated with casual contact with the waters of Eastern North Carolina under normal ecological conditions: that is, in the absence of fish kills, algae blooms, red tides, or other perceptible indicators of poor water quality. The negative news coverage regarding the ill health effects of waters known to have experienced fish kills with high concentrations of *Pfiesteria*-like organisms—based primarily on self-diagnosed, self-selected, and anecdotal accounts—appears to have been greatly exaggerated.

II. INTRODUCTION

During the summer of 1995, massive fish kills in North Carolina estuaries, combined with several anecdotal accounts of individuals afflicted with a variety of symptoms attributed to contact with the dinoflagellate known as *Pfiesteria piscicida,* encouraged the state of North Carolina to commission several studies of different aspects of water quality. These studies come at a time that toxic algae blooms and other incidents that suggest deteriorating conditions of water quality translate directly into deteriorating conditions of work for fishers, marine workers, and other coastal residents who interact with the marine environment regularly. A 1994 study of phytoplankton ecology in North Carolina concluded that, "In recent years, there has been a dramatic increase in reported fish kills attributed to toxic dinoflagellate blooms, particularly in nutrient-enriched estuarine areas" (Mallin 1994: 561). In addition, a conference held at Harvard in the spring of 1997 concluded that algae blooms and other symptoms of ailing estuarine environments had become a matter for world-wide concern; their collections of data on fish kills and various kinds of algae involved is, to date, one of the most comprehensive assembled, including data on North Carolina kills from scientists who have worked with *Pfiesteria*-like dinoflagellates (Epstein, et al. 1997).[1]

Others have expressed concern about potential negative human health effects of fish kills and algae blooms (Morris 1991), with a great deal of attention focused on the toxic dinoflagellate known as *Pfiesteria piscicida* (Burkholder, Glasgow, and Steidinger, 1994; Burkholder Noga, Hobbs, and Glasgow 1992). Several anecdotal accounts link this microorganism to ill health effects among individuals in laboratory contexts and among individuals directly exposed to fish kills, particularly marine construction workers and others working in the Neuse River. In the

laboratory cases and a few others, these accounts have been backed up by clinical examination and tests, particularly of cognitive skills, which have shown severe impairment among those exposed to laboratory samples and mild and reversible impairment among individuals exposed in the wild. To date, however, conclusive diagnoses have been difficult to document in the absence of a specific toxin. In the summer of 1997, a complex of dinoflagellates similar to *Pfiesteria piscicida* were found in association with a fish kill in the Pocomoke River in Maryland. For the first time, individuals who were known to have been in contact with the fish kill were tested and found to suffer cognitive impairment. Again, their conditions improved within a few days.

While the links between *Pfiesteria*-like organisms and ill health among humans have yet to be fully confirmed, the few correlations between illness and exposure to these organisms is enough to cause concern. These organisms' effects on non-human species has been better documented than their effects on humans. In fish and shellfish, ill effects of exposure to these microorganisms range from epidermal lesions to suffocation and death; several millions of fish have died in association with *Pfiesteria's* in the past few years (Burkholder and Glasgow 1997). Some marine biologists estimate that nearly one third of the fish kills in the Pamlico and Neuse estuaries between 1991 and 1993 were caused by *Pfiesteria*. Others, however, dispute the contention that *Pfiesteria* is a causal agent in fish kills, viewing it instead as a symptom of low oxygen levels that cause a variety of environmental maladies. Local environmental groups have referred to *Pfiesteria* and similar organisms as "the canary in the coal mine" (Rolles 1997). Marine construction workers whom we interviewed for this study provided extensive information regarding the problems that they developed after working in the vicinity of a fish kill, reporting dermatological and neurological symptoms that were similar to those of scientists exposed to high

concentrations of the dinoflagellate in laboratory settings; again, however, the specific causes of their illnesses have not been identified, and dermatologists argue that several agents associated with marine environments may have cause their illnesses (Burke 1997). Indeed, several volumes exist that are devoted to toxins in marine environments, suggesting that a wide range of factors could be responsible for human health problems among individuals coming in contact with estuaries and oceans.

II.a. Rationale for the Current Study

Given the potential threats to human health of an organism that seems so highly varied, resistant, and adaptive, and the severe health problems experienced by those working with the organism in the lab and in areas affected by fish kills attributed to *Pfiesteria,* we undertook a survey of the health of crabbers, using a sampling design that allows comparisons among Pamlico crabbers, Albemarle crabbers, and Community Controls. Such comparisons are strengthened with detailed data on the specific areas where crabbers set their crab pots and where fish kills, algae blooms, and other water quality problems have been known to occur.

The findings reported here derive from this study, a multidisciplinary effort to assess the health of crabbers who fish in North Carolina's waters as well as the health of individuals from coastal communities who do not have marine-related occupations. Specifically, the study addressed the question of the possible threats to human health posed by North Carolina's coastal waters under normal environmental conditions (that is, in the absence of fish kills, algae blooms, red tides, or other perceptible indicators of poor water quality), based on the reported health status of individuals who expose themselves to the water on a daily basis. *We emphasize at the outset that the data we present and the conclusions we draw from those data do not speak to the*

larger and far more complex question of the overall health of North Carolina's coastal environment, particularly the quality of the water in its rivers, its sounds, its creeks, bays, and so forth. Our study, simply, presents and interprets statistics about the health of individuals who have interacted with the water, directly, for long periods of time, daily, through the seasons of the year and the specific places that fish kills and other symptoms of deteriorating water quality are known to occur. These individuals, for the most part, are folk experts on water quality, having learned how to recognize and avoid poor water conditions through lifetimes of repeated and sustained observation (see Griffith 1996 for a discussion of folk knowledge among North Carolina fisher folk). As such, these individuals are likely to avoid working in the middle of a fish kill or algae bloom, yet are equally likely to expose themselves to poor quality water before and after such episodes occur. Often they are the first to witness, from observing the struggling of crabs in their pots, evident lowered levels of oxygen, an event that often precedes a fish kill or an algae bloom (Stanley 1992; Epstein, et al. 1997). They are, therefore, the ideal population to examine to determine how likely one is to become sick by working on the water both under normal conditions and immediately before and after an episode such as a fish kill. We do not mean to suggest by our findings that the incidence of health or sickness among this population, however, is in any way a barometer of the overall health of the environment. It is as ridiculous to extend the findings from a group of large mammals to an entire complex estuarine system as it is to extend findings based on fish to humans.

We make this early disclaimer because of widespread misrepresentation of our earlier versions of our work, particularly by the popular media. During the course of the study, several additional developments generated a great deal of misunderstanding about both the character of

the present study and the nature of the problems facing North Carolina estuaries and other bodies of water that have experienced algae blooms, fish kills, evasive grasses and other organisms, and other symptoms of a stressed environment. In particular, heightened awareness about *Pfiesteria* that accompanied the publication of And The Waters Turned To Blood (Barker 1997), a sensationalized account of the dinoflagellate's threat to human health based on several anecdotes, enabled some individuals to politicize water quality issues to a degree that it became difficult to present facts objectively. Attempts to present data that contest the numerous suggestions that *Pfiesteria* presents an *Ebola*-like[2] threat to people living and working in the marine environment have been met with charges of vested interests, political motives, or, at worst, claims of cover-ups conspiracy that are increasingly grist for the mills of yellow journalism. In light of this overly politicized environment, we begin our presentation by pointing out the strengths and weaknesses of our research methods and the data they have generated.

II.b. Methods

II.b.1. Questionnaire Development

Research into the health of crabbers in North Carolina began late in 1995, with the development of a questionnaire and its submission East Carolina University's Human Subjects Review panel. Questionnaire development relied heavily on the expertise of the project director and principal investigator, Griffith, co-principal investigator Borré, staff epidemiologist Shechter, occupational physician Marian Swinker, and sociologist and field director Vernon Kelley. Particularly important in the development of the questionnaire were our knowledge of previous studies of coastal North Carolina populations (e.g. Griffith 1993, 1996; Johnson and Griffith 1996; Johnson and Orbach 1996), works of medical anthropology that dealt with populations that

have uneven relations with the health industry (e.g. Balshem 1991; Chavez 1995; Garro 1988; Griffith 1995a, 1995b), and information about the potential threats of toxic dinoflagellates to humans (e.g. Morris 1991; Burkholder, Glasgow, and Steidinger, 1994; Burkholder Noga, Hobbs, and Glasgow 1992). Sociologist Vernon Kelley and a team of field workers pre-tested the instrument shortly thereafter, making certain that the questions were understandable to the sample population and that no questions would elicit misleading responses. Several meetings followed in which the questionnaire was refined into the version presented in Appendix A.

II.b.2. Sampling:

We received a list of crabbers from the state's Division of Marine Fisheries for sampling purposes. This list included all those commercially licensed crabbers who had landed more than 6,000 pounds of crab during the 1995 season. Based on this list, we developed a sampling strategy in which we would interview 250 (57.5%) of the 435 crabbers located in Beaufort (n=124), Pamlico (n=98), Hyde (n=124), and Carteret (n=89) Counties, later adding crabbers from Craven and Dare Counties.[3] Throughout this report, we refer to this group as the Pamlico Crabbers; this is the principal group of interest in the report, since they are the crabbers working in areas known to have experienced large numbers of large (100,000+) fish kills that were associated with *Pfiesteria*-like organisms.

In addition, we selected a control sample of 125 crabbers from the Albemarle region, distributed among Tyrell, Chowan, Perquimans, Pasquotank, Camden, and Currituck Counties. This is the group we refer to as the Albemarle crabbers. For both samples, we used the following inclusion criteria:

- Crabbers needed to be at least 18 years of age.

- Crabbers needed to have crabbed during the previous year.
- Crabbers are to have resided in Eastern North Carolina for five years and to have been a full-time resident of Eastern North Carolina.

Following data collection of Pamlico crabbers, we interviewed individuals from the communities in which these individuals were residing at the time of the interview, randomly selecting them from lists of neighbors developed by the Pamlico crabbers. We were careful to screen them based on three criteria:

- That they not be involved in any activities that involved daily exposure to the water.
- That they be within five years of age of the crabbers interviewed in their communities.
- That they lived in Eastern North Carolina as year-round residents.

II.b.3. Data

Interviewing began in the late spring of 1996 and lasted until November of the same year. We succeeded in interviewing 253 individuals whom we call Pamlico crabbers, 113 individuals whom we call Albemarle crabbers, and 125 individuals whom we call Community controls. While we use these three groups for most of the overall comparative work, detailed examinations of the cases required us to either drop or reassign three cases prior to the analysis. Two cases in the Pamlico crabber group, for example, reported setting no traps in 1996, and hence were excluded from the statistical tests on the relationship between exposure and levels of illness (because exposure was defined by number of pots pulled per day). One of the Albemarle crabbers was found to cross-over into the Pungo River to set crab traps during height of the crabbing season; he was therefore reassigned to the Pamlico (i.e., exposed) group for the comparative analyses. These changes, however, had no effect on the conclusions of the study.

Other interviews and observations supplemented this study, including interviews with individuals who self-diagnosed themselves as having been affected by *Pfiesteria,* physicians and other health care providers throughout the region, state officials who have been involved in water quality issues for several years, scientists throughout the UNC network of colleges and universities as well as scientists at other universities, and trips on crabbing vessels. In addition, for additional information on fishing and fishing behaviors, we relied extensively on the data collected in three recent studies on North Carolina's fishing industry, conducted for the North Carolina Moratorium Steering Committee in 1995-96 (Garrity-Blake 1996; Griffith 1996; Johnson and Orbach 1996).

The following section introduces our findings, providing basic demographic information on the crabbers and community controls we interviewed. The three samples are, in general, remarkably similar along a number of lines, reflecting the highly meticulous nature of our sampling strategy and the skills of our field manager, Vernon Kelley, and field assistants Patrick Stanforth, John Brown, Douglas Hobbs, and Brian Ellis. Kelley, Stanforth, Brown, and Hobbs, in particular, worked extensively on projects conducted for the Moratorium committee.

From the careful sampling strategy and the special skills and experiences of the research team, then, we have assembled the most comprehensive and reliable data to date on the presence of illness among people who work day-in, day-out throughout North Carolina's estuary. Nevertheless, they suffer from certain weaknesses, as well as possess certain strengths, which we point out here.

Strengths

1. Individuals who participated in the study were randomly selected. This is the primary strength of the data presented here as compared to other examinations of the relation between Pfiesteria-like dinoflagellates and health. To date, all other accounts have been based on anecdotal evidence from self-selected individuals. That is, individuals came forward after they already believed they had been exposed to Pfiesteria, a circumstance which introduces bias into the data that would lead to overstating the incidence of Pfiesteria-related illness (Johnson 1992).
2. Individuals were interviewed in person by field assistants who had had years of experience interviewing fishermen on a wide range of issues. They had thus already established some rapport with this population, and in person interviews are notoriously more reliable than either mail-out or telephone interviews, since the latter often result in low response rates.
3. The random sampling strategy insured that we included crabbers and community controls from several areas and thus assuring independent observations, a precondition for many statistical tests (Thomas 1977).
4. The sampling strategy assured that potential confounding variables, such as age, tobacco use, and alcohol use, were randomly distributed over the population, as well as informant errors in reporting, thus reducing the bias that might be introduced from these sources.

Weaknesses and Possible Sources of Bias

1. These are self-reported data. As such, they do not have the backing of clinical work,

but are respondents' own estimations of their health. This introduces the possibility that respondents will either overestimate or underestimate their health problems. Given the publicity given to this topic, we anticipated finding individuals attributing illness to Pfiesteria, with little basis for such self-diagnoses, as appears to be the case among many of the cases mentioned above (Morris 1995).

2. This is a retrospective study, based on crabbers' abilities to recall incidents of illness. Studies of this type suffer from respondents' inability to recall all illnesses they have experienced in recent years, particularly illness indicated by mild symptoms.
3. Crabbers may have had a vested interest in under-reporting their illness, given the possibility that high incidences of illness might conceivably lead to river or sound closures or other restrictions on crabbing. While this is a possibility, having worked with this population in other settings (Griffith 1996, 1993), we find it hard to believe that high percentages of crabbers would have deliberately misled us; in any case, it would have been difficult for them all to tell the same lie, and their reporting errors along these lines would have been randomly distributed.
4. Crabbers who have already been affected by deteriorating water quality were not available for study because they no longer crab (and thus would not have been included in the sampling frame). This is highly unlikely, given the long years of experience of crabbers in our sample (between 18 and 19 years, on average). In addition, neither of three recent studies of fishermen in North Carolina (Griffith 1996; Johnson and Orbach 1996)—the latter based on a representative sample and the former collecting more detailed and richer information from smaller groups of

fishers—found any reports of large numbers of crabbers dropping out of the population due to health problems. Indeed, many of North Carolina's problems in fisheries stem from few fishers dropping out while too many new fishers are joining their ranks.

5. Given long-term contact with the water, crabbers may have developed immunities to Pfiesteria and related water quality problems, thus predisposing them to low-levels of ill-health due to contact with the water. While this may have occurred, physicians in Eastern North Carolina interviewed for this study continue to treat crabbers and other fishermen for various water-related afflictions, suggesting that they have not developed immunities to all sources of marine illness. However, as we noted earlier, fishers have developed avoidance behaviors regarding what they refer to as "dead water," and hence stay away from working in fish kills. This may be interpreted, of course, as an adaptive behavioral strategy and, in this sense, a kind of immunity. They remain, under such conditions, the ideal group to study for information on what health problems are likely to result from interacting with the water under normal ecological conditions.

Again, while these problems exist with the data set, these data remain the most accurate and comprehensive to date regarding the probable effects of interacting with Eastern North Carolina's estuarine waters on human health. We welcome additional studies using similar methodologies, and remain confident that similar studies, such as those currently proposed by the Centers for Disease Control (CDC) will derive essentially the same conclusion regarding North Carolina's waters that we have: that, under normal ecological conditions, they do not pose a

serious threat to public health.

Following the section on demographics, there are two sections that discuss attributes of the crabbing community that impact their health: 1) the occupational hazards associated with crabbing and crabbers' use of protective gears and behaviors; and 2) the relations between the crabbing/ fishing community and the medical community in Eastern North Carolina.

III. FINDINGS

III. a. Demographic Characteristics

The crabbers in our sample population constitute a highly stable population, coming from an average of 18.63 years of experience on the water, a fact that should come as no surprise to those familiar with commercial fishing in the state. Fishing is an occupation that is composed primarily of long-time residents of the state; only recently—with net bans, declining stocks, and other factors negatively affecting fishing in other states—have new fishermen been entering North Carolina fisheries, and many of these were thwarted by the moratorium on commercial fishing licenses imposed in 1995. Many fishers we interviewed for this and other studies readily volunteer the information that they were born into a fishing family; often, their grandparents, great-grandparents, and other ancestors fished for a living.

While successful commercial fishing in the state does not depend on coming from a fishing family background, it certainly is an asset to learning about technical, social, and cultural dimensions of the enterprise. Long-time residents with parents and grandparents who fish often undergo long apprenticeships with their elder relatives, during which they learn about fishing territories, seasonal issues, how different substrates affect fishing and sailing, and other technical dimensions of the enterprise. They can compose crews and forge relationships with seafood

dealers and processors through social contacts and a knowledge of the cultural rules that influence effective business partnerships. They may, as well, select marriage partners from other fishing families who know about the critical supportive roles that women play in most North Carolina fishing families.

III. a. 1. Specific Aspects of Residence of the Three Groups

The selective advantages that native fishers have over fishers born elsewhere may account, in part, for the fact that over two-thirds of the Pamlico crabbers (69.7%) and a similar proportion of the Community Controls (67.2%) were born in one of five counties: Beaufort, Carteret, Craven, Hyde, or Pamlico. By contrast, only 54% of the Albemarle crabbers come from counties in that region, including Currituck, Pasquotank, Perquimmons, Tyrell, and Washington. Considerably more Albemarle crabbers than Pamlico or Community controls come from coastal regions outside the state, 17.7% compared to 5.7% and 7.2%, respectively. The higher numbers of foreign-born in the Albemarle include seven Vietnamese, accounting for between 6% and 7% of the total Albemarle population.

Fewer than 24% of the Pamlico crabbers and fewer than 30% of the Albemarle crabbers are new residents, living in their current area for under five years; among the Community Controls, this figure is slightly higher, with 32.5% living in the region for fewer than five years. By contrast, 57.3% of the Community Controls, 59.5% of the Pamlico crabbers, and 54.4% of the Albemarle crabbers have lived in the area for over ten years.

Reflecting this is the fact that 89.6% of all the respondents own their own homes, with home-ownership rates slightly higher (94.4%) among the Pamlico crabbers; 83.2% of the Community Controls own their own homes and 86% of the Albemarle crabbers.

Together, our data on birthplaces, length of residence, and home ownership suggest that the Pamlico crabbers are the most native, the Community Controls second, and the Albemarle crabbers the least native. None of the groups contain large numbers of what could be considered foreign born or even outsiders, however.

Most respondents live in houses as opposed to trailers, apartments, or other kinds of dwellings, although more community controls live in houses than the crabbers, 85.6% compared to 73% of the Pamlico crabbers and 64.9% of the Albemarle crabbers. The highest proportion of mobile home residence, 33.3%, is found among the Albemarle crabbers, compared to only 26.2% of the Pamlico crabbers and 12.8% of the Community Controls.

A few minor differences between the three groups emerge when we compare various ways in which respondents operate their homes. Briefly, although around one quarter of each of the groups uses electricity to heat their homes, of the remaining 75%, far fewer Albemarle crabbers depend on gas heat than the other two groups (only 7.9% compared to 38.5% of the Pamlico crabbers and 24% of the Controls), and more Albemarle crabbers (17.5%) and Community Controls (18.4%) depend on propane than Pamlico crabbers (.8%). In terms of water and sewer, statistically significant differences emerge.

Table 01: Sewer and Water Practices, Crabbers and Community Controls Compared

Variable	Comm. Controls	Pamlico crabbers	Albemarle crabbers
Septic Tank	74.4%	95.2%	98.2%
Sewer	25.6%	4.8%	1.8%
p<.001 (chi-square)			
Municipal Water	67.2%	34.6%	57.0%
Well water	32.8%	63.9%	36.8%
p<.001			

Note: totals may not add up to 100% because a few cases listed "other" sources

These rather mundane statistics reflect similarities between the two groups of crabbing populations in terms of two practices that could conceivably affect their health and that also reflect possible residential differences between the groups. Clearly, more crabbers use septic tanks than Community Controls. These differences, along with those that exist between the groups regarding municipal water, may reflect more bias toward living in the more urbanized areas of coastal communities among Community Controls, and more urban dwellers among the Albemarle crabbers than the Pamlico crabbers. We could find no way, however, that these differences affected the health statistics of the three groups.

Perhaps also indicating a bias toward rural areas, there were minor differences between the crabbers and controls regarding keeping and breeding animals. Around 80% of both crabber populations keep some animals, compared to only 65.6% of the Community Controls, and between two and three times as many Albemarle crabbers breed animals as the other two groups. Animal breeding was not common, however, with only 16.7% of the Albemarle crabbers engaging in this practice.

III. a. 2. General Characteristics of Respondents and Household Members

Table 02 describes the three sample populations in terms of statistics regarding selected demographic characteristics. These data show that our respondents were overwhelmingly white, middle aged, male, married, living with only one to two other people, and high school educated, characteristics that seem to describe most commercial fishers in the state (Johnson and Orbach 1996). There are few significant differences between the three groups in terms of these variables. In terms of the objectives of this study, this is fortunate, since variables such as age, ethnicity, and education these are frequently indicators of health status.

Table 02. Selected Demographic Characteristics

Variable	Pamlico	Albemarle	Controls
Percent Caucasian	98	91	95
Percent Married	78.2	76.3	71.2
Percent single/ never married	11.1	10.5	20.8
Percent Divorced	10.3	12.3	7.2
Mean age (s.d.)	44.43 (13.11)	45.11 (12.07)	44.17 (14.45)
Mean Household size (s.d.)	2.96 (1.29)	2.75 (1.19)	2.68 (1.10)
Mean Years of Education (s.d.)	11.97 (2.22)	12.93 (5.95)	13.06 (2.14)

Most of the fishers are married, and most spouses are gainfully employed, with only 15.9% of the Pamlico crabbers' spouses, 9.6% of the Albemarle crabbers' spouses, and 4% of the Community Controls' spouses falling into the "housewife" category. Common occupations include working in public education, either as teachers or teachers' aids, office or secretarial work, and working in one or another branch of the health industry. Crabbers' spouses also, according the crabbers, work in commercial fishing. It is not uncommon for spouses to operate crab shedding facilities, for example, and certainly it is the norm for spouses to perform several support services of readying catch for market (cleaning, sorting, etc.), weaving nets, cleaning gear, and serving as crew on vessels. Many commercial fishers' spouses have assumed roles as industry spokespersons, founding political organizations that are dedicated to preserving the fishing way of life. Because fishers, in North Carolina and elsewhere, consider fishing more of a way of life than merely an occupation, one which involves entire households and families, it is nearly impossible for spouses not to take a highly active role in the household fishing operation (Durrenberger 1996; Maril 1983, 1996; Doeringer, et al. 1987; Griffith and Dyer 1996; Griffith 1996).

In addition to working spouses, a sizable minority of fishers themselves earn extra incomes

either throughout the year or on a seasonal basis. Nearly one quarter of the Pamlico crabbers (23.3%) and one fifth of the Albemarle crabbers (19.3%) work outside crabbing as well, sometimes in marine or fishing related occupations such as operating ferries or running crab shedding facilities. More commonly, however, fishers list seasonal work among their other occupations, including other fishing, which 16.7% of the Pamlico crabbers and 22.8% of the Albemarle crabbers reported among their seasonal occupations. Another occupation common among fishers, perhaps related to the myriad tasks associated with maintaining and operating fishing vessels and gear, is construction.

Other time-honored traditions in Eastern North Carolina, which enable households to reduce their consumption costs, are hunting, recreational fishing, and trapping (Forrest 1988). Given the low income levels of many crabbers and the similarities between hunting and fishing, we should not be surprised to learn that considerably more crabbers hunt than Community Controls, 58.2% of the Pamlico crabbers and 63.2% of the Albemarle crabbers compared to only 37.6% of the Controls (chi-square analysis $p<.001$). Slightly under two thirds of all three populations reported fishing for recreation, and very few individuals in our sample engage in trapping. As with breeding dogs, the largest number of trappers are in the Albemarle sample, accounting for 12.3% of that group.

III. b. Occupational Hazards of Crabbing

III. b. 1. Work of Crabbers

Two recent studies of North Carolina fisheries conferred that the primary gears in the Pamlico and Albemarle regions are crab pots and stationary gill and pound nets; Albemarle fishermen also set eel pots (Griffith 1996; Johnson and Orbach 1996). Full time fishers fish these

daily; it isn't uncommon, from March or April until November or December, weather permitting, to see crabbers in their wide flat boats puttering along a string of floats, pulling pots and shaking crabs into tall plastic buckets the size of garbage cans. The process itself is laborious, especially for those fishers who do not use winches. We accompanied crabbers aboard vessels, spending whole days with them to learn the daily routines of crabbing and to enable us to identify potential areas for health hazards as well as estimate levels of exposure to water.

III. b. 2. Daily and Seasonal Routines

As is common among fishermen in many locations around the world, North Carolina crabbers tend to rise early, usually an hour or so before dawn, and fish into the early afternoon. Readying the vessel for launching may have been accomplished the previous evening, yet there remain several routine operational tasks to be performed, including checking fuel and oil levels, loading any additional traps or other gear, and stocking bait for the traps that will be fished through the day. Primary protective gears include the following, listed in order of frequency of use within the two crabber groups; those marked with an asterisk were more important among one group of crabbers than the other:

Rubber Boots

Oil Skins or Bib

Cap*

Long Pants

Rubber Gloves*

Sunglasses

These were the primary pieces of protective equipment, worn by at least half of both

groups of crabbers. Because their frequency of use varies little across the two crabber populations, they seem not to influence exposure levels; indeed, the discussion below makes the point that crabbers tend to be wet throughout the working day, despite their use of protective equipment.

Considerably more Albemarle crabbers use gloves than Pamlico crabbers, 81.6% vs. 61.1%, and slightly more Pamlico crabbers wear long pants than Albemarle crabbers, 66.3% vs. 57.6%. Other gears and protective equipment listed, but less commonly used, were: aprons, sun block, long sleeved shirts, back braces, cotton gloves, coats, and hip waders. Fewer than 20% of all crabbers reported utilizing these.

Often the first task of the day on the water is visiting a bait net, although some fishers purchase their bait from the same dealer/ processor to whom they will sell their catch later in the day. Checking a bait net involves more time on the water early in the morning and, hence, increased levels of exposure to potential threats from microorganisms like *Pfiesteria.*

Most crabbing, conducted primarily within state waters (inside the barrier islands), is a near-shore, daily activity, not requiring either long journeys to fishing grounds and never overnight or multiple days at sea. Traveling to one's crab pots usually takes under an hour and for some fishers only a few moments, although this varies through the season as crabbers move their pots from territory to territory, following crab migrations or for other reasons discussed below. Despite brief journeys to crab pots, the pace of travel is swift and crabbers begin getting wet within minutes after leaving their docking locations, their faces misted from the spray of the vessel's passage through the water and gradually exposed to more and more water as the day wears on and the hauling and dumping of traps adds to the accumulating pools in the bottom of

the vessel. Those of us who made trips with crabbers observed that they were exposed to water at some level throughout the entire trip, beginning with wet faces and necks and usually ending the day with wet arms and legs as well.

Once a crabber reaches his line of pots, the actual work is repetitive and straightforward. Pulling up to one of the floats, the experienced crabber reaches underneath with a hook attached to a long pole, pulls the line out of the water, grabs the line and lifts the trap up and into the vessel, opening its top and letting the crabs scatter into the vessel, then baits it and returns it to the water. The entire process usually takes under a minute, yet it may be repeated between 300 and 350 times a day, every day, from around five in the morning until two in the afternoon, for six days a week. Between clusters of pots, crabbers sort and cull their crabs. Because the number of traps a crabber pulls per day determines the amount of time he spends on the water, we use this variable (pots pulled per day) as a proxy for exposure in one of the comparative analytical sections below.

Crabbers in our study reported setting an average of 381.11 pots (s.d. = 205.92; mode = 400) and, in good weather, checking an average of 330 per day (s.d. = 146.60; mode = 400), suggesting that most crabbers in our study set no more traps than they are able to check daily. This is in line with other recent studies of crabbing in these areas, as can be seen in the additional data presented in Table 03, based on Johnson and Orbach (1996). In the two regions, the mean number of pots in the water varied significantly, yet the mean number of pots fished per day in good weather did not vary across the two groups.

Table 03: Average Numbers of Pots in Water and Pots Fished per Day, Pamlico and Albemarle Crabbers Compared

Variable	Pamlico (n=252)	Albemarle (n=114)	ANOVA level	All Crabbers*
Mean Pots in Water	355.10 (sd=187.61)	438.6 (sd=232.26)	.000	353.6 (256.38)
Mean Pots per Day	326.65 (sd=152.54)	339.91 (sd=137.72)	.432	n.d.

*Johnson and Orbach (1996: 98)

Observations of crabbers pulling, lifting, emptying, and rebaiting their crab pots show that this process involves a great deal of sustained daily exposure to the waters of the estuary and other occupational hazards. First, most obviously, the process of lifting the pot into the boat, especially with crabs and occasional fish flapping around, results in water both spraying and dripping into the boat and onto the crabber. The forearms of crabbers, moistened from water trickling off their gloves and down their arms, are particularly exposed during this time. Second, crabbers tend to lean against the edge of the vessel while lifting the pot, rubbing their upper thighs against the gunwale and sometimes causing chafing and the development of open sores. Third, lifting the pot stresses the lower back muscles. Finally, the process of sorting and handling the catch after the crabs and associated fish and debris have been dumped into the boat is always potentially dangerous, both because of the notorious hostility of the crabs and their sharp shells and in part because of the speed of the work (Warner 1976; Griffith 1994, 1996).

The hazards associated with crabbing may be reduced with other crew in one's vessel, sharing the work load, although work may increase with more than one crew aboard as well. About half of the crabbers crab alone and about half use one another crew, a finding which is consistent with other recent data on crabbers in North Carolina:

Table 04: Crew Size on North Carolina Crabber Vessels

Crew Size	Pamlico crabbers	Albemarle crabbers
1	53.2	43.9
2	40.9	49.1
More than 2	3.6	7.0

Breaking the day's monotony is an early morning visit to the pound net for bait fish and a visit at the end of the day to the dealer's or processor's dock. These remain more or less constant across the two populations, making the number of pots pulled per day the principal variable to use to differentiate between crabbers in terms of levels of exposure to the water. Crabbers spend between twenty minutes and an hour at the dock, but generally simply off-load their crabs before heading back home. The lifting and weighing of crabs at the processor's or dealer's facility represents one of the principal occupational hazards of crabbing, that of strained back muscles.

While they are fishing, pots constitute stationary gear, of course, but crabbers move their pots through the season as the crabs migrate across the estuary and as it becomes illegal to block certain channels to navigation with lines of floats and pots; they thus encounter many different water depths and water qualities. In fact, one reason crabbers might move their traps is to avoid "dead water"—water with so little oxygen that the crabs struggle to the tops of the traps in shallow water, trying to break the surface to breathe. About this a Pamlico crabber reported, "Me, I got to where my water got in so bad shape, from June right on through September, the water's dead. I mean, you got to get up in two or three foot of water, because the crabs can't live out there in that. I mean, they might live out there, but if you pot them out there in that deep water and they can't move, there's no oxygen there and they die... The water gets in such bad shape out here they start dying."

The phenomenon known as "dead water" is widely known among crabbers in our sample, with 85.3% of the Pamlico crabbers reporting having been affected by it and 86.8% of the Albemarle crabbers. The phenomenon specifically known as *Pfiesteria*, however, was far less well known across the estuary. Only 28.9% of the Albemarle crabbers had heard of it, while nearly half (48%) of the Pamlico crabbers and 32% of the Community Controls had heard of it. In addition, 62.7% of the Pamlico Crabbers had witnessed a fish kill and 65.8% of the Albemarle crabbers. Neither knowledge of *Pfiesteria* nor experience with a fish kill resulted in crabbers' reporting higher levels of illness, however, as we will present in more detail below.

III. b. 3. Additional Occupational Tasks

Fishing involves a wide variety of tasks in addition to the actual business of fishing, most of which are related to the construction and maintenance of vessels and gear. Many, if not most, fishers in North Carolina have work spaces or workshops set up in or near their homes that resemble the automobile mechanic's garages in smell and appearance. The similarity is not mere coincidence, of course. Boat and automobile engines, sometimes identical, require the same or similar solvents, oils, lubricants, and tools for their maintenance and operation. Commonly, fishers breathe the fumes of gasoline, paint, and various lubricants on a daily basis. In addition, most fishermen construct at least some of their own gear, particularly building traps and weaving nets, and some fishermen construct their own vessels. Table 05 presents some of these data:

Table 05: Percent of Pamlico and Albemarle Crabbers who Build Their Own Boats and Maintain their own Engines

Variable	Pamlico crabbers	Albemarle crabbers
Build boats (%)	88.1	91.2
Maintain engine (%)	79.8	60.5

Often, crabbers do not perceive as chemicals many of the chemical substances they deal with on a daily basis chemicals. In one instance, a fisher we interviewed while he was painting his vessel and using gasoline as a cleaning fluid, responded "no" to and early version of the question about chemicals, leading us to change it from, "Do you use chemicals in your work?" to "Do you use chemicals such as bleach in your work?" In response to this new question, we found that 64.9% of the Albemarle crabbers use chemicals regularly in their work, while only 50% of the Pamlico crabbers responded to this question in a positive way.

III. b. 4. Common Occupational Hazards of Crabbing

Several occupational hazards confront crabbers, besides those that may derive from marine toxins. The following table shows those injuries that were reported with any frequency, comparing the two groups:

The only significant differences between the two populations were in regards to the first two injuries on table 06, with significantly higher number of Albemarle crabbers reporting both cuts and their own designation of "sea needle in the eye." The first four kinds of injuries are not uncommon among both populations, affecting upwards of one fifth to one half of crabbers, but the table clearly suggests that most crabbers do not report experiencing a large number of hazards.

Injuries may be underreported, as is evident from the discrepancy between rows one and two in the Albemarle column in table 06, crabbers may not consider minor cuts and sprains as injuries, but simply as common aches and pains associated with their job. Knowing how common it is for crabs to attack and pierce the skin of crabbers, it is almost inconceivable that most crabbers have not been cut at least once during their years on the water.

Table 06: Percent Reporting Injuries, Pamlico and Albemarle Crabbers Compared

Injury	Pamlico crabbers	Albemarle crabbers
Ever been injured while crabbing?	53.2%	39.5%
Types of injuries reported		
Cuts	38.9	50.9
Sea needle in eye	13.5	36.0
Back sprains	25	29.2
Muscle sprains	21.8	25.4
Bruises	11.9	6.1
Fractures	4.0	1.8
Head injury	1.2	2.6

III. c. Relations Between Fishing Communities and Health Providers in North Carolina

III. c. 1. Health in Eastern North Carolina: A Context for the Health Status of Crabbers

As can be seen in Appendix A, our survey elicited information on nervous, gastrointestinal, dermatological, cardiovascular, respiratory, and uro-genital complaints or problems. In addition, we asked about other information that might influence the health of crabbers, such as alcohol and drug use or the prevalence of diabetes and cancer. No descriptive health studies of North Carolina fishermen are available in the medical literature with which to compare these data, and much of the recent reporting of environmental illness related to *Pfiesteria* is based on a relatively few self-diagnosed,[4] self-selected cases, primarily of marine construction workers and scientists working in laboratory settings. Therefore, we briefly describe the general health status of the population living in Eastern North Carolina below as a context for interpreting our findings. This section draws on data from interviews with coastal physicians concerning the health of their fishermen patients, information from marine construction workers in

New Bern who reported symptoms they attributed to *Pfiesteria,* and interviews with physicians from the Pamlico region.

The *Health Atlas of Eastern North Carolina* (Wilson 1997) reports the morbidity and mortality statistics for adults living in a 41 county region, including the seven counties of the Albemarle and Pamlico sounds. The region suffers from a shortage of health providers compared to other regions in the state. According to the *Atlas,* as well as local physicians, coastal populations neither seek nor receive adequate health care. State funds for health departments are limited; some communities are located two hours from primary care services. Many are health under-insured or uninsured and some private physicians cannot afford to treat self-pay, uninsured patients. Some of these either refuse to see the uninsured or limit care options according to what the patient can pay.

Rural health clinics are located in Columbia, Tyrell County; Vanceboro, Craven County; and Ocracoke, Hyde County; these clinics offer limited primary care services and triage. All have had trouble maintaining a physician on staff. Eight county hospitals funded originally through Hill Burton funds struggle to serve the fisherman's counties and survive financially. Several small hospitals in eastern North Carolina are currently under negotiation for buy-outs. In addition, barriers to health care delivery include language and cultural differences, poverty, transportation, functional illiteracy, and inadequate structural organization of rural health care services.

In Eastern North Carolina, mortality rates for heart disease, stroke and cancer not only rank as major causes of death but are renowned as some of the worst in the state. Morbidity rates for diabetes and hypertension, the major contributing secondary diseases to these causes of death, also are some of the highest in the nation. In addition to diabetes and high blood pressure, certain

infectious diseases appear to be increasing in Eastern North Carolina, including sexually transmitted diseases and tuberculosis. However, only 7.2% of the survey respondents experienced diabetes, and under 20% of the crabbers we interviewed reported suffering from cardiovascular problems such as high blood pressure.

III. c. 2. Health Providers' Views of Fishers' Health

For this study, we interviewed primary care providers in Chowan, Pasquotank, Beaufort and Craven Counties to determine their perceptions of health problems of fishermen in their communities; we also interviewed pharmacists, public health workers, nurses, and others familiar with health issues along the coast.[5] All primary care providers interviewed concurred that fishermen tended to be low-income, uninsured, and non-compliant. They reported poor compliance particularly in caring for open sores, in taking prescribed medications, and in changing risk behaviors. Pharmacists we interviewed supported this. Physicians also felt that uninsured fishermen made it a habit to "physician-hop," rotating among several primary care providers rather than staying with one. The reason for this was best expressed by one provider in Chowan County, "They come to you and never pay their bill. Then, they are too embarrassed to come back because they owe money." When asked if fishermen differed from other uninsured people, another physician working in Tyrell County replied, "Not really, all the uninsured follow that pattern. People are proud and feel ashamed when they can't pay. We have a sliding fee scale, but some people can't even afford that."

When asked what the major health problems of fishermen were, most believed there were no differences between fishermen, farmers, and farmworkers. They reported that the most widespread problems for all their patients were high blood pressure, heart disease and diabetes,

also noting that they were not sure how many of their patients were professional fishermen. Two physicians reported that they knew fishermen in their communities and knew they did not go to a doctor except when very sick. As evidence of the perceptual nature of physicians' accounts, our data show lower levels of diabetes and heart disease among crabbers than are found in the population at large.

When asked specifically about skin problems, all physicians reported that fishermen have many bacterial infections in their skin due to several different reasons. First, the sores don't heal and get reinfected because the fishermen don't keep them dry. Second, fishermen, like many other rural patients, do not take a full course of their antibiotic nor use antibiotic cream as prescribed. Crabbers, in particular, are known to get an infection from crabs puncturing the skin. This infection is particularly stubborn to heal. Twelve physicians reported that fishermen's main health problem was skin cancer. A dermatologist at PCMH confirmed that skin cancer rates are very high as well as psoriasis and skin rashes.

While there seems to be no shortage of sick individuals and lay persons who have been attributing illnesses to *Pfiesteria*, physicians have yet to diagnose a case of illness caused by *Pfiesteria* in the region. Of course, there is no diagnostic test for the dinoflagellate as yet, and physicians currently have no standardized definition of the illness; this is rare in epidemiological research, because epidemiology usually *follows* rather than precedes human mortality and a cluster of symptoms associated with that mortality. So far, no one has died from exposure to *Pfiesteria,* and the only symptom clusters that exist have come from the principal cases that occurred in the laboratory.

Causes of skin problems in fishermen were not attributed to water quality or toxin

exposure but to bacterial infection, injury, lack of regard for water safety, and use of caustic chemicals. All physicians agreed that healing problems were aggravated by failure to rest the injured area, continual exposure of skin to sun and salt water and use of rubber gloves, boots and protective outer clothing that does not permit air to get to the skin. Our observations of crabbers on their vessels indicated that much of their bodies stay wet through the day, despite the protective gears discussed earlier.

III. c. 3. Fishers' Views of Health Care Delivery

Views of the medical profession from the general population are, at times, as jaded as those of physicians toward fishermen and the rural poor. As is common in small, rural, close-knit communities, patients' perceptions may derive from a handful of anecdotes, such as the following:

Case 1: During a June, 1996 interview, a crabber's wife, who also works on the crab vessel when needed, reported that she had not had a pap smear in 13 years. She stopped visiting doctors when one "made her sicker." The family doctor who treated her in a small medical center told her that she had a vaginal infection. When she complained of abdominal pains and painful intercourse, she responded the doctor told her she did not need to be having sex anyway because she couldn't afford the kids she already had. He told her that because, she said, her husband was "just a fisherman" and, as such, they could not pay for tests to diagnose the infection. To treat her infection he gave her samples of "about 20 kinds of medicine" and told her to take one of each either twice or three times a day to get rid of the problem. She became sick, she believes from the medication, and had to be treated in the hospital at Washington, NC.

Cases 2 & 3: Two other wives related their childbirth experiences. Both had two children;

neither had any prenatal care until the third trimester. One, from Englehard, went into premature labor and drove two hours to Beaufort County Hospital emergency room. The ER nurse sent her on to Pitt County Memorial Hospital at Greenville because the physician did not want to have her give birth there. She delivered at the PCMH emergency room and the baby was normal. They returned to Englehard the next day. Her perception was that the doctors in Beaufort did not want to take care of her baby if she was born sick. She and her husband had no insurance and she thought that made a difference in how she was cared for by physicians.

Opened ended interviews with a few crabbers about their treatment of hypertension further support the idea that crabbers health practices conflict with those of established medical practice. Three crabbers, one each from Beaufort, Hyde, and Tyrell counties, and two crabbers' wives from Carteret and Beaufort counties, discussed health problems they experienced due to high blood pressure. The three male crabbers and the husbands of the women all had been diagnosed with high blood pressure. Currently, 4 of the 5 were not taking prescribed medication due to its cost. Two stopped taking the medication once it brought blood pressure down. Four of the five smoked and also refused to return to a physician. One wife said (paraphrased from field notes), "I know one day he's not comin' home. He'll just drop over dead and no one will find him or his boat." One husband also had "kidney trouble," which he defined as pain in the lower back, nausea, and inability to urinate. He passed kidney stones twice in the last 12 years. He felt his kidney trouble was due to the kidney stones, but said his doctor warned him that uncontrolled blood pressure could destroy his kidneys. That is why he takes his medicine.

Risk behaviors associated with heart disease, stroke, high blood pressure, diabetes and

cancer prevalent in Eastern North Carolina include tobacco and alcohol abuse, high fat diets low in fruits and vegetables, lack of exercise and obesity. Table 07 compares the three groups regarding varying aspects of their drug, tobacco, and alcohol use:

Table 07: Comparisons of Use of Tobacco, Alcohol, and Drugs by Sample Population

Percent Who Use	Community Controls	Pamlico Crabbers	Albemarle Crabbers
Tobacco	56.6	57.8	46.6
Alcohol	60.8	63.8	57.5
Prescription Drugs	26.4	24.1	20.4
OTC medications*	49.6	44.7	75.2
Home remedies**	14.4	25.4	36.6
Recreational drugs	5.6	9.5	7.1

*=statistically significant (chi-square=30.034; df=2; p=.000) **= statistically significant (chi-square=15.490; df=2; p=.000).

While the three populations are similar with regard to tobacco and alcohol use, this table shows that Albemarle crabbers use significantly more over-the-counter drugs and home remedies than the other two groups, and that the use of home remedies is twice as common among all crabbers, 28.8% of whom use home remedies, than among the 14.4% of Community Controls who use home remedies (chi-square=10.314; df=1; p=.001). Behind these statistics lie some of crabbers' the mistrust of the medical community and their lack of ability to afford its services as well as common practices for treating their own wounds. In our interviews with crabbers during this and other studies, we found that a common treatment for various kinds of skin ailments among crabbers is the use of chlorine bleach; the effect of this, according to medical personnel on our research team, is that it removes layers of skin, evidently sometimes along with infections, rashes, and so forth, because most crabbers who report this practice report also that it is effective.

The higher use of over-the-counter (OTC) drugs among Albemarle crabbers may be

related to their higher reported levels of allergies and respiratory ailments, which we present in more detail below.

Indicators of the populations' interactions with the health care delivery system are presented in Table 08:

Table 08: Percent of Use of Health Care Systems by Sample Population

Percent Who Have:	Community Controls	Pamlico Crabbers	Albemarle Crabbers
Health insurance	79.8	59.8	63.7
Regular source of medical care	74.4	70.2	75.2
Seen a doctor in the past year	63.2	66.0	69.9
Been hospitalized	64.8	69.2	63.7
Been in an emergency room in the past two years	32.8	26.1	25.7

Again, we found no statistical differences between the three groups in terms of the above variables, which indicate that all three groups engage the health care delivery system with equal frequency. Any health differences between groups, therefore, cannot be attributed to these factors.

Eastern North Carolina's environmental health problems have attained national notoriety due to publicity over animal waste spills. Just as significant, more widespread but not frequently publicized are problems with high water tables, heavy clay soils, dysfunctional septic tank systems and bacterial and fungal spore growth due to seasonal flooding. Agricultural run-off and soil leaching are major problems both for farmers trying to protect nutrient poor soils to give the best possible yields and to fishermen who believe that phosphate run-off contributes to "dead water"

and fish kills. As with most environmental groups in Eastern North Carolina, many fishermen believe that high concentrations of *Pfiesteria* are due to animal waste effluent spilling into tributaries feeding the sounds.

III. c. 4. Patterns of Self-Reported Health Status Among The Sample Populations

The interviewers asked study participants to report their medical history concerning including cancer, diabetes, and infectious diseases. While the response rate to the question about infectious disease was too low to allow any comparison across the three populations, table 09 shows, again, that the incidences of cancer and diabetes are similar across the three groups (differences were not found to be statistically significant):

Table 09: Incidence of Cancer and Diabetes by Sample Population

% Who Have Had	Community Controls	Pamlico Crabbers	Albemarle Crabbers
Cancer	8.0	10.4	8.0
Diabetes	7.2	8.0	5.4

Our analysis compares the three sample populations in terms of 6 major groups of symptoms, all of which have been associated with *Pfiesteria* exposure:

1. neurological symptoms such as changes in personality or memory; cognitive impairment; problems with coordination, nerves, movement or sensation; and headaches, seizures or visual changes.
2. respiratory problems
3. dermatological problems such as rashes, sores and cancer.
4. symptoms associated with eating, bowel habits and stomach problems.
5. heart/circulation problems and high blood pressure.
6. urination, bladder and prostate problems.

After eliciting data on these types of symptoms, we asked crabbers to report any problems with physical activities and symptoms associated with general malaise, fatigue, unexplained fever or other non-specific conditions defined by the fisherman as being "unwell." While these are

vague categories, they were included because many of the symptoms associated with *Pfiesteria* have been expressed in such vague terms. As noted earlier, these symptom categories were based on articles and information related to toxic dinoflagellate exposure and consultation with an occupational physician. Table 10 presents these data.

Table 10: Percent who Reported Symptoms: Crabbers and Community Controls Compared

Symptom	Com. Controls	All Crabbers	Pamlico	Albemarle
Personality or Memory Changes	9.6	7.3	7.9	5.3
Problems with nerves, etc.	5.6	7.0	7.5	6.2
Headaches, seizures, etc.	8.8	14.1	12.6	16.8
Respiratory Problems*	24.8	23.7	19.4	35.4
Dermatological problems*	11.2	24.6	25.3	23.0
Digestion, stomach problem	8.0	9.3	9.5	9.7
Heart, circulation problems	20.0	16.1	17.0	15.8
Urination, bladder problems	4.0	7.0	7.1	6.2
Felt unwell, fatigued	6.4	6.5	8.3	3.5
Problems with physical activities	8.0	5.9	7.5	3.5

* = statistically significant

III. c. 5. Discussion

These statistics reveal very low levels of illness in our sample populations, suggesting that extreme health problems along the coast are uncommon and that crabbers are more likely than non-crabbers to suffer from only a few types of symptoms. Of all these comparisons, we discovered highly significant differences between community controls and crabbers only in terms of dermatological problems (chi-square=10.322; df=2; p=.006), and slightly significant differences in terms of problems with fatigue and general feelings of illness. On closer examination, however, we can see that skin problems are not restricted to crabbers in the Pamlico region, but occur with equal frequency among crabbers in the Albemarle region. This suggests that skin problems are an occupational hazard of crabbing itself and not necessarily the result of toxic dinoflagellate poisoning. We support this with closer scrutiny of the cases, showing in table 11 the specific

disorders suffered by crabbers and the principal reasons given for their disorders.

Table 11: Selected Variables Related to Skin Disorders Reported by Crabbers

Percent Who Report Cause/ Character of Skin Disorder as:	Crabbers With Skin Disorders (24.6% of All Crabbers; N=90)
"Being on the water"	27.8
"Exposure to the sun"	23.3
Exema/ Skin rashes	34.4
Skin Sores	35.6
Skin Cancers	32.2
Boils/ Cysts	18.9
Loss of Pigment	13.3
Cuts that don't heal	13.3
Fungal infection	12.2
Other	8.9

Note: totals do not add up to 100% because some crabbers reported more than one ailment.

General skin sores and rashes, listed by 70% of the crabbers who suffered skin disorders, are the two afflictions that have been linked, in the popular media and among a few lay observers, to exposure to *Pfiesteria*. These judgments are primarily due to suspect comparisons between lesions on fish and lesions on humans, yet bear some attention here because of the common perception in many individuals' minds that *Pfiesteria* causes skin disorders such as these. If that is indeed the case, again, we must conclude that these disorders are not common among crabbers. While these disorders were cited by 70% of crabbers who suffered skin disorders, only 17.2% of the total population of crabbers reported suffering from these kinds of skin disorders.

That over one-third of Albemarle crabbers reported respiratory problems was somewhat alarming, given that it was a statistically significant difference (chi-square = 10.822; df=2; p=.004). Examining these 40 cases in more detail, we found that problems due to allergies (hay fever, sinus congestions, etc.) were most common, perhaps in part due to the broad stretches of

lumbering and farming areas along the north and south shores of the Albemarle sound. Table 12 presents data on these cases. Given the low levels of respiratory ailments found among the Pamlico group, however, we cannot conclude that these ailments are due to toxic dinoflagellate exposure or casual contact with the estuary.

Table 12: Most Common Reasons for Respiratory Problems Among Albemarle Crabbers Who Reported Respiratory Problems (n=40)

Problem	Percent Reporting
Sinus congestion	62.5
Has fever	52.5
Sinus infection	25.0
Asthma/ Bronchitis	20.0

Note: totals exceed 100% because some crabbers listed more than one reason.

Besides these two sets of symptoms, differences between the three groups were not statistically significant, which suggests that these levels of illness are common among individuals in this age group, this region of the country, with common smoking and drinking habits, etc. Overall, levels of illness were low and cannot be attributed to casual exposure to the waters of Eastern North Carolina.

One final set of comparisons involves the probable effect of heightened awareness about toxic dinoflagellates on propensity to report health problems by the three groups. This issue is important in determining whether or not those who have heard about *Pfiesteria* are more likely to report illness than those who have not, giving some indication of the hypochondria surrounding this issue. It is no secret that the press coverage devoted to this issue has been, at times, hysterical, especially with claims of *Pfiesteria's* similarity to deadly contagious rainforest illnesses. Did this have any effect on respondents' reports of health? As table 13 shows, evidently not. That is, those who had heard of the dinoflagellate (39.8% of the total sample, including all three

groups) did not report significantly higher incidences of symptoms than those who had not heard of the organism (60.2% of the total). None of the figures were found to be statistically significant

Table 13: Respondents' Reported Symptoms by Knowledge of *Pfiesteria*

Symptom Reported	Have not heard of *Pfiesteria*	Have heard of *Pfiesteria*
Personality or memory changes	24 (8.2%)	14 (7.2%)
Problems with nerves, etc.	24 (8.2%)	8 (4.1%)
Headaches, seizures, etc.	36 (7.4%)	26 (13.4%)
Respiratory problems	72 (24.5%)	48 (24.7%)
Dermatological problems	54 (18.4%)	48 (24.7%)
Digestion, stomach problems	26 (8.8%)	19 (9.8%)
Heart, circulation problems	53 (18%)	33 (17%)
Urination, bladder problems	14 (4.8%)	16 (8.2%)
Felt unwell, fatigue	16 (5.4%)	17 (8.8%)
Problems with physical activity	21 (7.1%)	12 (6.2%)

III. d. Summary of Comparisons Among the Three Populations

Again, few reported health differences exist among the three populations and, in general, the three populations report relatively low levels of illness. Crabbers seem no more or less healthy than the general population, despite that they interact, daily, with a resource that has been portrayed, recently, as highly contaminated. While our interviews with marine construction workers, the recent Johns Hopkins findings from the Pocomoke River in Maryland, and the widespread information from afflicted laboratory workers certainly suggest that *Pfiesteria* poisoning is particularly alarming, the data presented here do not support the proposition that exposure to the highly toxic form of *Pfiesteria* is common among individuals who interact with the water on a daily basis throughout their lives. Instead, these data suggest that, under normal ecological conditions (e.g. outside of a fish disease event, a fish kill, an algae bloom, etc.), the

waters of Eastern North Carolina are not dangerous to human health.

We point out, however, that from other studies (Griffith 1996), we know that crabbers have a sophisticated knowledge of the marine environment and most have experiences with what they call "dead water" and other problems of poor water quality. Thus their ability to detect subtle differences between water that appears to be contaminated and water that appears to be clean may lead them to avoid areas where toxic forms of *Pfiesteria* are likely to lurk, a factor which could account for their low levels of illness associated with an occupation that requires daily contact with the estuary. The following two sections focus specifically on the two groups of crabbers, leaving the community controls out of the comparative analysis.

III. e. In-Depth Examination of Crabbing Samples

Having made broad comparisons among the three populations, we turn to a more detailed examination of the incidence of health and illness within the crabbing population. These comparisons proceed along three lines: reported illness by experience with a fish kill; reported illness by different levels of exposure, as indicated by numbers of crab pots pulled per day; and reported illness by crabbing territory, based on map data provided by 295 of the 366 crabbers (that is, around 20% of the crabbers either refused or could not provide data on where they placed their traps from season to season).

III. e. 1. Reported Illness By Exposure to A Fish Kill

First, we explore whether or not experience with a fish kill, as indicated by a positive response to the question, "Have you ever seen a fish kill?" influences reported health. Table 14 shows that this does seem to be the case in at least one reported symptom: that of respiratory ailments and allergies. In other words, while in most cases witnessing a fish kill does not

differentiate crabbers from one another, in the case of respiratory ailments, those who witnessed fish kills (64.9% of all crabbers) are more likely to report suffering from such ailments than those who have not witnessed fish kills (35.1% of all crabbers). This may lend some additional support to the Maryland case, where individuals found working in and around fish kills were found to suffer health problems; in any case, this finding would suggest a recommendation that coastal residents exercise caution in the vicinity of a fish kill.

Table 14: Crabbers' Reported Symptoms by Experience with Fish Kills

Symptom Reported	Have not Experienced Kill	Have Experienced Kill
Personality or memory changes	10 (7.8%)	16 (6.8%)
Problems with nerves, etc.	10 (7.8%)	16 (6.8%)
Headaches, seizures, etc.	17 (13.3%)	33 (13.3%)
Respiratory problems*	19 (14.8%)	70 (29.5%)
Dermatological problems	33 (25.8%)	57 (24.1%)
Digestion, stomach problems	8 (6.3%)	26 (11.0%)
Heart, circulation problems	19 (14.8%)	42 (17.7%)
Urination, bladder problems	8 (6.3%)	17 (7.2%)
Felt unwell, fatigue	5 (3.9%)	19 (8.0%)
Problems with physical activity	7 (5.5%)	16 (6.8%)

*statistically significant (chi-square=9.73; df=1; p=.002)

III. e. 2. Reported Illness by Levels of Exposure

For the following analysis, we divided up the population into tertiles according to the number of traps per day they reported pulling: group one (low) pull 50-272 traps per day (n=113), group two (medium) 273 to 400 per day (n=152) and group three (high) pull more than 400 traps per day (n=75). We determined this variable to be an accurate reflection of exposure despite potential mediating factors such as protective gear. Based on our direct observations of crabbers on the water, we determined that protective gear seem not to prevent exposure to water. Number of pots a crabber pulls per day, however, determines the amount of time a crabber spends

on the water daily and reflects the degree to which they are likely to come into direct contact with the water. In addition, as crabbers lean toward the water to pull crab pots, usually while the vessel is still in motion, they risk breathing any toxins that might occur in aerosol form and have been stirred up by the passage of the vessel. Table 15 presents the results of these comparisons:

Table 15: Percent Reporting Symptoms By Level of Exposure: All Crabbers/ Pamlico Crabbers

Reported Symptom	Group 1 (Low)			Group 2 (Med.)			Group 3 (High)		
Personality or memory changes	7.4	/	10.3	7.9	/	8.5	5.1	/	4.1
Problems with nerves, etc.	5.7	/	7.7	7.3	/	6.8	8.9	/	8.2
Headaches, seizures, etc.	9.8	/	12.8	13.3	/	12.0	21.5	/	14.3
Respiratory problems	26.2	/	17.9	24.2	/	19.7	21.5	/	14.3
Dermatological problems	26.2	/	28.2	23.6	/	23.9	24.1	/	22.4
Digestion, stomach problems	6.6	/	5.1	9.7	/	10.3	13.9	/	12.2
Heart, circulation problems	18.9	/	16.7	20.0	/	17.9	6.3	/	10.2
Urination, bladder problems	4.1	/	5.1	10.9	/	11.1	2.5	/	2.0
Felt Unwell, Fatigue	8.2	/	9.0	4.8	/	6.0	8.9	/	10.2
Problems with physical activity	6.6	/	7.7	6.1	/	6.8	6.3	/	6.1

None of these comparisons was found to be statistically significant; in other words, as exposure to the water increased, we did not find a corresponding increase in the incidence of reported symptoms among either all the crabbers or the Pamlico crabbers. The lack of statistical significance in all of these comparisons suggests that the illnesses that do plague the crabbers are not directly related to exposure to the water as defined by numbers of pots pulled per day, again supporting the overall conclusion that exposure to the waters of Eastern North Carolina does not pose a serious threat to public health.

III. e. 4. Fish Kills and Crabbing Territories

Perhaps our most compelling evidence that *Pfiesteria* poses no serious threat to public health outside of a fish kill or disease incident comes from data we collected on the actual places

crabbers set their traps as compared to regions that have been associated with fish kills and, more specifically, with *Pfiesteria.* For this analysis, during our interviews with crabbers, we asked them to indicate, on nautical maps of the rivers and sounds of Eastern North Carolina, where they set their crab pots and fished through the course of the year. Because crabbers do move their traps through the year, we had them indicate territories by months and by seasons, and then assigned territories to crabbers based on where they crabbed during the summer months and for most of the year. As a result of these comparisons, we reassigned one of the Albemarle crabbers to the Pamlico group, since although he lived in the Albemarle region, he crabbed during the height of the season in the Pungo River, which is known for fish kills and for *Pfiesteria* infestation.

We were, in addition, able to compile information on fish kills from several sources, including data from the state of North Carolina,[6] from the National Marine Fisheries Service's HEED project, and from published sources on fish kills and other incidents (Pietrafesa and Miller 1997; Stanley 1992). From these sources, located 96 fish kills that had been associated with concentrations of *Pfiesteria.* While there have been many more kills with high concentrations of *Pfiesteria* (e.g. greater than 20,000 cells/ml), these 96 were sufficient to determine areas of the estuary that, year after year, have experienced fish kills in association with *Pfiesteria.* We emphasize that most data on fish kills are either incomplete or biased toward certain areas over others, since all fish kill data depend on someone reporting the kill. Smaller kills, or kills that occur during times of the year or season that there are fewer individuals on the water, are more likely to go unreported than others.

According to existing data, areas that are particularly prone to such kills are the Pamlico River, primarily between South Creek and Bayview and around Blounts Bay, the Pungo River

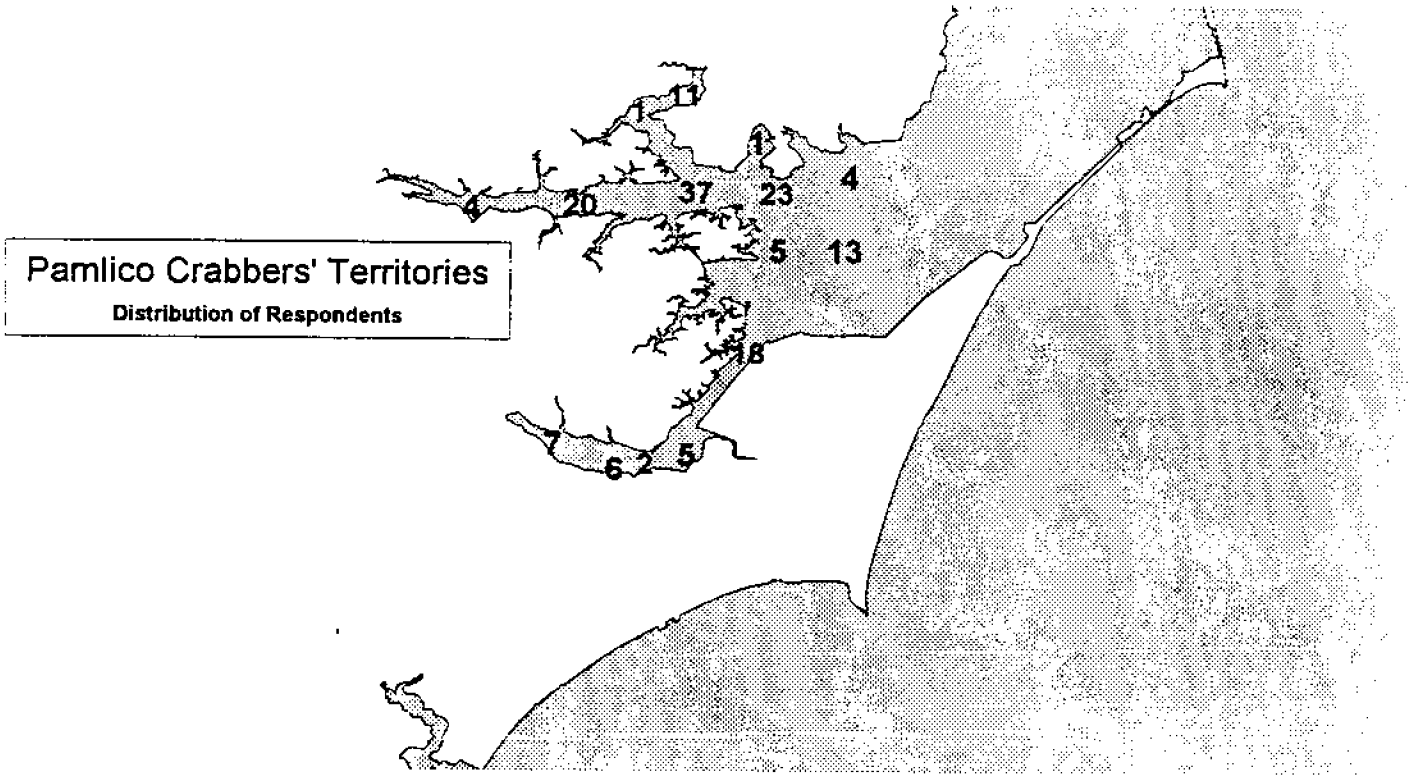
area, and the lower Neuse River around Minnesott Beach and between Flanner Beach and Cherry Point Landing. On the following maps, we have listed several of the more infamous locations for fish kills associated with *Pfiesteria,* and below that shown the distribution of crabbers we interviewed over the same area (see Maps 1 and 2). Clearly, our interviewed included several individuals who routinely fish in and around these kill sites.

We grouped the crabbing territory data into six regions, two of which correspond to these areas of frequent and intense fish kills associated with *Pfiesteria.* These are:

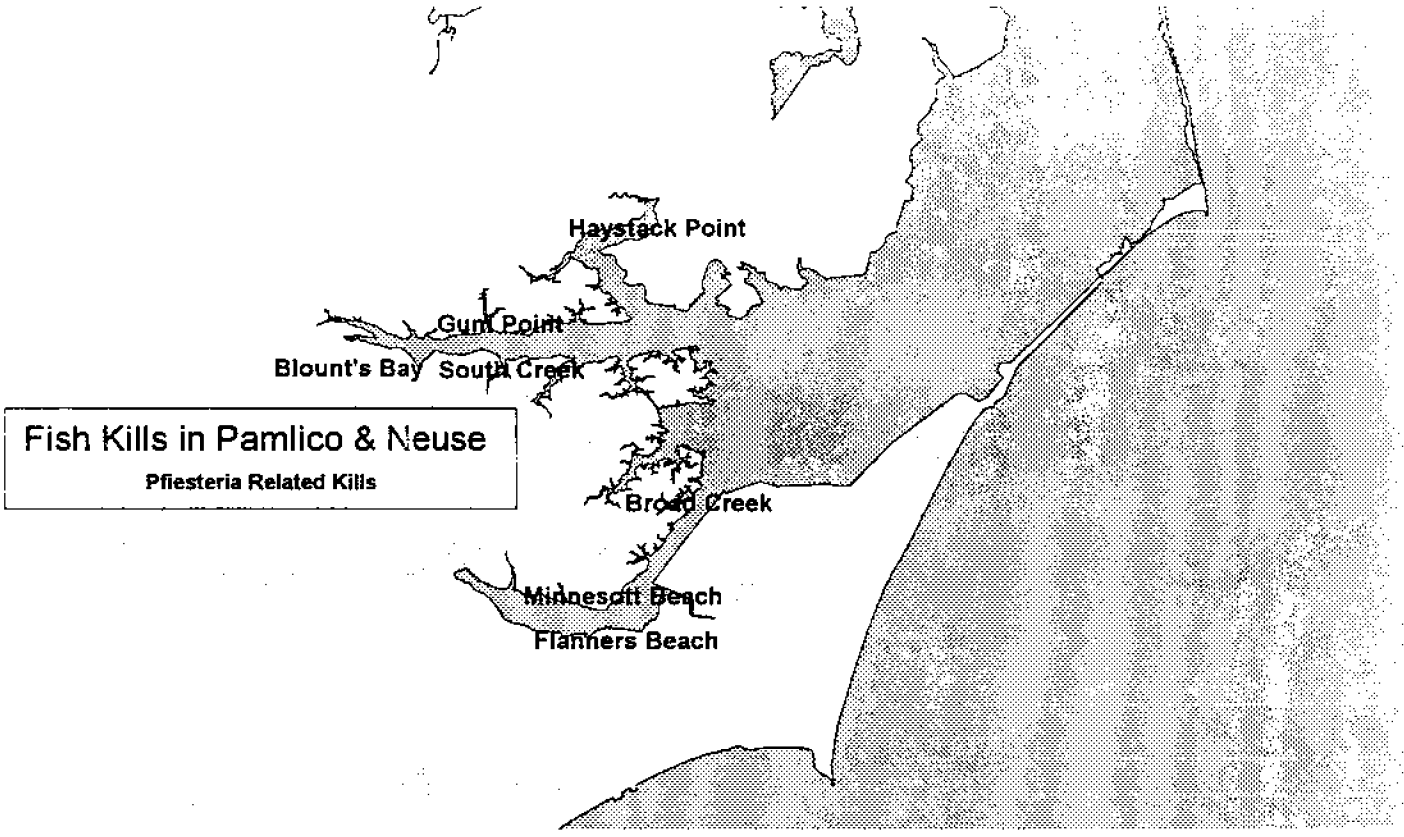
1. **Upper Albemarle, consisting of the rivers around Elizabeth City and the Currituck Sound.**
2. **Albemarle Sound, consisting of the area from the mouth of the Roanoke River to the Alligator River.**
3. **Pamlico and Pungo Rivers, consisting the Pamlico and Pungo Rivers, from Washington to where the Pamlico River meets the Pamlico Sound.**
4. **Pamlico Sound.**
5. **Neuse River, from New Bern to Minnesott Beach.**
6. **Core Sound/ Onslow Bay, or the area from Core Sound to the mouth of the New River.**

Clearly, regions three and five are the areas most prone to reported fish kills, particularly kills associated with *Pfiesteria.* If interaction with these areas were more dangerous to public health than interaction with other areas that were less prone to reported fish kills, we would expect to find high levels of sickness in these areas compared to the other areas. We find some limited support that the Pungo and Neuse regions are in fact less healthy than the other regions, although we hesitate to suggest that any of these areas pose very serious threats to public health.

Map 1


Pamlico Crabbers' Territories
Distribution of Respondents
11
1
1
4
4
20
37
23
5
13
18
6
2
5

Map 2


Haystack Point
Gum Point
Blount's Bay
South Creek
Broad Creek
Minnesott Beach
Flanners Beach
Fish Kills in Pamlico & Neuse
Pfiesteria Related Kills

However, when we examine the ratios of well crabbers to ill crabbers (that is, those reporting no symptoms vs. those reporting some symptoms) by region, we can rank the areas in the following manner:

Table 16: Ratios of Well People to Ill People by Crabbing Region, Ranked from Lowest to Highest (n=295)

Crabbing Region	Ratio of Well to Ill Individuals (Well : Ill)
Neuse	5.4 : 1
Pamlico & Pungo Rivers	6 : 1
Ablemarle Sound	7 : 1
Upper Albemarle	8.35 : 1
Core Sound/ Onslow Bay	8.6 : 1
Pamlico Sound	9: 1

These figures show that, indeed, those regions that have been associated with many fish kills (Neuse and Pamlico & Pungo Rivers) do in fact have lower levels of well crabbers for every ill crabber, while the Pamlico Sound, with its ratio of 9 to 1, has the most well crabbers for every ill crabber. While these data do provide some limited cause for concern regarding working on these two bodies of water as compared to the others, comparisons across the groups using the appropriate statistical tests show that, with one exception, the numbers of well vs. ill crabbers do not vary significantly by region. The one exception is that of fatigue, which we discuss further below. For these comparisons, we grouped the Neuse and Pamlico Regions together and grouped all the other regions together, since the cell sizes were too small for comparisons across all regions.

Again, with the exception of fatigue, neither region is associated with statistically significant higher levels of illness than the other. The chi-square figures for fatigue are significant at the .05 level, which is a fairly low level of significance, yet these figures bear additional

investigation. Given the small number of cases (9), we can easily examine them in more detail.

Table 17: Crabbers' Reported Symptoms by Region for Crabbers' With Map Data (N=244)

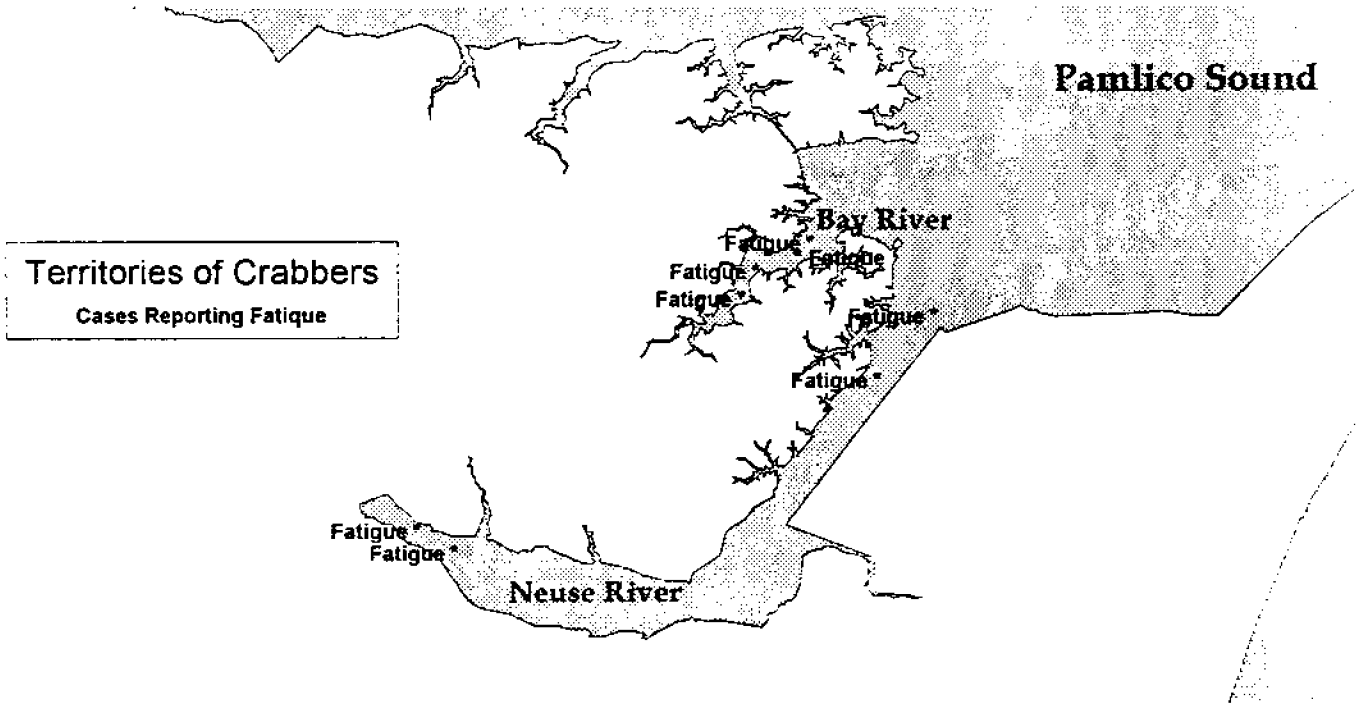
Symptom Reported:	Neuse & Pamlico Regions	All Other Regions
Personality or memory changes	12 (12.5%)	9 (6.1%)
Problems with nerves, etc.	6 (6.3%)	11 (7.4%)
Headaches, seizures, etc.	18 (18.8%)	23 (15.5%)
Respiratory problems	25 (26%)	41 (27.7%)
Dermatological problems	28 (29.2%)	33 (22.3%)
Digestion, stomach problems	8 (8.3%)	14 (9.5%)
Heart, circulation problems	16 (16.7%)	22 (14.9%)
Urination, bladder problems	8 (8.3%)	11 (7.4%)
Felt unwell, fatigue*	9 (9.4%)	5 (3.4%)
Problems with physical activity	7 (7.3%)	7 (4.7%)

*statistically significant (chi-square = 3.872; df=1; p=.049)

Examining the reasons for the fatigue among these nine crabbers, we find that most (4 of the 9) cite "overwork" or simply the work of crabbing, one related it to stress or tension, one to a heart problem, one to the fact that he works another job as well as crabs, and one to diabetes (one of the 9 could not give a reason). This information suggests that causes other than water quality are responsible for these crabbers' cases of fatigue. Further, when we examine their crabbing territories, we find that they are not clustered in fish kill areas but that instead tend to be clustered in the Bay River areas (see Map 3).

In general, then, data on crabbers' territories, combined with data on fish kills, supports our overall conclusion that the waters of Eastern North Carolina are not dangerous to public health under normal ecological conditions, even in areas that have been known to have experienced massive fish kills and high concentrations of *Pfiesteria.* We do note, however, that the ranking data may cause some concern that the two regions known for fish kills are somewhat

Map 3


Territories of Crabbers
Cases Reporting Fatique
Pamlico Sound
Bay River
Fatigue *
Fatigue *
Fatigue *
Fatigue *
Fatigue *
Fatigue *
Fatigue *
Fatigue *
Neuse River

more associated with sick individuals (as the ratios above show) than the other regions.

IV. SUMMARY AND CONCLUSIONS

In the above analysis, we organized the survey data along three lines: 1) a comparative discussion of general demographic and health characteristics of the two crabber populations and the community controls; 2) a comparative discussion of the three populations in terms of the health problems attributed to exposure to *Pfiesteria* and related organisms; and 3) an examination of crabbers' health in terms of exposure to the water and crabbing territories/ fish-kill areas. Combined, the findings from these comparisons do not support the widely held position that the waters of Eastern North Carolina pose serious threats to public health. Quite the contrary: the waters of Eastern North Carolina are, quite simply, not dangerous places to be, despite the fact that high concentrations (e.g. >20,000 cells/ml) of *Pfiesteria* and other organisms that are deadly to fish and other marine organisms are found throughout the estuary, in active forms, primarily through the summer months.

Our findings did include, however, a ranking of different areas of the estuary in terms of the ratios of well crabbers to ill crabbers, and this ranking revealed that the two areas known to host the most fish kills during the summer months, over the past ten years—specifically, the Pamlico and Pungo Rivers and the Neuse River—have worse well to ill crabber ratios (between 5:1 and 6:1) than those areas, such as the Pamlico Sound, that have not experienced as many reported fish kills (whose ratio is 9:1). This is slim evidence that a problem exists, given that comparisons that benefited from appropriate statistical tests found only one symptom that was more likely to be reported by crabbers in these areas. This symptom was fatigue, and close analysis of the specific individuals who reported this found that most were concentrated in an area

not prone to fish kills (Bay River) and most could give explanations for their fatigue that were not related to water quality. Consequently, our overall conclusion—that, outside of fish kills and other disease incidents, the waters of Eastern North Carolina pose no serious threats to public health—remains the primary finding of this analysis.

Additional evidence supporting this conclusion is relatively abundant, even when we only consider the years since *Pfiesteria* was identified. Interviews with physicians revealed that most agree with the conclusion that fishers are prone to skin disorders, either from skin cancer or from cuts and puncture wounds that fishers either treat themselves or fail to treat adequately. Physicians also admitted having no way of identifying the sickness known as *Pfiesteriosis,* despite the handful of individuals who have diagnosed themselves as so afflicted.

That only a few individuals have claimed to have been stricken by *Pfiesteria* is itself fairly telling evidence of the low risk associated with this dinoflagellate. A brief exercise in simple mathematics may help clarify this. Thousands of individuals swim, fish, boat, and otherwise recreate along North Carolina's Rivers and Sounds annually. Yet between 1991 and 1997, no one has died from exposure to *Pfiesteria* (even those individuals exposed to high concentrations in laboratories) and under thirty individuals working in natural settings have come forward with *Pfiesteriosis,* most of them either self-diagnosed or diagnosed by a river keeper, a limnologist, or one of a handful of newspaper, radio, or television journalists. Assuming that these individuals are qualified to diagnose illness in humans, and that there actually are 30 cases of *Pfiesteriosis,* this still means that one's chance of contracting *Pfiesteriosis* while swimming or boating in Eastern North Carolina are quite low. To estimate the actual risk, we would first need to complete a census of all the individuals who had come in contact with the water over the six year period since

Pfiesteria has been identified and divide that by the 30 self-diagnosed cases. Such as census is outside the scope of our project (if even possible), but we can derive a very conservative estimate using vessel license data in the counties around and near the affected Pamlico, Pungo, and Neuse Rivers: Beaufort, Carteret, Craven, Dare, Hyde, Pamlico, and Pitt. The total number of commercial fishing and sportfishing vessel licenses for these counties is 8,912 (North Carolina Division of Marine Fisheries). Assuming that each one of these vessels contained one individual who came into contact with the water at least once each year during the previous six years, this would equal 53,472 exposures (8,912 x 6). Dividing this figure by 30 cases of self-reported *Pfiesteriosis* yields a one in 1,782 chance of contracting *Pfiesteriosis.* This is, of course, an extremely conservative estimate, only including licensed vessels and assuming only one trip per year per vessel and excluding the thousands of vacationers, visitors, and seasonal residents who swim, boat, fish, and otherwise use the waters the Neuse, Pamlico, and Pungo Rivers on an annual basis. If we were to include these individuals, the risks would be far, far lower.

As a final note, we address the issue of low-level cognitive impairment, which has been one of the most often cited symptoms of *Pfiesteriosis.* While we performed no cognitive tests with watermen for the current study, during previous studies we taped several hundred hours of conversations with watermen who have worked on the waters of Eastern North Carolina for their entire lives. These tapes, from which many quotes are included in Griffith's 1996 report to the Moratorium Committee, show North Carolina's watermen to be articulate, witty, thoughtful individuals—hardly the characteristics of a cognitively impaired population. While this is not strict clinical evidence, it does suggest that years on the waters of Eastern North Carolina do not evidently affect one's ability to express oneself in thoughtful and creative ways.

We conclude by reiterating an earlier emphasis: that this report in no way constitutes an overall clean bill of health for the waters of Eastern North Carolina. It is clear to even casual observers, and particularly clear to North Carolina's watermen, that water quality problems persist along the rivers and sounds of the state. This report simply points out that these problems have not yet, apparently, reached a point where they pose severe health risks to human health.

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[1] Unfortunately, actual trends in fish kills and related incidents are difficult to determine with available data, since these are *reported* accounts. Increases in the reporting of fish kills may in fact be simply an artifact of increases in awareness about estuarine health issues or increased state monitoring in response to public outcries from increased awareness, much in the same way neighborhood crime watch programs often result in a statitical increase in crime, due not to actual increases in criminal activity, but increases in the reporting of criminal activity.

[2] *Ebola* is a rainforest virus that kills over 90% of its victims within days of exposure, recently profiled in the popular book, The Hot Zone, by Richard Preston (1995).

[3]See Johnson and Orbach (1996) for information about using licensing data as a sampling frame.

[4] In some cases, the diagnosis was made by a lay person, such as a river keeper, based on the similarity between, say, a sore on a human and a lesion on a menhaden; repeated conversations with ECU dermatologist William Burke revealed that diagnosing skin problems are difficult even for physicians who are not trained in dermatology.

[5] We emphasize the the interviews with physcians and other health providers were based primarily on their *perceptions* of the health of fishers. That is, few of these individuals consulted actual patient records during interviews, although many were well versed in controversies surrounding *Pfiesteria*. In many cases, they were as caught up in the hysteria surrounding the organism's presence as members of the general public, despite having little or not idea what to expect in terms of its health effects and despite having the benefit of a toxin or a diagnostic test to detect exposure to the organism (Burke, personal communication).

[6] Contrary to some reports by the media and by noted scientists speaking throughout the state, North Carolina does collect data on water quality issues, including data on fish kills and on the geographic distribution and density of *Pfiesteria*, which they referred to as *Gymnodinium breve* prior to its being identified by Noga and Burkholder around seven years ago.

APPENDIX A:

RESEARCH INSTRUMENTS

CRABBERS HEALTH STUDY
Contact Log and Disposition Cover Sheet

Name: FIELD(3), FIELD(2) **Phone:** ( ) ____-________

Address: FIELD(4)
FIELD(5), FIELD(6) FIELD(7) **Alternate:** ( ) ____-________

Boat Location: ______________________________

Final Dispostion: (circle one) CI BO RF CNL CO NE Dead Other:__________

Date: ___/___/___ Time: ___:___ am/pm Day: M T W Th F S Su Interviewer: ______	Comments______________________ ______________________ ______________________ Temp. Disposition: BZ NA CB AM W# NS	Call Back: PH Int Date: ___/___/___ Time: ___:___ am/pm
Date: ___/___/___ Time: ___:___ am/pm Day: M T W Th F S Su Interviewer: ______	Comments______________________ ______________________ ______________________ Temp. Disposition: BZ NA CB AM W# NS	Call Back: PH Int Date: ___/___/___ Time: ___:___ am/pm
Date: ___/___/___ Time: ___:___ am/pm Day: M T W Th F S Su Interviewer: ______	Comments______________________ ______________________ ______________________ Temp. Disposition: BZ NA CB AM W# NS	Call Back: PH Int Date: ___/___/___ Time: ___:___ am/pm
Date: ___/___/___ Time: ___:___ am/pm Day: M T W Th F S Su Interviewer: ______	Comments______________________ ______________________ ______________________ Temp. Disposition: BZ NA CB AM W# NS	Call Back: PH Int Date: ___/___/___ Time: ___:___ am/pm
Date: ___/___/___ Time: ___:___ am/pm Day: M T W Th F S Su Interviewer: ______	Comments______________________ ______________________ ______________________ Temp. Disposition: BZ NA CB AM W# NS	Call Back: PH Int Date: ___/___/___ Time: ___:___ am/pm

BZ= Busy NA= No Answer AM= Answering Machine CB=Callback W#=Wrong Number NS= No Show
CI= Completed Interview BO= Breakoff RF= Refusal CNL= Could Not Locate CO= Close out NE= Not Eligible

CONSENT FORM

An Exploratory Study of Potential Human Health Effects of Deteriorating Water Quality Among North Carolina Crabbers

East Carolina University is conducting research on some of the occupational health and safety problems that crabbers experience while working in the waters of Eastern North Carolina. The purpose of this research is to find out what health and safety problems and concerns are unique to crabbers in North Carolina, and how the state's medical facilities may address those problems and concerns. To conduct this research, people who live in the same communities and neighborhoods as crabbers, but who are not crabbers, will be interviewed. I will be asked a few questions about lifestyles that may not seem related to health and safety, but that are necessary to understand the reasons health and safety problems arise. I will be asked potentially sensitive and embarrassing, and specific questions about alcohol and illegal drug use, and whether I have been treated for various sexually transmitted diseases. I will also be asked to answer detailed information about my health. This study is not a health evaluation. The investigators and interviewers are not physicians and they will not give me medical advice regarding my condition or any suspicious symptoms. If I have any questions about my health or are suffering any suspicious symptoms, I should consult with a medical doctor.

For this research, I will spend between on half-hour and two hours answering questions about my work and life in Eastern North Carolina's coastal region. I understand I may be asked to do a follow-up interview which will be more casual and allow me to explain my life and health concerns, and work in more detail. I also may be asked to allow one of the researchers to observe my work as a fisherman/crabber so they may be better able to assess how my working conditions relate to my health.

I am free to ask questions at any time during the interview and I may refuse to answer any questions that I do not wish to answer. My participation in this research is entirely voluntary. I am free to end the interview at any time I so desire without explanation for whatever reason I see as appropriate.

The information that will be collected from me will be kept confidential. As soon as the information o this form is coded an checked for accuracy, the page that includes my name and address will be removed. The information collected from me will be added to information collected from other participants in the study and will presented in aggregate (group) form only; I will not be identified in the results of the study.

I will be compensated for my time with a payment of $10.00. If I have any questions about this research or its results, I may contact either Aaron Schechter or Kristen Borré, Ph.D. at the Department of Family Medicine, East Carolina University, Greenville, NC (919) 816-2587 or David Griffith, Ph.D. at the Institute for Coastal and Marine Resources, East Carolina University, Greenville, NC (919) 328-1748. Also, if questions arise about my rights as a research subject, I may contact the Chairman of the University Policy and Review Committee on Human Research at phone number (919) 816-2914 (days). The policy of East Carolina University does not provide for compensation or medical treatment for subjects because of physical or other injury resulting from this research activity. However, every effort will be made to make the facilities of the School of Medicine available for treatment in the event of such physical injury.

CONSENT TO PARTICIPATE

I certify that I have read all of the above, asked question and received answers concerning areas I did not understand and have received satisfactory answers to these questions. I willingly give my consent for participation in this research study. (A copy of this consent form will be given to the person signing as the subject.)

_____

Respondent Name (Print)

_____

Signature of Respondent Date

Study ID #

FISHERMAN AND HEALTH SURVEY [ID ____]

Interviewer Code _____ __

Todays Date: __/__/__ ______

Time Start: __:__ *Record in Military Time* ____

Time Stop: __:__ *Record in Military Time* ____

DEMOGRAPHICS

1. Could you please tell me your place of birth? _____ ____
2. What is your date of birth? __/__/__ ______
3. What is your current age? __ __ __
4. What is your self-defined ethnicity? To which of these groups do you belong?

   1 Caucasian 2 African-American 3 Hispanic 4 Southeast Asian 5 Other _____ _
5. What is your marital status? 0 Single/Never Married 1 Married 2 Separated/Divorce 3 Widowed 4 Other _____ _
6. Who lives in your household other than you?

	Name	Age	Gender	Education	
	Respondent				____
					____
					____
					____
					____
					____

7. Total number of persons living in the household? _____ __
8. What is your current occupation? _____ __

Study ID #

9.	What is your spouse's current occupation? _____	__
10.	What is your current phone number? (___) ___-____	
11a.	In which of these structures do you live? 1 House 2 Trailer/Mobile Home 3 Apt./TH/Duplex Other _____	_
11b.	Do you currently: 1 Own 2 Rent/Lease 3 Other _____	_
11c.	What do you heat your home with? 1 Electricity 2 Gas 4 Wood 8 Oil 16 Propane 32 Kerosene 64 Other _____	__
11d.	What is your waste disposal system? 1 Septic Tank 2 Sewer 3 Other _____	_
11e.	Describe your water supply? 1 Municipal 2 Well Water 4 Bottled 8 Cistern well 16 Other _____	__
12.	Do you keep any animals where you live? 0 No *(Skip to 14)* 1 Yes 9 Don't Know	_
13.	Do you breed animals? 0 No 1 Yes 8 NA 9 Don't Know	_
14.	How long have you lived at this address? 1 (less than one year) 2 (1 - 3 years) 3 (3 - 5 years) 4 (5 - 10 years) 5 (10 or more years)	_
OCCUPATIONAL HISTORY		
15.	How long have you been a _____(answer to Q8) ___ years	__
16.	What are your job responsibilities and tasks?	
17.	Where do you work? 1 Outside 2 On a boat 4 At home 8 Factory/Plant 16 Office Building 32 Other _____	__
18a.	Do you participate in any seasonal labor? 0 No 1 Yes 9 Don't Know	_

Study ID #

18b.	What kind of seasonal work?	Agriculture/Farming	0 No	1 Yes	8=N.A.	__
		Auto body repair	0 No	1 Yes	8=N.A.	__
		Construction	0 No	1 Yes	8=N.A.	__
		Manual Labor	0 No	1 Yes	8=N.A.	__
		Fishing	0 No	1 Yes	8=N.A.	__
		Painting	0 No	1 Yes	8=N.A.	__
		Cleaning/Janitorial	0 No	1 Yes	8=N.A.	__
		Boat Building	0 No	1 Yes	8=N.A.	__
		Welding	0 No	1 Yes	8=N.A.	__
		Other_____	0 No	1 Yes	8=N.A.	__
		Other	0 No	1 Yes	8=N.A.	__

18c. What type of fishing do you engage in?

SEASON (mon-mon)	SPECIES (Code)	GEAR (Codes)			
			__ __	__ __	__ __ __ __
__ __ -- __ __	99=D.K		__ __	__ __	__ __ __ __
__ __ -- __ __	99=D.K		__ __	__ __	__ __ __ __
__ __ -- __ __	99=D.K		__ __	__ __	__ __ __ __
__ __ -- __ __	99=D.K		__ __	__ __	__ __ __ __
__ __ -- __ __	99=D.K		__ __	__ __	__ __ __ __

19. Has you catch ever been effected by dead water? 0 No 1 Yes 9 Don't Know __

20. Have you ever heard of pfisteria dinoflagellete or "Joann's micro-organisms"? 0 No 1 Yes 9 DK __

21. What protective equipment do you use when out on the water crabbing or fishing?

Sunglasses	0 No	1 Yes	__
Cap/Hat	0 No	1 Yes	__
Rubber Gloves	0 No	1 Yes	__
Trousers	0 No	1 Yes	__
Long Sleeve Shirt	0 No	1 Yes	__
Oil Skins/Bib	0 No	1 Yes	__
Apron	0 No	1 Yes	__
Skirt	0 No	1 Yes	__
Coat	0 No	1 Yes	__
Rubber Boots	0 No	1 Yes	__
Hip Waders	0 No	1 Yes	__
Sunblock/Sunscreen	0 No	1 Yes	__
Other_____	0 No	1 Yes	__

Study ID #

22.	Is your protective gear washed/rinsed daily? 0 No 1 Yes 9 DK	_
23.	Are you out on the water as the weather permits? 0 No 1 Yes 9 DK	_
24.	What is the total number of pots you fish/soak? _____ pots	_ _ _
25.	In good weather, how many pots do you work per day? _____ pots	_ _ _
26a.	Have you ever been injured while working on a crabbing boat or as a fisherman? 0 No 1 Yes 9 DK	_
26b.	What type of injuries have you had? **[We are only interested in fishing-related injuries]** Infected Lacerations /cuts 0 No 1 Yes	_
	Fracture/dislocations 0 No 1 Yes	_
	Muscle/tendon pull/sprain 0 No 1 Yes	_
	Back sprain/herniated disk 0 No 1 Yes	_
	Head injury 0 No 1 Yes	_
	Bruises 0 No 1 Yes	_
	Other 0 No 1 Yes	
27.	What other symptoms or medical conditions do you have which are a result of being a crabber/fisherman?	
28.	What are your current hobbies or recreational activities??	
	Hunting 0 No 1 Yes	_
	Recreational fishing 0 No 1 Yes	_
	Trapping 0 No 1 Yes	
29.	Do you use any chemicals such as bleach on a regular basis? 0 No 1 Yes 9 DK *[Probe: ask about household chemicals like toilet cleaner that they may use to clean their boats, geat, etc.]*	_
30.	Do you build your own boats? 0 No 1 Yes 9 DK	_
31.	Do you repair and maintain your own engines? 0 No 1 Yes 9 DK	_
32.	How many crew members work on your boat including yourself? _____	_ _
33.	How many days/week do you pull your pots? _____	_

Study ID #

MEDICAL HISTORY			
34.	Are you currently taking any prescription medications or medications that the doctor told you to take? 0 No 1 Yes 9 DK		—
	What medications do you take and why do you take them?		
	Medication (Name Dose Frequency)	Reason	

35.	Please tell me about the kinds of over the counter medications(those you bought in the store) that you use regularly? 0 No 1 Yes 9 DK		
	What medications do you take and why do you take them?		
	Medication (Name Dose Frequency)	Reason	

36.	Do you ever use any home remedies such as teas, salves, ointments, tonics or other home preparations? 0 No 1 Yes 9 DK *[Probe: ask about anything they might use for medical purposes that they got from an elderly neighbor or another local person who wasn't a health provider]*	—
	What do you take/do and why do you take/do them?	
	Medication (Name Dose Frequency) / Reason	
37.	Have you ever smoked cigarettes? 0 No 1 Yes 9 DK	—
38.	At what age did you start smoking on a regular basis? _____ 88 NA	— —
39a.	Do you currently smoke? 0 No *(Quitter GOTO 39c)* 1 Yes 8 NA 9 DK	—
39b.	How many cigarettes per day do you smoke? _____ cig/day 88 NA	— —
39c.	At what age did you quit smoking? _____ 88 NA	— —
40.	If you use smokeless tobacco, how much do you use or buy? _____ # of tins /week # of pouches /week	— —
41.	Do you currently smoke cigars or pipes? 0 No 1 Yes Pipes 2 Yes Cigars 3 Both 9 DK	—
42a.	Have you ever used alcohol? 0 No 1 Yes Current drinker 2 Yes Ex-drinker 9 DK	—
42b.	What type of alcohol do you usually drink? 1 Beer 2 Wine 4 Fortified wine 8 Liquor 88 NA 99 DK	— —
42c.	If you currently drink, How many cans/ bottles/glasses (circle one) do you consume per week? _____ 88 NA	— —
43a.	Have you ever had a problem because of alcohol? 0 No 1 Yes 9 DK	—
43b.	Has anyone in your family ever told you that you have a problem with alcohol? 0 No 1 Yes 9 DK	—
44.	Have you ever used recreational drugs? 0 No 1 Yes 9 DK	—

Study ID #

45.	Has anyone in your family ever told you that you have a problem with drugs? 0 No 1 Yes 9 DK	—
46.	Within the last year, have you been told by a nurse or doctor that you have an infectious or contagious disease? 0 No 1 Yes Please describe and give name(s) of disease or conditions	—
47.	Do you have any environmental allergies such as hay fever, animals, food or dust? 0 No 1 Yes 9 DK	—

In responding to the following health questions, we are not interested in symptoms related to alcohol consumption, recreational drug use, or minor sicknesses like colds or flu (unless your frequency of minor illness has gone up dramatically in the past year). Interviewer: ask bold faced first, before asking questions about specific symptoms.

	Conditions	Ever No	Yes	DK	N/A	Can you relate it to a particular cause? No	Yes - Specify	Who have you seen about it? Use code list and record sum	____
1a.	**In the past year, have you had any personality changes or memory changes?**	0	1	9	8	0	1		_ ____ ____
1b.	**In the past year, have you had any problems with nerves, movement, sensation, coordination?**	0	1	9	8	0	1		_ ____ ____
1c.	**In the past year, have you had any problems with headaches, seizures, or visual changes?**	0	1	9	8	0	1		_ ____ ____
48a.	headache	0	1	9	8	0	1		_ ____
48b.	loss of memory	0	1	9	8	0	1		_ ____
48c.	uncoordination	0	1	9	8	0	1		_ ____
48d.	unsteadiness/dizziness	0	1	9	8	0	1		_ ____
48e.	fainting/blacking out	0	1	9	8	0	1		_ ____
48f.	blurred vision/loss of vision	0	1	9	8	0	1		_ ____
48g.	muscle weakness/paralysis	0	1	9	8	0	1		_ ____
48h.	difficulty concentrating	0	1	9	8	0	1		_ ____
48i.	mood swings	0	1	9	8	0	1		_ ____
48j.	tremors/shakiness	0	1	9	8	0	1		_ ____ ____

0= None, 1=Physician, 2=Nurse, 4=Pharmacist, 8=Chiropracter, 16=Alternative Healer/Herbalist, 32=Emergency Room, 64=Physicians Assit/Nurse Practicioner
[Probe: Ask whether or not they consult a neighbor or local person who is considered to have a lot of medical knowledge. If so, code as Alternative Healer]

	Conditions	Ever No	Yes	DK	N/A	Can you relate it to a particular cause? No	Yes	Specify	Who have you seen about it? Use code list and record sum	____
48k.	seizures/epilepsy	0	1	9	8	0	1			____
48l.	rage attacks	0	1	9	8	0	1			____
48m.	depression/crying/hopelessness/loss of pleasure	0	1	9	8	0	1			____
48n.	numbness or tingling in arms or legs	0	1	9	8	0	1			____
48o.	hallucinations	0	1	9	8	0	1			____
48p.	feeling disoriented	0	1	9	8	0	1			____
48q.	unable to remember how to do things	0	1	9	8	0	1			____
48r.	teeth itch	0	1	9	8	0	1			____
11.	**In the past year, have you had problems with breathing, coughing, sinuses, allergies, or other respiratory problems?**	0	1	9	8	0	1			____ ____
48s.	chest pain	0	1	9	8	0	1			____
48t.	shortness of breath	0	1	9	8	0	1			____
48u.	cough ± mucous	0	1	9	8	0	1			____
48v.	cough up blood	0	1	9	8	0	1			____
48w.	**wheezing**	0	1	9	8	0	1			____ ____

0= None, 1=Physician, 2=Nurse, 4=Pharmacist, 8=Chiropracter, 16=Alternative Healer/Herbalist, 32=Emergency Room, 64=Physicians Ass it/Nurse Practicioner

	Conditions	Ever No	Yes	DK	N/A	Can you relate it to a particular cause? No	Yes	Specify	Who have you seen about it? Use code list and record sum	
48x.	out of breath with exertion	0	1	9	8	0	1			
48y.	bronchitis	0	1	9	8	0	1			
48z.	pneumonia	0	1	9	8	0	1			
49a.	allergies/hay fever	0	1	9	8	0	1			
49b.	sinus infection	0	1	9	8	0	1			
49c.	sinus congestion	0	1	9	8	0	1			
49d.	frequent colds	0	1	9	8	0	1			
49e.	asthma	0	1	9	8	0	1			
49f.	eyes burn/itches/waters	0	1	9	8	0	1			
49g.	nose burns/itches/waters	0	1	9	8	0	1			
49h.	throat burns/itches/drains	0	1	9	8	0	1			
49i.	throat infection	0	1	9	8	0	1			
III.	**In the past year, have you had skin problems, rashes, sores, or skin cancer?**	0	1	9	8	0	1			
49j.	skin rashes/eczema	0	1	9	8	0	1			
49z.	sores on skin	0	1	9	8	0	1			

0= None, 1=Physician, 2=Nurse, 4=Pharmacist, 8=Chiropracter, 16=Alternative Healer/Herbalist, 32=Emergency Room, 64=Physicians Assit/Nurse Practicioner

	Conditions	Ever No	Yes	DK	N/A	Can you relate it to a particular cause? No	Yes	Specify	Who have you seen about it? Use code list and record num	
49l	loss of skin pigment	0	1	9	8	0	1			
49m	cuts that don't heal	0	1	9	8	0	1			
49n	fungal infection/ringworm	0	1	9	8	0	1			
49o	nail infection	0	1	9	8	0	1			
49p	mouth ulcers	0	1	9	8	0	1			
49q	hair hurts	0	1	9	8	0	1			
49r	skin cancer/moles or tags	0	1	9	8	0	1			
49s	boils/skin infections/cysts	0	1	9	8	0	1			
IY	**In the past year, have you had stomach or digestion problems, problems eating, or problems with bowels?**	0	1	9	8	0	1			
49t	problems swallowing/food sticks	0	1	9	8	0	1			
49u	heartburn	0	1	9	8	0	1			
49v	indigestion	0	1	9	8	0	1			
49w	nausea	0	1	9	8	0	1			
[redacted]	vomiting	0	1	9	8	0	1			

0= None, 1=Physician, 2=Nurse, 4=Pharmacist, 8=Chiropracter, 16=Alternative Healer/Herbalist, 32=Emergency Room, 64=Physicians Assit/Nurse Practicioner

Study ID #

	Conditions	Ever No Yes DK N/A	Can you relate it to a particular cause? No Yes Specify	Who have you seen about it? Use code list and record sum	———
49y	weight loss	0 1 9 8	0 1		— ———
49z	abdominal pain/cramping	0 1 9 8	0 1		— ———
50a	blood in bowel movement	0 1 9 8	0 1		— ———
50b	diarrhea (more than one episode on one day)	0 1 9 8	0 1		— ———
50c	ulcers	0 1 9 8	0 1		— ———
50d	gall bladder disease	0 1 9 8	0 1		— ———
V	**In the past year, have you had heart/circulation problems? High blood pressure?**	0 1 9 8	0 1		— ——— ———
50e	chest pain	0 1 9 8	0 1		— ———
50f	pressure in chest	0 1 9 8	0 1		— ———
50g	irregular heartbeat/palpitation	0 1 9 8	0 1		— ———
50h	heart attack	0 1 9 8	0 1		— ———
50i	bypass surgery for heart or legs	0 1 9 8	0 1		— ———
50j	stroke	0 1 9 8	0 1		— ———
50k	bad circulation	0 1 9 8	0 1		— ———
50	**varicose veins**	0 1 9 8	0 1		— ——— ———

0= None, 1=**Physician**, 2=Nurse, 4=Pharmacist, 8=Chiropracter, 16=Alternative Healer/Herbalist, 32=Emergency Room, 64=Physicians Assit/Nurse Practicioner

	Conditions	Ever No	Yes	DK	N/A	Can you relate it to a particular cause? No	Yes	Specify	Who have you seen about it? Use code list and record sum	____
50m	rheumatic fever	0	1	9	8	0	1			__ ____
51n	failing spells	0	1	9	8	0	1			__ ____
VI.	**In the past year, have you had any urination, bladder, or prostate problems?**	0	1	9	8	0	1			__ ____ ____
50o	prostate problems	0	1	9	8	0	1			__ ____
50p	loss of bowel or bladder control	0	1	9	8	0	1			__ ____
50q	difficulty starting urination	0	1	9	8	0	1			__ ____
50r	urinating frequently	0	1	9	8	0	1			__ ____
VII.	**In the past year, have you had problems with physical activities?**	0	1	9	8	0	1			__ ____
50s	unable to climb one flight of stairs without stopping to rest	0	1	9	8	0	1			__ ____
50t	unable to walk one block on level surface	0	1	9	8	0	1			__ ____
50u	difficulty walking	0	1	9	8	0	1			__ ____
50v	arthritis	0	1	9	8	0	1			__ ____
50w	bursitis	0	1	9	8	0	1			__ ____ ____

0= None, 1=Physician, 2=Nurse, 4=Pharmacist, 8=Chiropracter, 16=Alternative Healer/Herbalist, 32=Emergency Room, 64=Physicians Assit/Nurse Practicioner

Study ID #

	Conditions	Ever No Yes DK N/A	Can you relate it to a particular cause? No Yes Specify	Who have you seen about it? Use code list and record num	
50	given up activities you used to do	0 1 9 8	0 1		
50	muscle aches/pains	0 1 9 8	0 1		
VIII	**In the past year, have you felt unwell, fatigued, or had unexplained fever?**	0 1 9 8	0 1		
50	given up activities you used to do?	0 1 9 8	0 1		
51	fatigue	0 1 9 8	0 1		
51	fevers	0 1 9 8	0 1		

0= None, 1=Physician, 2=Nurse, 4=Pharmacist, 8=Chiropracter, 16=Alternative Healer/Herbalist, 32=Emergency Room, 64=Physicians Assit/Nurse Practicioner

51c. Do you have diabetes (high sugar)? 0 No 1 Yes

51d. Have you ever been diagnosed with cancer? 0 No 1 Yes

51e. If yes, what type? _____

HEALTH CARE UTILIZATION

52	Do you have a regular source of medical care? 0 No 1 Yes 9 DK	
53	Where did you go the last time you had a medical problem? 1 Private Doctor 2 Health Department 3 Local Clinic 4 Emergency Department 5 Other	
54	Are you covered by health insurance? 0 No 1 Yes 9 DK	
	What type do you have? 88 NA	
	Have you seen a physician in the past year? 0 No 1 Yes 9 DK	
56	**Have you ever** been hospitalized? 0 No 1 Yes 9 DK 88 NA	
	If yes, Why?	
	1st hospital visit _____	
	2nd hospital visit _____	
	3rd hospital visit _____	

Study ID #

57.	Have you been to the emergency department in the last two years? 0 No 1 Yes 9 DK 88 NA If yes, Why? 1st emergency visit _____ 2nd emergency visit _____ 3rd emergency visit _____	___ ___ ___ ___
58.	When was your last complete physical or checkup (done to check on health not to treat illness)? _____ mon/year 0 None 1 prostate 1 pap (women) 2 cholesterol 4 blood pressure 8 NA	__/___ ___

Daily routine: Please describe, briefly, your daily routine:

Do you eat your catch? 0 No 1 Yes ___

Where do you crab?

Have you ever experienced:

A Fish Kill?	Y/N	___
Red or Brown Tide?	Y/N	___
An Algae bloom?	Y/N	___

□□□□

Study ID #

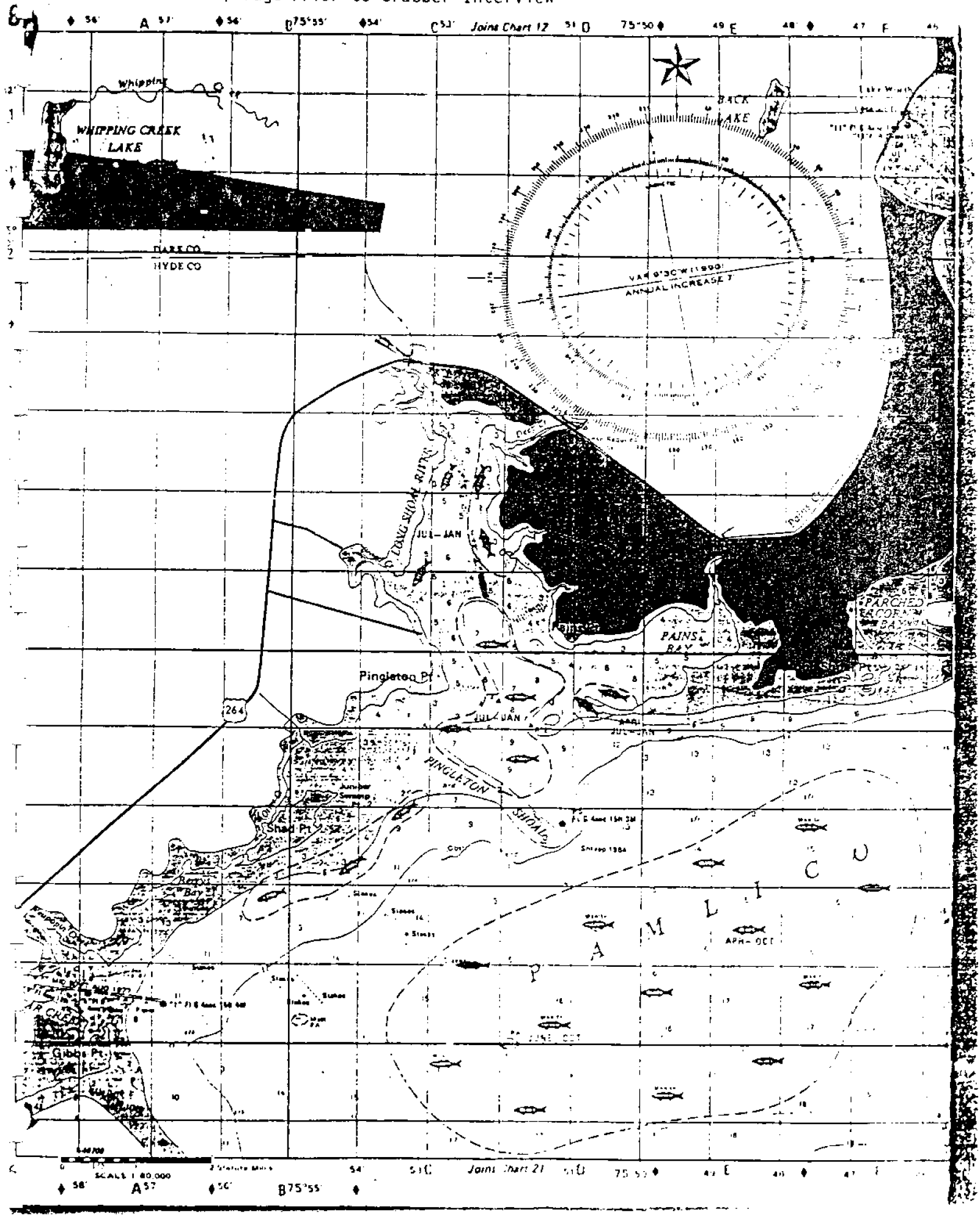
Crabbing Location Data Collection

Could you please show me where you usually set your pots? You can identify up to 5 different locations if you crab in different locations during different times of the year.

Location	Description	Map # (eg. 1A)	Longitude (East West)	Latitude (North South)	Time Month- Mouth	% of season you crab at this location
1			___ ° ___ ‘ ___ ° ___ ‘	___ ° ___ ‘ ___ ° ___ ‘	___-___	
2			___ ° ___ ‘ ___ ° ___ ‘	___ ° ___ ‘ ___ ° ___ ‘	___-___	
3			___ ° ___ ‘ ___ ° ___ ‘	___ ° ___ ‘ ___ ° ___ ‘	___-___	
4			___ ° ___ ‘ ___ ° ___ ‘	___ ° ___ ‘ ___ ° ___ ‘	___-___	
5			___ ° ___ ‘ ___ ° ___ ‘	___ ° ___ ‘ ___ ° ___ ‘	___-___	

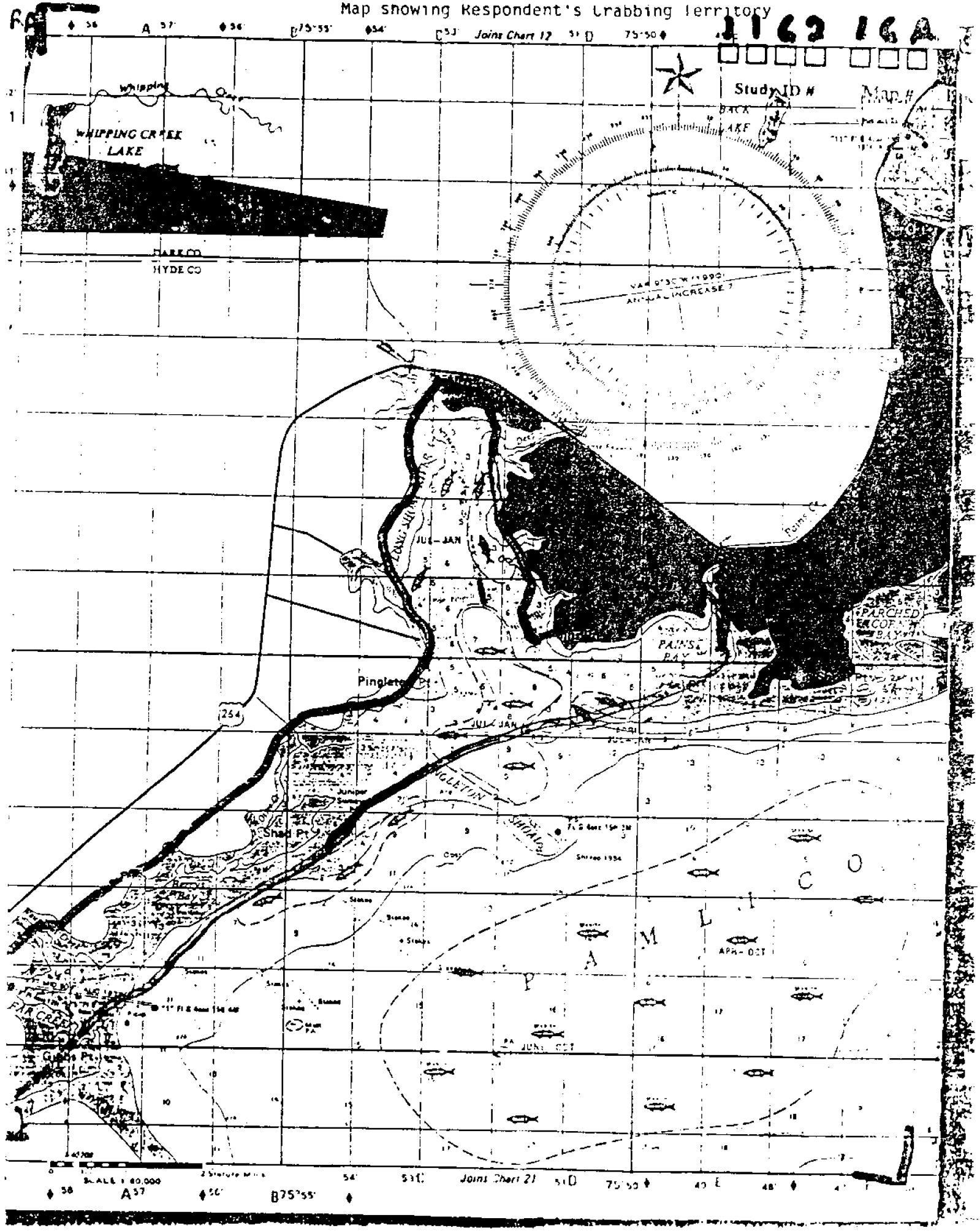
Place a transparency over the appropriate Map and Shade in the area identified by the Subject. Before removing the overlay record the **Subject ID #**, the location #, and the map number on the transparency. Also draw an outline of all four corners of the map onto the **overlay.**

Map Page Prior to Crabber Interview



Map showing Respondent's Crabbing Territory

2162 16A



COMMUNITY CONTROLS

Can you recommend three individuals in your community—similar to you in age, ethnic background, and so forth—**three individuals who are not crabbers**, who might agree to be interviewed?

We will be putting all the names that crabbers give us onto a list and only selecting a random sample of them, so we probably won't contact all three individuals. We would like three names, however, to have a large enough list to select a sample from. Those we interview, of course, will also be paid $10.00.

Name # 1: _____

Address: _____

Phone _____
_____

Name # 1: _____

Address: _____

Phone: _____
_____

Name # 1: _____

Address: _____

Phone: _____
_____

APPENDIX B

Fish Kill Data

	Month/Year	Duration (Days)	Source	Place
1.	7/94	1	NCDWC	Pungo/Haystack Point (14B)
2.	7/94	1	NCDWC	Pamlico River/Gum Point (19A)
3.	7/94	2	NCDWC	Neuse River/Flanners Beach (23B)
4.	7/94	1	NCDWC	Pamlico River/Blounts Bay (18B)
5.	8/94	1	NCDWC	Chocowinity Bay (18B)
6.	9/94	1	NCDWC	Crawford Creek
7.	9/94	1	NCDWC	Silas Creek
8.	7/95	90	Tester	Neuse River Estuary (23-24)
9.	7/95	90	Tester	Pamlico River Estuary (18-19)
10.	6/93	30	Tester	Bogue Sound (32-33)
11.	6/93	nd	Burkholder	Neuse: Minnesot Beach (29A)
12.	7/93	nd	Burkholder	Neuse: Flanners Beach (23B)
13.	7/93	nd	Burkholder	Flanners →Cherry Pt. (23B-28B)
14.	7/93	nd	Burkholder	South Creek (19A.B)
15.	9/93	nd	Burkholder	Neuse: Coffee Creek (23B-28B)
16.	8/93	nd	DEHNR	Broad Creek (23B)/Upper Broad
17.	9/93	nd	DEHNR	Coffee Creek
18.	2/94	nd	DEHNR	Neuse: Oriental (24A)
19.	7/94	nd	DEHNR	Neuse: Goose Creek (28B)
20.	7/95	nd	DEHNR	Neuse-1 (23B) 28B
21.	9/95	nd	DEHNR	Neuse-1 (23B)
22.	9/27/95-10/25/95		DEHNR	35 recorded incidents from Trent River to Broad Creek (23B→29A→24B) Camp Don Lee
23.	7/93		DEHNR	
24.	7/15/93		DEHNR	Pamlico River at TG-1
25.	2/01/94		DEHNR	Pamlico River (19B)
26.	7/05/94		DEHNR	Pungo River A1
27.	7/06/94		DEHNR	Pamlico River (19A)
28.	7/19/94		DEHNR	Pamlico River (18B)
29.	7/19/94		DEHNR	Pamlico River (19B)
30.	8/31/97		DEHNR	Chocowinity Bay (18B)
31.	9/14/94		DEHNR	Crawford Creek (18B)
32.	9/19/94		DEHNR	Silas Creek (18B)
33.	7/14/95		DEHNR	Pungo River, Jarvis Pier
34.	7/27/95		DEHNR	Pamlico River, Pam. Ferry-C
35.	7/27/95		DEHNR	Pamlico River, Durham Cr. (18B)
36.	7/27/95		DEHNR	Pamlico River, Barge Basin-B

37.	6/21/94		DEHNR	New River (31B)
37.	8/17/93		DEHNR	Neuse River (23B)
39.	9/15/93		DEHNR	Coffee Creek (Trib B)
40.	2/16/94		DEHNR	Neuse River (29B)
41.	7/12/94		DEHNR	Neuse River NEU131X
42.	7/27/95		DEHNR	Neuse River NEU-1
43.	9/20/95		DEHNR	Neuse River, Neuse-1
44.	10/19/95		DEHNR	Neuse River, M2
45.	10/19/95		DEHNR	Neuse River, M3
46.	10/19/95		DEHNR	Neuse River, M4
47.	10/19/95		DEHNR	Neuse River, M5
48.	10/23/95		DEHNR	Neuse River, Don Lee-1 (24B)
49.	10/23/95		DEHNR	Neuse River, Cherry Br-2 (29A)
50.	10/23/95		DEHNR	Neuse River, Neuse-3
51.	10/23/95		DEHNR	Neuse River, Neuse-5
52.	10/23/95		DEHNR	Neuse River (29A)
53.	10/25/95		DEHNR	Neuse River (28B)
54.	10/25/95		DEHNR	Neuse River (24A)
55.	10/30/95		DEHNR	Neuse River, N4
56.	10/30/95		DEHNR	Neuse River, N5
57.	10/30/95		DEHNR	Neuse River, N6
58.	10/30/95		DEHNR	Neuse River, N7
59.	10/30/95		DEHNR	Neuse River, N8
60.	10/30/95		DEHNR	Neuse River, N9
61.	10/30/95		DEHNR	Neuse River, N10
62.	10/30/95		DEHNR	Neuse River, Don Lee-5 (24B)

Neurobehavioral Effects of Dinoflagellate Toxins in Rats

Final Report of the Project

October 6, 1997

Edward D. Levin

Neurobehavioral Effects of Dinoflagellate Toxins in Rats

Edward D. Levin, Ph.D.

Duke University

Principal Investigator

North Carolina Sea Grant Final Report

October 6, 1997

Results of the Sea Grant College Funded Research

Abstracts

Levin ED, DE Schmechel, HB Glasgow, Jr., JM Burkholder and NM Deamer-Melia. *Pfiesteria piscicida* effects on cognitive performance in rats. Southeastern Estuarine Research Society, Atlantic Beach, NC, Nov. 7, 1996.

Levin ED, DE Schmechel, HB Glasgow Jr and JM Burkholder Cognitive effects seen in rats exposed to the dinoflagellate *pfiesteria*. Society of Toxicology Annual Meeting, Cinncinati, OH, March 9-13, 1997, The Toxicologist, 36:62, 1997.

Levin ED, DE Schmechel, HB Glasgow Jr, NJ Deamer-Melia, JM Burkholder, VC Moser, and GJ Harry. *Pfiesteria piscicida* exposure in rats results in persisting learning deficits. NIEHS Workshop on Hazardous Marine/Freshwater Microbes and Toxins. Research Triangle Park, NC, August 26-27, 1997

Levin ED, DE Schmechel, HB Glasgow, Jr., NM Deamer-Melia and JM Burkholder. *Pfiesteria* toxin. In: Experimental and Clinical Neurotoxicology (2nd Edition). P.S. Spencer, H.S. Shaumberg and A.C. Ludolph (eds.), Oxford University Press, New York, 1997.

Paper

Levin ED, DE Schmechel, JM Burkholder, HB Glasgow, Jr., N Deamer-Melia, VC Moser and G.J. Harry. Persisting learning deficits in rats after exposure to *pfiesteria piscicida*. Environmental Health Perspectives, in press, 1997.

ABSTRACT

The dinoflagellate, *Pfiesteria piscicida,* is implicated as a cause of fish kills in North Carolina estuaries and elsewhere. Accidental exposure of humans to *Pfiesteria* has been reported to result in a complex syndrome including cognitive deficits. This series of studies was conducted to experimentally assess possible cognitive effects of *Pfiesteria* exposure in rats. Samples of water from aquaria in which *Pfiesteria* were killing fish were frozen. Then, thawed samples were injected into female Sprague-Dawley rats. Significant learning impairments were documented in rats administered recently frozen samples of *Pfiesteria.* No effect was seen in the recall of a previously learned task, but when the rats were called upon to learn a new task, the Pfiesteria-treated animals showed a significant learning deficit. This effect persisted up to at least 10 weeks after one injection. The *Pfiesteria*-induced learning deficit was not associated with generalized debilitation or health impairment. Deficits in habituation of arousal and rearing behavior were seen using a functional observational battery (FOB). No *Pfiesteria*-induced effects on blood count or in a standard pathological screening of the brain, liver, lungs, kidneys and spleen were seen. These studies document a persistent learning impairment in rats after exposure to *Pfiesteria.* This effect may be analogous to the cognitive deficits humans have shown after *Pfiesteria* exposure.

INTRODUCTION

Pfiesteria piscicida, (Steidinger and Burkholder), is a toxic dinoflagellate which inhabits estuarine waters of the eastern United States. It was first discovered swarming in a major fish kill in May 1991 *(3). Pfiesteria piscicida* has a complex life cycle with at least 24 distinct life stages *(4).* The small flagellated vegetative form is associated with the most lethal effects *(3). Pfiesteria* has been implicated as a major cause of recent massive fish kills in North Carolina estuaries *(5).* Exposed fish appear to be narcotized and show poor fright response*(6).* The chemical identity of the toxin(s) in *Pfiesteria piscicida* has not been determined. Preliminary *in vitro* studies have shown neurons to be more sensitive than endothelial cells to toxic damage as measured by lactate dehydrogenase leakage and lowered ATP levels *(7).* Adverse health effects have been reported after accidental laboratory exposure *(8)* including cognitive disturbance, fatigue, mood lability and dermal lesions. These experimental studies was to determine whether *Pfiesteria* would cause cognitive deficits in a rodent model.

The radial-arm maze, chosen to assess spatial learning and memory after *Pfiesteria* exposure, is sensitive to the adverse effects of a variety of neural lesions, drug treatments and toxicant exposures*(9-13).* The Functional Observational Battery (FOB) was used as a broad screen used to identify potential neurobehavioral effects *(14-16).* Tissues taken from exposed rats were evaluated for associated pathology. The goal of these studies was to document in an animal model the effects of *Pfiesteria* exposure on cognitive processes and overall health. The goal of these experimental neurobehavioral studies with *Pfiesteria* would help lay the groundwork for future studies to identify the critical toxin or toxins produced by *Pfiesteria*..

METHODS

Subjects: Adult female Sprague-Dawley rats were housed in groups of 2-4. In Study 1, the rats had *ad lib* access to food. In the other studies they were fed daily after testing to keep their weights at 80-85% of free-feeding levels.

Radial-Arm Maze Training: Testing was conducted on a radial 8-arm maze with a central arena 50 cm in diameter and eight 10 x 60 cm arms extending radially. The maze was 30 cm above the floor in a testing room which contained many extra-maze visual cues. Before each session, all the arms of the maze were baited with a piece of sugared cereal. The accuracy measure was the number of entries before an error (entries to repeat). The latency measure was the session duration divided by the number of arms entered (seconds per entry). For repeated acquisition testing, three of the arms of the 8-arm radial maze were baited before each trial. Five trials were run each session. The same arms were baited for all of the trials

of a session, but the arms baited were changed between sessions. Total errors to select the three baited arms were counted for each trial.

The Functional Observational Battery (FOB): The FOB is a series of observations and tests used to evaluate the sensorimotor integrity of the rat. Abnormal motor movements, activity level, lacrimation, salivation, piloerection and handling reactivity were. The rat was placed on the top of a table and allowed to freely explore for three minutes. Gait abnormalities, arousal, activity level, rearing, abnormal motor movements and excretion level (urination, defecation) were noted. The rat's reactions to the sound of a metal clicker, a pinch near the end of the tail, approach of a pen, and touch on the rump were rated, and the aerial righting reflex and pupillary response to light were tested. Finally grip strength, landing foot splay, rectal temperature and weight were measured.

Pathology: Automated complete blood counts white cell differential counts were made of samples from control and exposed rats. The brain, liver, lungs, kidney and spleen were excised and placed in 10% formalin. Tissue cellularity was visualized with Hematoxylin and Eosin (H&E). In brain, astrocytes were identified by immunohistochemistry using polyclonal anti-GFAP antibodies.

Pfiesteria samples were collected directly from aquaria at the North Carolina State University laboratory of Dr. Burkholder in which *Pfiesteria piscicida* cultures were actively killing fish. The aquarium water was injected with no additives into sealed glass test tubes. These tubes were frozen at -80° for at least one hour. In all the studies the tubes holding the samples were warmed at room temperature until no ice crystals remained before injection.

Study 1 (Pilot Study) was an initial pilot evaluation of the acute and persisting behavioral effects of *Pfiesteria* exposure. Six rats were administered subcutaneously (S.C.) *Pfiesteria* samples which had contained a range of 35,600 to 961,200 *Pfiesteria* cells per kg of rat body weight. The *Pfiesteria* samples for were kept frozen (-4° C) between 23-43 days as the rats were administered *Pfiesteria* on a staggered schedule. Beginning two days after exposure, the rats were tested in the win-shift radial-arm maze task for 18 sessions over six weeks.

Study 2 (Repeat Study) was a more focused evaluation of the *Pfiesteria* samples which had contained 106,800 cells/kg using the sample employed in Study 1. This sample of *Pfiesteria* had been stored sealed and frozen at -4° C for 7 weeks before use in this study. Ten rats were injected with *Pfiesteria* and ten were injected with saline (S.C.). Win-shift radial-arm maze training began two days after administration.

Study 3 (Fresh Sample Study) evaluated the effects of a fresh sample of *Pfiesteria* collected. The sample was only frozen at -4° C overnight and thawed only once, just before injection. Ten rats were injected with *Pfiesteria* samples which had contained 106,800 cells of *Pfiesteria./*kg of rat body weight compared to ten controls injected with control aquarium water collected by the same method except that the tanks did not contain *Pfiesteria*. The rats began training in the win-shift radial-arm maze task two days after *Pfiesteria* exposure. After behavioral testing the rats were sacrificed and the brain, lungs, liver, kidneys and spleen were collected for pathological assessment.

Study 4 (Pretraining Study) determined if the deficits seen in radial-arm maze performance in the previous studies were due to impairments in learning or memory. Rats were pretrained for 18 sessions on a radial-arm maze win-shift task before *Pfiesteria* administration. Then they were administered *Pfiesteria* samples which had contained 0, 35,600 or 106,800 cells/kg. As in Study 3, a fresh sample of *Pfiesteria* collected which had been frozen at -4° C only overnight was used and the control dose was aquarium water without *Pfiesteria*. Two days after exposure, testing on the radial-arm maze win-shift task resumed. The rats were tested for the following 6 weeks for 18 sessions. The rats were then tested for six sessions over four weeks using a repeated acquisition task in the same 8-arm radial maze. The rats in Study 4 were assessed at timepoints of one hour, one week, four weeks and nine weeks post-exposure using a standardized Functional Observational Battery (FOB).

RESULTS

Study 1, Pilot Study: The *Pfiesteria*-treated rats had significantly lower average entries to repeat scores ($p<0.005$) than controls averaged over 18 sessions of testing. The controls averaged 5.5±0.2 entries to repeat, while the *Pfiesteria*-treated rats averaged 4.8±0.1 entries to repeat. Latency was not significantly affected by *Pfiesteria* exposure.

Study 2, Repeat Study: In this study ten rats were injected with the *Pfiesteria* solution used in Study 1. There was no significant effect of *Pfiesteria* exposure in this study using a Pfiesteria solution that had been stored for seven weeks. Over 18 sessions of training the controls averaged 6.1±0.3 entries to repeat while the *Pfiesteria*-exposed rats averaged a slightly lower 5.7±0.3 entries to repeat.

Study 3, Fresh Solution Study: A fresh solution of *Pfiesteria* was used in Study 3. There was a significant effect of *Pfiesteria* exposure impairing choice accuracy in the radial-arm maze (Fig. 1). The main effect of *Pfiesteria* exposure was significant ($p<0.025$), with the controls averaging 6.2±0.2 entries to repeat and the *Pfiesteria*-exposed rats averaging 5.4±0.2 entries to repeat over the 24 sessions of testing. There was a significant session block x *Pfiesteria* interaction ($p<0.025$). Analyses of the simple

main effects of *Pfiesteria* at each session block showed significant *Pfiesteria* induced deficits during session blocks 10-12 ($p<0.05$), 13-15 ($p<0.005$) and 16-18 ($p<0.005$). The *Pfiesteria*-treated rats showed some improvement during the extended phase of training. No significant effects of *Pfiesteria* exposure were seen in terms of response latency. When analyzed together Studies 1, 2 and 3 showed a significant Pfiesteria-induced deficit in radial-arm maze choice accuracy during the first 18 sessions of training ($p<0.005$) with 26 *Pfiesteria*-treated rats and 26 controls.

No significant effects of *Pfiesteria* exposure were seen in the complete blood count assessment and white blood cell differential counts (Table 1). Gross and microscopic examination of H & E stained sections revealed no clearly observable lesions or signs of pathology. GFAP (glial fibrillary acidic protein) immunoreactivity was not increased in the brains of *Pfiesteria*-exposed animals.

Study 4, Pretraining Study: To differentiate the effects of *Pfiesteria* on learning and memory, rats were pretrained on the radial-arm maze win-shift procedure used in the previous studies for 18 sessions prior to *Pfiesteria* administration. Beginning two days after dosing, the rats were tested for maintenance of working memory choice accuracy for an additional 18 sessions. *Pfiesteria* exposure did not significantly impair neurobehavioral function required for maintaining accurate performance of the radial-arm maze task after the rats were pretrained. There was a significant ($p<0.05$)*Pfiesteria* effect reducing response latency in the higher dose Pfiesteria group (Controls=25.9±3.3, lower dose *Pfiesteria*=24.2±2.9 and higher dose *Pfiesteria*=16.4±1.3 seconds per entry.

To assess the effects on learning, the rats were switched to the repeated acquisition procedure in the radial-arm maze. As shown in figure 2 there was a significant deficit in learning by rats given the higher *Pfiesteria* ($p<0.05$). This learning deficit was seen approximately 10 weeks after *Pfiesteria* exposure. There were significant *Pfiesteria* effects on response latency in both Session Block 1-3 ($p<0.025$) and Session Block 4-6 ($p<0.01$). Dunnett's comparisons showed significant differences between controls and the high dose group during both session block 1-3 ($p<0.05$) and session block 4-6 ($p<0.01$), and a significant difference between controls and the low dose group only during session block 4-6 ($p<0.05$) (Table 2).

The functional observational battery was also sensitive to the effects of *Pfiesteria* exposure. The one week and four week FOB timepoints corresponded to the periods of win-shift radial-arm maze retesting. The nine week FOB time point corresponded to the period of repeated acquisition radial-arm maze testing. Across repeated testing sessions there was a significant habituation seen in the controls ($p<0.005$) with regard to the arousal and rearing measures (Fig. 3). Animals receiving the high dose of *Pfiesteria* showed significantly less habituation than the control group. The linear trends analysis

across test sessions showed a significant *Pfiesteria*-induced difference in arousal and rearing ($p<0.05$). Post-hoc Dunnett's tests showed significant differences between controls and the high dose but not the low dose group for both arousal and rearing ($p<0.05$). There were no observed differences with the other measures of sensorimotor function, no abnormal motor movements, and no changes in physiological parameters (e.g., body temperature). Increased body tone approached statistical significance, with an overall main effect of dose of $p<0.06$. Collapsed across time, the analyses showed a trend ($p<0.10$) towards increased tone in both treatment groups. At all time points except the last, there were 2-4 more rats in either group showing an apparent increased tone. Defecation also showed a significant dose effect ($p<0.05$), due to slightly lower defecation in the high-dose group which was most apparent at the 9-week test.

CONCLUSIONS

- σ *Pfiesteria* impaired learning as assessed by both the win-shift and repeated acquisition tasks on the radial-arm maze.

- σ A significant learning deficit was seen 10 weeks after acute *Pfiesteria* exposure.

- σ No deficit was seen in radial-arm maze performance when the rats were pretrained prior to exposure, demonstrating that *Pfiesteria*-treated rats were able to perform the task when no new learning was required.

- σ The learning deficit was attenuated when the Pfiesteria samples were kept frozen for two months prior to administration.

- σ No overt health impairments or gross behavioral dysfunction was seen during the period immediately after injection or for the rest of the course of the study which extended for up to 10 weeks after Pfiesteria exposure.

- σ The Functional Observational Battery (FOB) testing showed little overt behavioral change in the Pfiesteria-treated rats except for impairments of habituation which is a simple form of learning.

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Table 1

Blood Analysis for Study 3 (mean±sem)

	Control	*Pfiesteria* (106,800 cells/kg)
White Blood Cells	2.45±0.35 x 10^3/mm^3	2.64±0.17 x 10^3/mm^3
Red Blood Cells	6.65±0.14 x 10^6/mm^3	6.67±0.18 x 10^6/mm^3
Hemoglobin	13.5±0.3 g/dL	13.3±0.3 g/dL
Hematocrit	36.5±0.9 %	36.1±1.0 %
Mean Corpuscular Volume	54.9±0.4 μ^3	54.2±0.3 μ^3
Mean Corpuscular Hemoglobin	20.2±0.2 %	20.0±0.2 %
Platelets	815±28 x 10^3/mm^3	878±38 x 10^3/mm^3
Differential White Blood Cell Count		
Segmented Neutrophils	11.9±1.4 %	11.3±2.3 %
Banded Neutrophils	0.5±1.4 %	0.0±0.0 %
Lymphocytes	86.3±1.5 %	85.7±3.4 %
Monocytes	0.9±0.3 %	2.2±1.2 %
Eosinophils	0.4±0.4 %	0.8±.04 %

Table 2

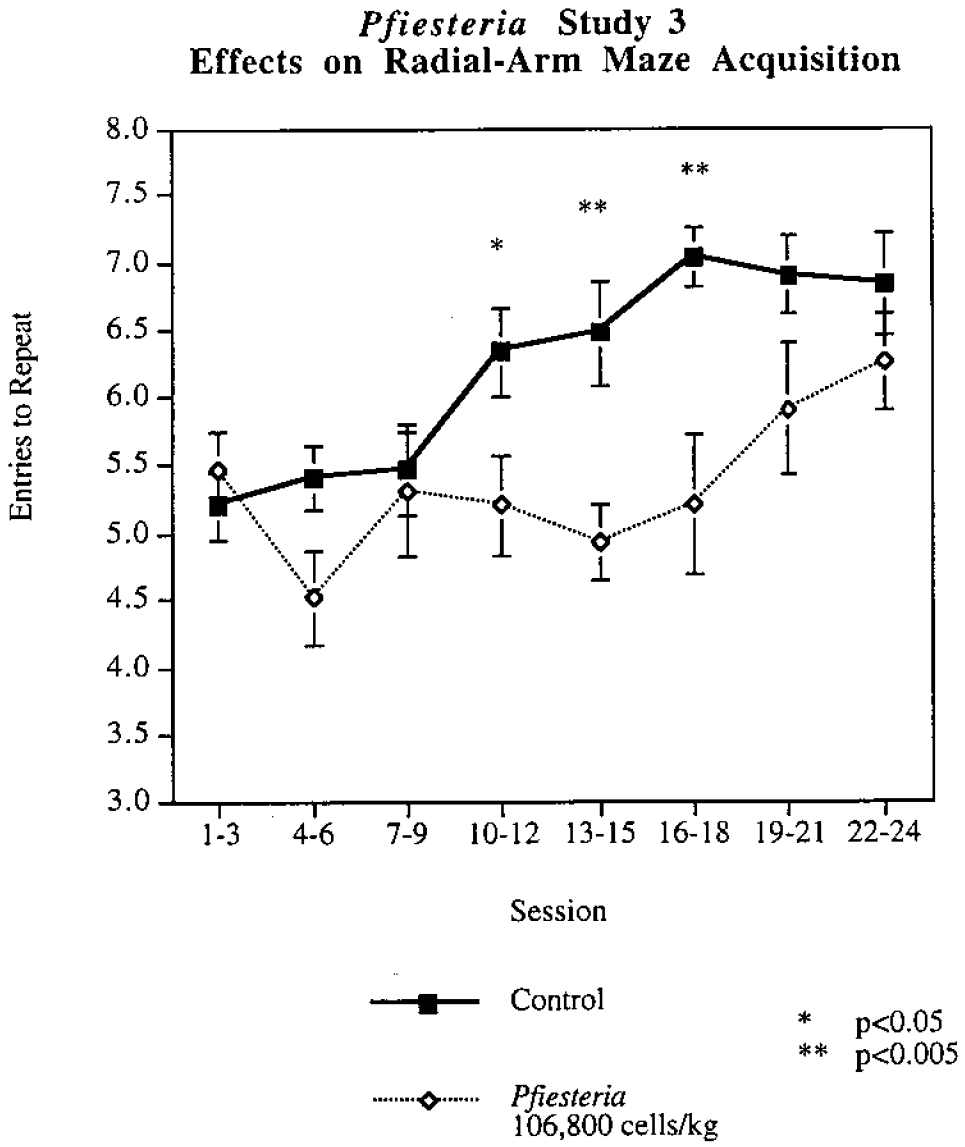
Response Latency for Repeated Acquisition in Study 4 (mean±sem)

Pfiesteria Dose (cells/kg)	Session Block 1-3	Session Block 4-6
0	53.2±9.6 sec./entry	53.4±11.1 sec./entry
35,600	33.0±7.2 sec./entry	25.2±5.3 sec./entry *
106,800	23.4±4.0 sec./entry *	19.1±3.4 sec./entry **

* $p<0.05$ vs. control

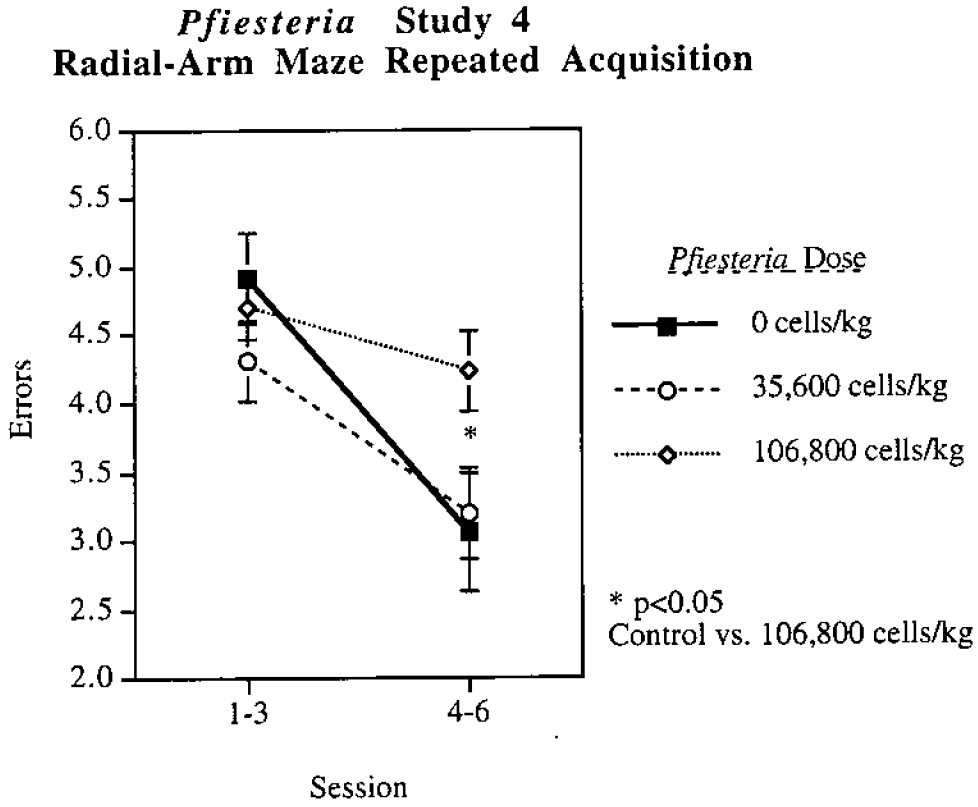
** $p<0.01$ vs. control

Figure 1



Study 3, Acquisition training in the win-shift radial-arm maze task (mean±sem), *Pfiesteria* main effect $p<0.05$

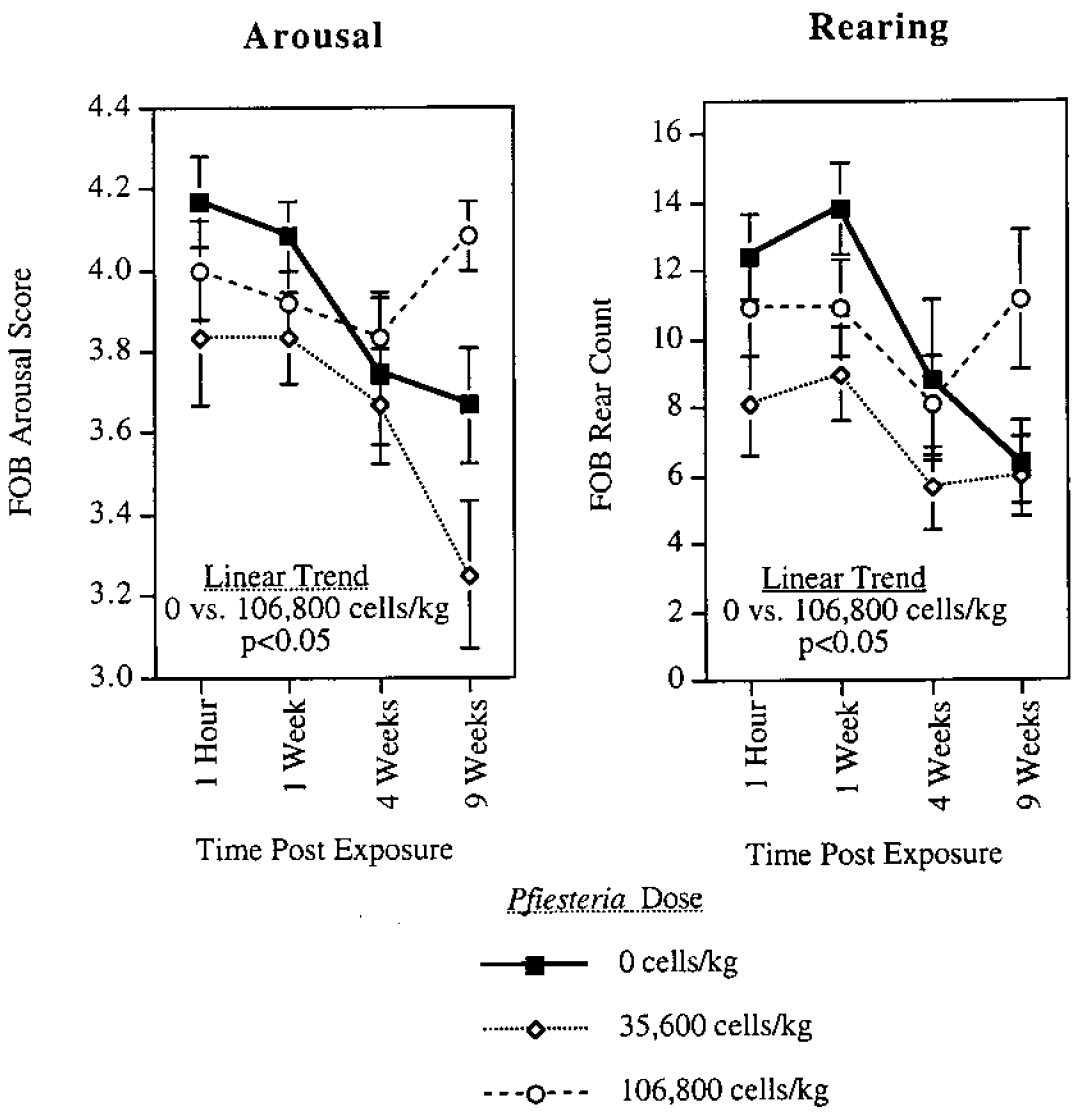
Figure 2



Study 4, repeated acquisition training on the radial-arm maze (N=12 per group), *p<0.05 Dunnett's test Control vs the group administered *Pfiesteria* samples which had contained 106,800 cells/kg

Figure 3

Pfiesteria Study 4
Functional Observational Battery



Study 4, Functional Observational Battery, arousal and rearing measures, linear trend over sessions *Pfiesteria* effect, $p<0.05$, Dunnett's test Control vs. the group administered *Pfiesteria* samples which had contained 106,800 cells/kg, $p<0.05$ for both arousal and rearing.

Southeastern Estuarine Research Society, Nov. 7, 1996.

PFIESTERIA PISCICIDA EFFECTS ON COGNITIVE PERFORMANCE IN RATS. E D Levin[1], D E Schmechel[2,3], H B Glasgow, Jr.[4] J M Burkholder[4] and N M. Deamer-Melia[4]. Depts of Psychiatry[1] and Medicine[2], Duke University Medical Center, Durham, NC., Durham VA Medical Center[3] and Dept of Botany[4], North Carolina State University, Raleigh, NC

The dinoflagellate Pfiesteria piscicida is suspected as a cause of fish kills in North Carolina estuaries. Accidental exposure of humans to Pfiesteria was found to result in a complex syndrome including cognitive impairment. The current project was conducted to assess the persisting cognitive effects of Pfiesteria exposure in rats. Adult female Sprague-Dawley rats (N=26) were injected (SC) with doses of ranging from 35,600 to 961,200 Pfiesteria cells per kg of rat body weight. Observations for six hours after acute injection documented no overt behavioral disturbances. Starting two days after injection, the rats began testing on the radial-arm maze, a test of spatial memory. They underwent 18 sessions of testing over the next six weeks. Compared to controls (N=26), the rats exposed to Pfiesteria had significantly lower choice accuracy scores ($F(1,46)=9.49$, $p<0.005$) in terms of the number of correct entries made before an error occurred. The controls averaged 5.93±0.16 (mean±sem) entries to repeat while the Pfiesteria-exposed rats averaged 5.27±0.16 entries to repeat. These studies documented a persistent cognitive impairment in rats after exposure to the dinoflagellate Pfiesteria. This may be related to the cognitive impairments humans have shown after Pfiesteria exposure. Further research will be directed at determining the behavioral nature and neural mechanisms of this effect. (Supported by the National Science Foundation and North Carolina Sea Grant College.)

Society of Toxicology, March 9-13, 1997

COGNITIVE EFFECTS SEEN IN RATS EXPOSED TO THE DINOFLAGELLATE PFIESTERIA. E D Levin[1], D E Schmechel[2], H B Glasgow, Jr.[3] and J M Burkholder[3]. Depts of Psychiatry[1] and Medicine[2], Duke University Medical Center, Durham, NC., Durham VA Medical Center[2] and Dept of Botany[4], North Carolina State University, Raleigh, NC

The dinoflagellate Pfiesteria piscicida inhabits estuarine waters and is suspected as a cause of fish kills in North Carolina. After accidental exposure of humans to Pfiesteria, a complex syndrome including cognitive impairment and short term memory loss has been documented (Glasgow et al, J. Toxicol. Environ. Health, 46:501-522, 1995). The current study was conducted to determine the persisting cognitive effects of Pfiesteria exposure in rats. Adult female Sprague-Dawley rats (N=6) were injected (SC) with doses of ranging from 35,600 to 961,200 Pfiesteria cells per kg of rat body weight. Observations of behavior for six hours after acute injection documented no overt behavioral disturbances. Starting two days after injection, the rats began testing on the radial-arm maze, a test of spatial memory. They underwent 18 sessions of testing over the next six weeks. Compared to controls (N=6), the rats exposed to Pfiesteria had significantly lower choice accuracy scores ($p<0.005$) in terms of the number of correct entries made before an error occurred. The controls averaged 5.5±0.2 (mean±sem) entries to repeat while the Pfiesteria-exposed rats averaged 4.8±0.1 entries to repeat. This study documented a persistent memory impairment in rats after exposure to the dinoflagellate Pfiesteria. This may be related to the memory impairments humans have shown after Pfiesteria exposure. Further research will be directed at determining the character and mechanism of this effect. (Supported by the National Science Foundation and North Carolina Sea Grant College.)

PFIESTERIA PISCICIDA EXPOSURE IN RATS RESULTS IN PERSISTING LEARNING DEFICITS

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ABSTRACT

The dinoflagellate, *Pfiesteria piscicida,* is implicated as a cause of fish kills in North Carolina estuaries and elsewhere. Accidental exposure of humans to *Pfiesteria* has been reported to result in a complex syndrome including cognitive deficits. This series of studies was conducted to experimentally assess possible cognitive effects of *Pfiesteria* exposure in rats. Samples of water from aquaria in which *Pfiesteria* were killing fish were frozen. Then, thawed samples were injected into female Sprague-Dawley rats. Significant learning impairments were documented in rats administered recently frozen samples of *Pfiesteria.* No effect was seen in the recall of a previously learned task, but when the rats were called upon to learn a new task, the Pfiesteria-treated animals showed a significant learning deficit. This effect persisted up to at least 10 weeks after one injection. The *Pfiesteria*-induced learning deficit was not associated with generalized debilitation or health impairment. Deficits in habituation of arousal and rearing behavior were seen using a functional observational battery (FOB). No *Pfiesteria*-induced effects on blood count or in a standard pathological screening of the brain, liver, lungs, kidneys and spleen were seen. These studies document a persistent learning impairment in rats after exposure to *Pfiesteria.* This effect may be analogous to the cognitive deficits humans have shown after *Pfiesteria* exposure.

Consumer Health Risks Due to Incidental Exposure of Fish to *Pfiesteria piscicida*

Final Report of the Project

February 1, 1998

P.D. McClellan-Green , L.A. Jaykus and D.P. Green

Pfiesteria piscicida, a dinoflagellate first identified and described in 1991, has been implicated as a causative agent in major fish kills in estuaries of the southeastern United States. The organism goes through a number of life stages during which the flagellated vegetative phase produces a toxic compound (Burkholder *et. al.,* 1992). This putative exotoxin, now thought to be composed of at least two toxins, has been described as highly lipophilic and is thought to be secreted into the estuary rather than being retained within the algal cells. While laboratory and field exposure studies have demonstrated the apparent toxicity of exposure to *P. piscicida* toxin (Pptx) by various finfish and shellfish no information is currently available regarding the consumer health risks due to incidental exposure of fish or shellfish harvested in close proximity to fish kills.

In 1992, scientists culturing *Pfiesteria* in the laboratory reported significant health effects due to exposure to the toxin. Major symptoms included tingling of the extremities, joint pain, weakness, headaches, nausea, abdominal cramps, eye irritation, mood changes, memory loss and skin lesions (Glasgow *et. al.*, 1995). It has since been determined that the scientists were exposed to high doses of the toxin(s) due to poor ventilation in the culture facility and improper handling techniques. Attempts to determine whether serious health effects have occurred in individuals exposed to the toxin outside the laboratory is still under investigation.

Recently, Dr. Glenn Morris and his laboratory at Johns Hopkins University in their report to the US Congress indicated that watermen exposed to a related organism in Maryland might have suffered adverse health effects. This study is still under investigation and complete details are unavailable at this time. Anecdotal reports in NC have suggested that *Pfiesteria* may be responsible for the production of skin lesions in crabbers and fishermen working on the Neuse River, N. C. However, a recent epidemiological study funded by NC Sea Grant found no

significant increase in illness in persons working in impacted areas and those working in clean environments. Therefore, while accidental laboratory exposures have demonstrated the apparent toxicity of this compound in humans no definitive evidence has been observed in the field especially through the consumption of fisheries products. Therefore, the goal of this project was to address the issue of public health and whether an individual could be at risk from consuming fish caught during an active fish kill.

Methodology: The initial phase of this study involved identification of morphological and cytotoxicological changes to tissue culture cells that had been exposed to the partially purified toxin. Dr. Ed Noga from the North Carolina State University College of Veterinary Medicine supplied the crude toxin. Due to the unknown nature of this toxin it was shipped bound to C_{18} cellulose in a 20% acetonitrile buffer. The toxin was eluted with 100% acetonitrile, dried under a stream of N_2 at room temperature, resuspended in dimethylsulfoxide (DMSO) and frozen. Human colon (Caco-2) epithelial cells and mouse neuroblastoma (Neuro 2A) cells were grown in culture to near confluence then exposed to increasing concentrations of the crude toxin.

Cells were grown in T75 flasks at 37°C with 5% CO_2 using Eagles Minimum Essential Media supplemented with Earle's salts, 0.1 mM non-essential amino acids and 10 % fetal bovine serum. The cells were subcultured by treatment with 0.05 % Trypsin, 0.53 mM EDTA. Twenty-four well culture dishes were seeded at a density of 100,000-200,000 cells per well. These subcultures were allowed to adhere overnight and the density visually checked prior to beginning the assay. Extracts of the crude toxin were added in an appropriate volume of dimethylsulfoxide (DMSO). Control wells were treated with DMSO. Triplicate wells were analyzed for each

sample.

Each assay was evaluated on the basis of cell viability, morphology and biochemical endpoints such as alterations in ATP content or lactate dehydrogenase leakage. Each plate was examined for rounding, blebbing of the membranes, graininess and the presence of visibly dead cells. These parameters were scored from 1 to 4 based on the degree of severity, 4 being the worst, for each well. At the end of the exposure period, the media was removed and saved for the determination of lactate dehydrogenase leakage. The cells were trypsinized to remove them from the flasks. The cells were resuspended in one milliliter of fresh culture media and the absorbance of the titurated sample measured at 800 nm (McClellan-Green *et. al.*, 1997). This absorbance was plotted against a standard curve for live Neuro 2A cells as determined by counting with a hemocytometer. The absorbance of unstained cell suspensions at 800 nm is linearly proportional to cell density (Mohler *et. al.*, 1996). A linear regression equation was calculated and the slope used to predict the number of cells present in each well following exposure to fish tissue extracts. These cells were then analyzed for their ATP content by the method of Adams, 1963, using phosphoglycerate kinase. LDH leakage into the media was determined by monitoring the conversion of pyruvate to lactate as described by Amador *et. al.*, 1962.

The major goal of this study was to determine whether consumers could be at risk due to consumption of fishery products exposed to the toxin during a fish kill. We contacted Mr. Ken Eagleson of the Department of Environmental Health and Natural Resources. He agreed to inform our laboratories if and when a fish kill occurred so samples could be taken immediately. The Neuse River Rapid Response team has not reported any *Pfiesteria* related kills on the river this year. This was recently confirmed in a phone conversation on September 25, 1997. The last major fish kill reported on either the New River or Neuse River in which *P. piscicida* may have played a

role in the death of the fish occurred on September 1, 1996. At that time a major fish kill was reported in Northeast Creek, a tributary located in the upper portions of the New River. On September 3, 1996, Dr. David Green and his laboratory collected live, apparently healthy fish from Northeast Creek with the aid of N. C. Marine Fisheries Division personnel. It should be noted that dead fish were still apparent on the river at that time and that some of the fish collected had visible sores, i.e. some menhaden. Species of fish sampled include Spot (*Leiostomus xanthurus*), Striped mullet (*Mugil cephalus*), Croaker (*Micropognius undulatus*), Spanish mackeral *(Scombermouus maculatus*), Silver perch (*Bairdiella chrysaura*), Pinfish (*Lagodon rhomboides*), and Atlantic menhaden (*Brevoorita tyrannus*). Southern Flounder (*Paralichthys lethostigma*) were added as an internal negative control. The Southern Flounder were collected from Bogue Sound and held in filtered seawater for several months prior to their analysis. No shellfish were sampled. The fish were transported to the laboratory on ice and immediately frozen at -20°C. The frozen fish were removed from the freezer and the muscle tissue removed in a manner similar to commercial filleting. The fish remained frozen during this process and were not allowed to thaw.

Four-gram aliquots of tissue were removed from the lateral muscle of the fish and placed in sterile 50 ml disposable tubes. Methanol was added and the mixture was homogenized with a Polytron in a biosafety fume hood. After centrifugation, the supernatant was transferred to a clean tube and 2 ml of nanopure water added. The mixture was then extracted with ten milliliters of hexane and the upper phase discarded. Four milliliters of nanopure water and 16 milliliters of chloroform were then added to the methanol phase and the mixture vortexed. Following centrifugation the lower chloroform was transferred to a clean tube and the methanol phase re-extracted with a second portion of chloroform. The two chloroform phases were combined and gently dried under a stream of nitrogen at room temperature (Quilliam *et. al.*, 1993). The dried

extract was resuspended in an appropriate volume of DMSO and stored at –80°C until analyzed. Analysis of tissue extracts was carried out as described above for the crude toxin.

Results: Both Neuro 2A and Caco 2 cells were exposed to decreasing concentrations of the crude toxin(s). These cells were examined microscopically for cytotoxic effects by monitoring for any morphological alterations such as blebbing, rounding, graininess or lysis (see Figure 1 and Table 1). Cells exposed to concentrations of Pptx greater than 1 X 10^{-16} g/ml exhibited extreme morphological changes. The cells began rounding within a few seconds of exposure to the toxin(s). Additionally, severe blebbing of the cell membrane occurred followed by the formation of what appeared to be lipid droplets in the media. The resulting loss of membrane integrity resulted in the appearance of a grainy texture within the cells followed by cell lysis. Cells exposed to 1 X 10^{-17} g/ml to 1 X 10^{-21} g/ml exhibited fewer or no changes in their morphology. Figure 1 shows light micrographs of both Caco 2 and Neuro 2A cells exposed to the toxin.

The cells were then examined for retention of cell viability as analyzed by the Live/Dead Eukolight Viability/ Cytotoxicity Assay (Molecular Probes, Inc.) (see Figure 2) and quantified via the method of Mohler *et. al.*, 1996 (Figure 3). The Live/Dead Viability Assay functions on the presence of ubiquitous intracellular esterase activity that is determined by the enzymatic conversion of a non-fluorescent compound, calcein AM. Viable cells convert this compound to the fluorescent compound calcein. The calcein is retained in viable cells producing an intense green fluorescence in live cells. EthD-1, a second dye utilized in this assay, enters damaged membranes and upon binding to nucleic acids produces a bright red color. As shown in Figure 2, cells exposed to Pptx at high concentrations, 1 X 10^{-5} g/ml, do not exhibit esterase activity indicative of living

viable cells. Additionally, the presence of EthD-1 can not be detected via the occurrence of a bright red color. Indeed, there are very few cells from the original 100,00 cells remaining intact at this concentration. Therefore, instead of being able to determine viability using green and red color indicators, we were presented with ambiguous collections of yellow and orange along with a high background staining. This indicated that Pptx possessed the capacity to disrupt both the cellular membrane and the nuclear membrane resulting in the complete destruction of the cell. This allowed an apparent mixing of the dye components and an increase in the background fluorescence. Additionally, it appears that DMSO at high concentrations, 5%, damaged the cells sufficiently to increase the background staining of the control cells. Therefore, a smaller sample size would have been necessary to implement this protocol for toxicity screening and therefore another method for quantifying the toxicity of Pptx was employed in these studies.

Mohler *et. al.* (1996) demonstrated that the absorbance of unstained cells in suspension is linearly proportional to the cell density at 800 nm. This method which was originally used to standardize the number of cells seeded into each well for the Live/Dead Eukolight Viability/Cytotoxicity assay was employed to quantify the viability of cells exposed to the toxin. A dose response curve for Pptx was established by monitoring the total number of live cells present in the wells after a 24hour exposure (Figure 3). Previous *in vitro* cell bioassays have been established for a variety of marine toxins. These assays are largely based on the conversion of various dyes to a fluorescent product or on color development assays such as the reduction of tetrazolium compounds (Manger *et. al.* 1993) and rely heavily on the mechanism of action of the toxins. As seen in Figure 3 our crude preparation of Pptx(s) exhibits an apparent LC_{50} of 1×10^{-16} g/ml in Neuro 2A cells using the Mohler method. This was compared to the effect of tetrodotoxin (TTX) in the same assay. Here TTX exhibited an apparent LC_{50} of 1×10^{-11} g/ml or 10pg.

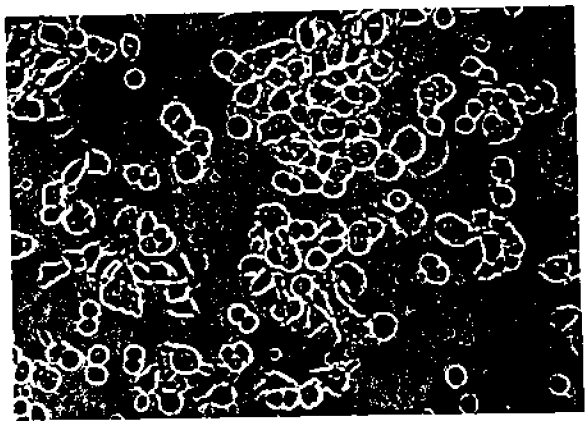
Previous reports using an *in vitro* cell based bioassay reported a detection limit of 3 nM for TTX (Kogure *et. al.,* 1988) while others have indicated the ability to detect this toxin in the picogram range.

Table 1. Morphological Characterization of Cells Exposed to Pptx (Neuro 2A/Caco 2)

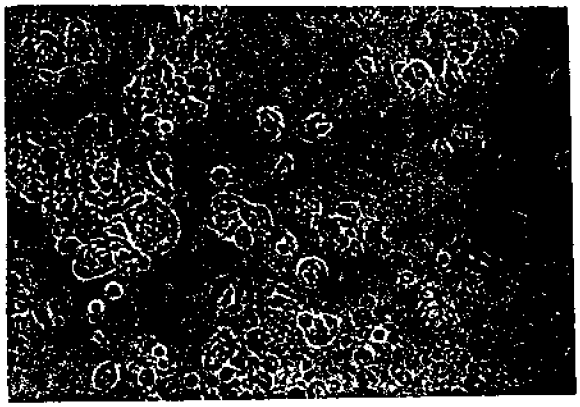
Concentration (g/ml)	Roundness	Blebbing	Graininess	Lysis
$1 X 10^{-4}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-5}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-6}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-7}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-8}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-9}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-10}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-11}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-12}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-13}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-14}$	++++/++++	++++/++++	++++/++++	++++/++++
$1 X 10^{-15}$	++++/+++	++++/+++	++++/+++	++++/+++
$1 X 10^{-16}$	+++/+++	+++/+++	+++/+++	+++/+++
$1 X 10^{-17}$	++/++	++/++	++/++	++/++
$1 X 10^{-18}$	+/+	+/+	+/+	+/+
$1 X 10^{-19}$	-/-	-/-	-/-	-/-
$1 X 10^{-20}$	-/-	-/-	-/-	-/-
$1 X 10^{-21}$	-/-	-/-	-/-	-/-

Therefore, our assay for the presence of marine toxins is extremely sensitive and should be sufficient for the detection of *Pfiesteria* toxin. A similar LC_{50} was obtained for Pptx using the Caco 2 cells (Figure 4). The dose response curve for the Caco 2 cells does not show as dramatic a shift in absorbance as the Neuro 2A cells and visual examination of the titurated suspension revealed a large amount of cell clumping by the Caco2 cells. Therefore, the use of the Neuro 2A

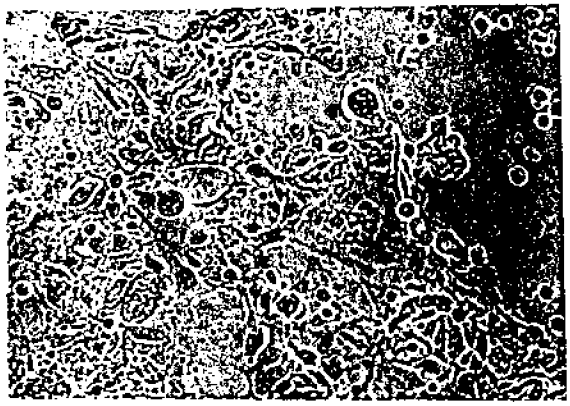
cells for the quantification of cell viability as per Mohler *et. al.*, (1996) is preferred over the Caco 2 cells due to the prevalence of clumping by the latter cell type.



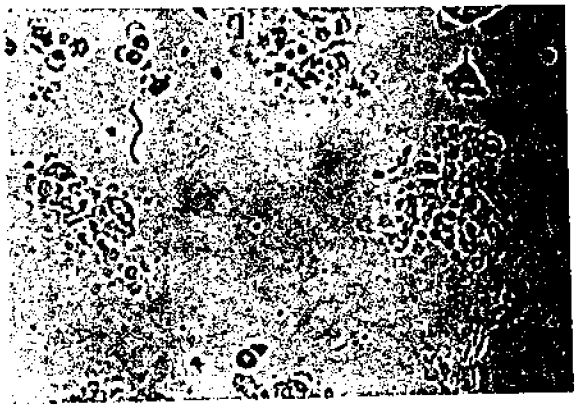
DMSO Dosed Neuro 2A



Pptx Dosed Neuro 2A

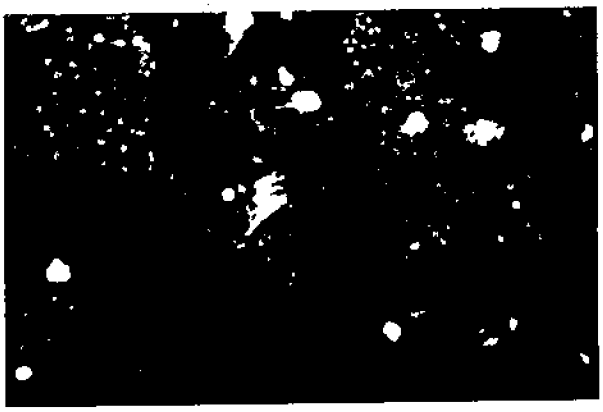


DMSO Dosed Caco2

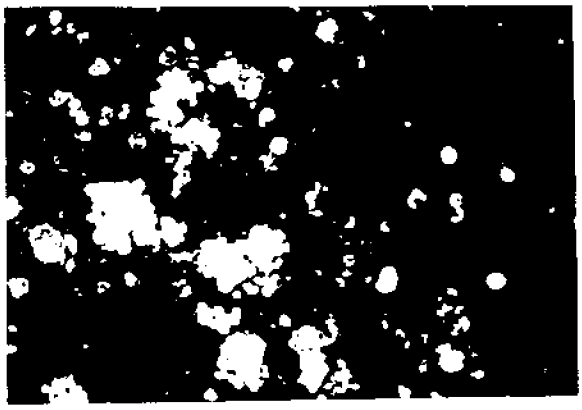


Pptx Dosed Caco2

Figure 1. Light photomicrograph (10X) of Pptx exposed Neuro 2A and Caco 2 cells. Cells were exposed to 1 X 10-5 g/ml of crude toxin from P. piscicida. The cells were then examined for morphological changes such as blebbing (B), rounding (R), graininess (G) and lysis (L).



DMSO Dosed Neuro 2A



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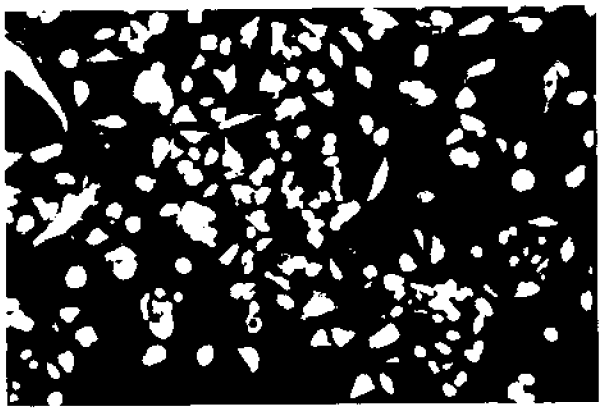
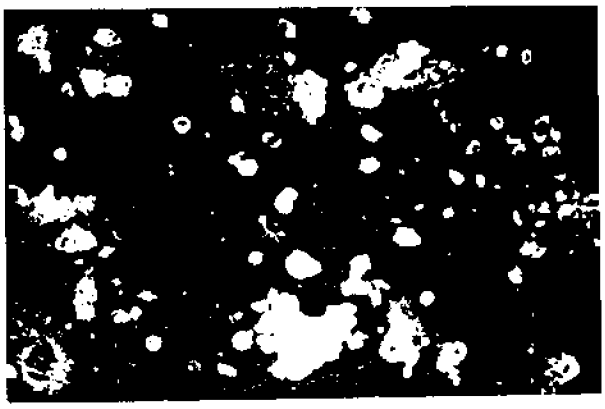


DMSO Dosed Caco2



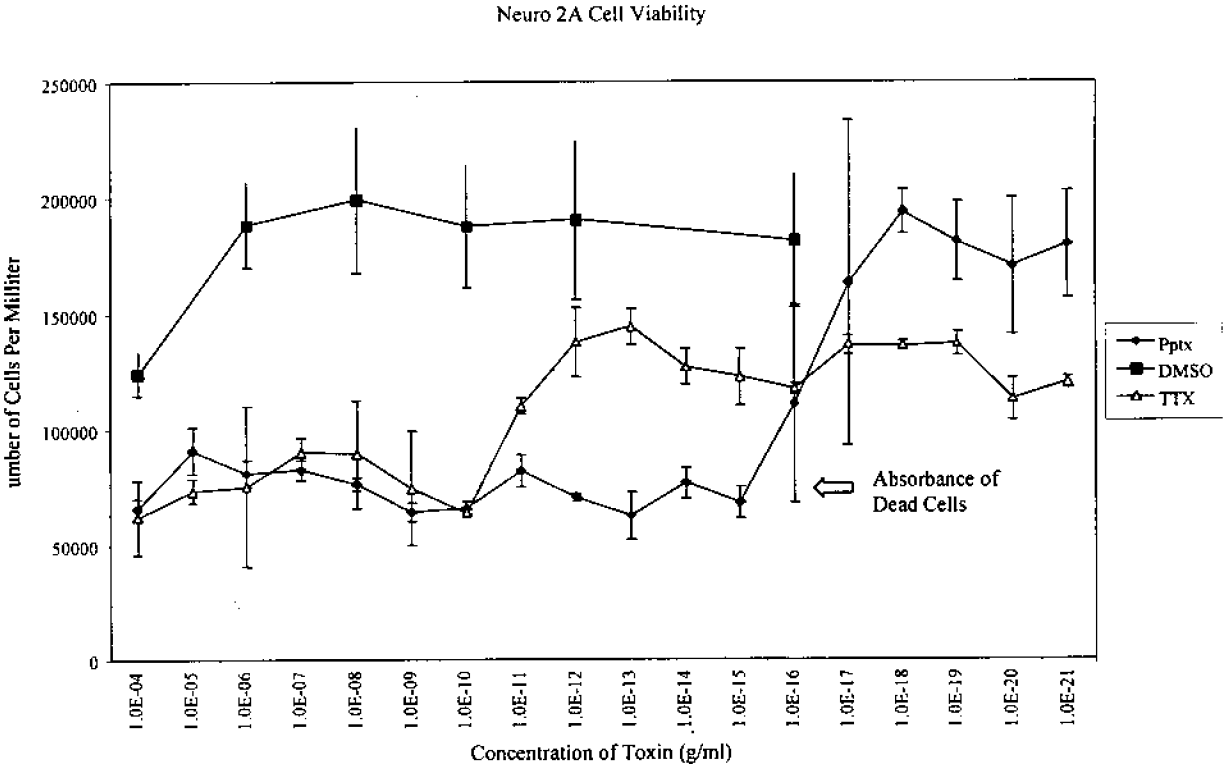
Pptx Dosed Caco2

Figure 2. Fluorescent micrograph of control cells exposed to 1 X 10-5 g/ml of Pptx. Live , viable cells actively retain calcein to produce a bright green color. Damaged but intact cells allow EthD-1 to enter the cell where it binds to nucleic acids producing a bright red color.

Analysis of the ATP content and lactate dehydrogenase leakage into the surrounding media did not produce any significant results. An extremely high variability was seen in the levels of ATP present in the cells following Pptx exposure (Figure 5). It is possible that the release of cellular proteases and enzymes from the lysed cells inhibited this assay. Indeed it has been reported that

other nucleoside triphosphates such as GTP, ITP and UTP can interfere with the assay (Bishop et. al., 1959).

Figure 3. Dose Response Curve for Neuro 2A cells exposed to decreasing concentrations of toxins. Tetrodotoxin was used as a control to validate the sensitivity of the assay. Pptx assays used 200,000 cells per ml and the TTX assays utilized 150,000 cells per assay. Samples are mean ± the standard deviation of triplicate samples.



The analysis of lactate dehydrogenase (LDH) leakage into the surrounding media likewise produced insignificant results. As seen in Figure 6, the level of LDH activity in the media was extremely variable and could not be correlated to the level of toxin exposure. Therefore, these biochemical assays are not suitable for the determination of cell viability using the *Pfiesteria* toxin.

After establishing the utility of the *in vitro* cell bioassay for the determination of the presence of the Pptx, our next objective was to examine environmentally exposed samples. On September

3, 1996, during a major fish kill Dr. David Green and his laboratory collected live, apparently healthy fish from Northeast Creek with the aid of N. C. Marine Fisheries Division personnel.

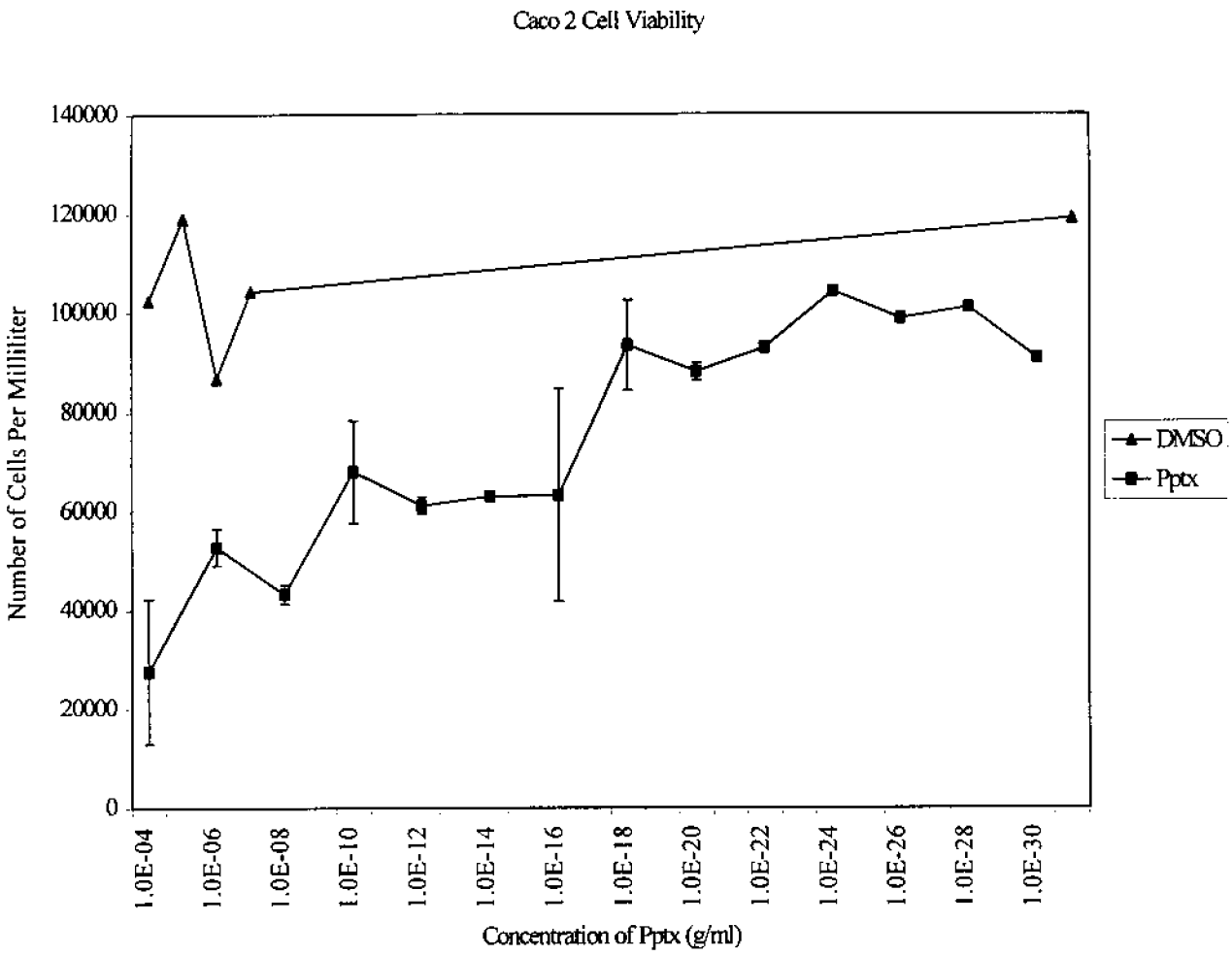
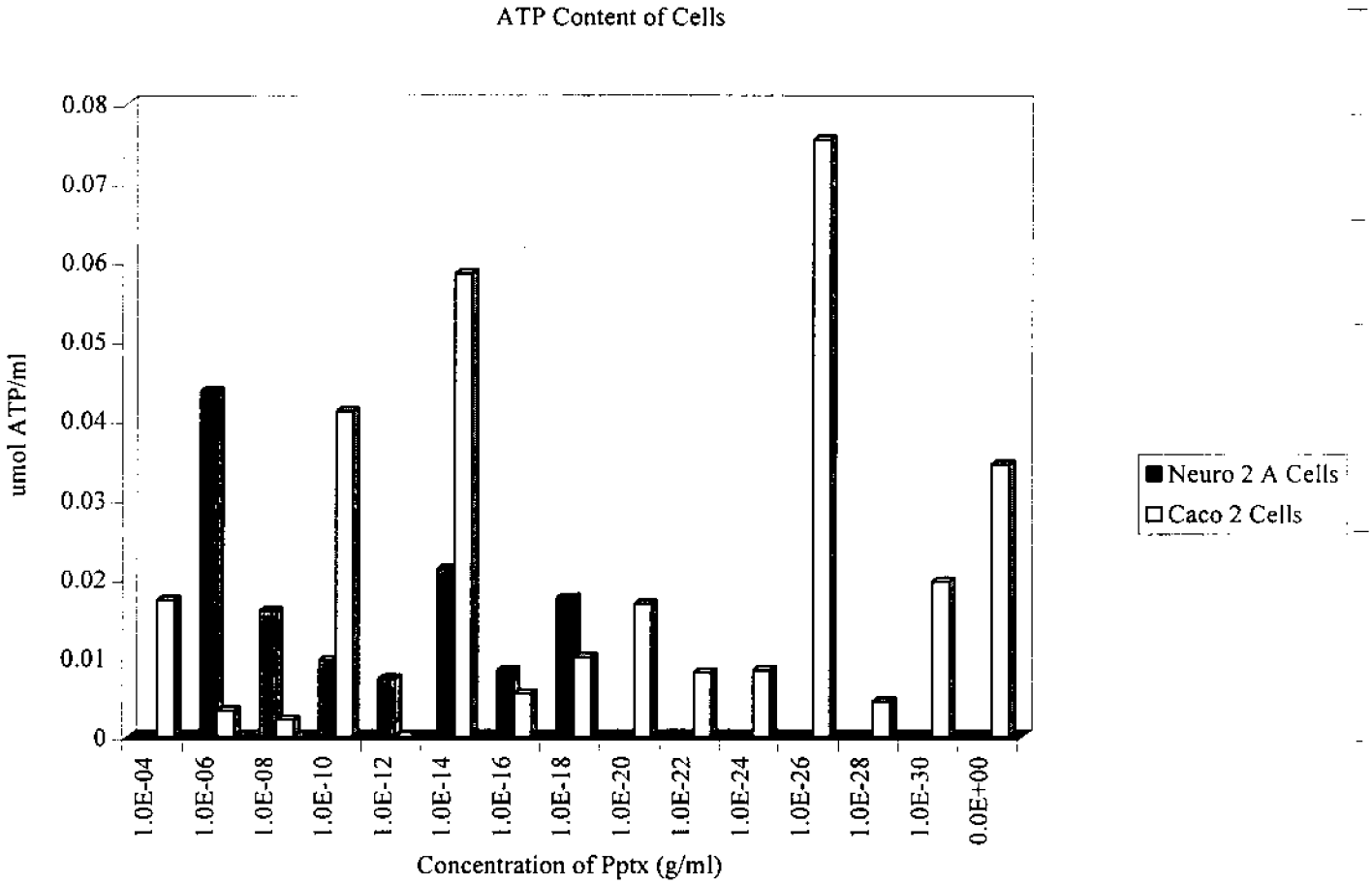


Figure 4. Dose Response Curve for Caco 2 cells exposed to decreasing concentrations of toxins. Pptx assays used 200,000 cells per ml. Samples are mean ± the standard deviation of triplicate samples.

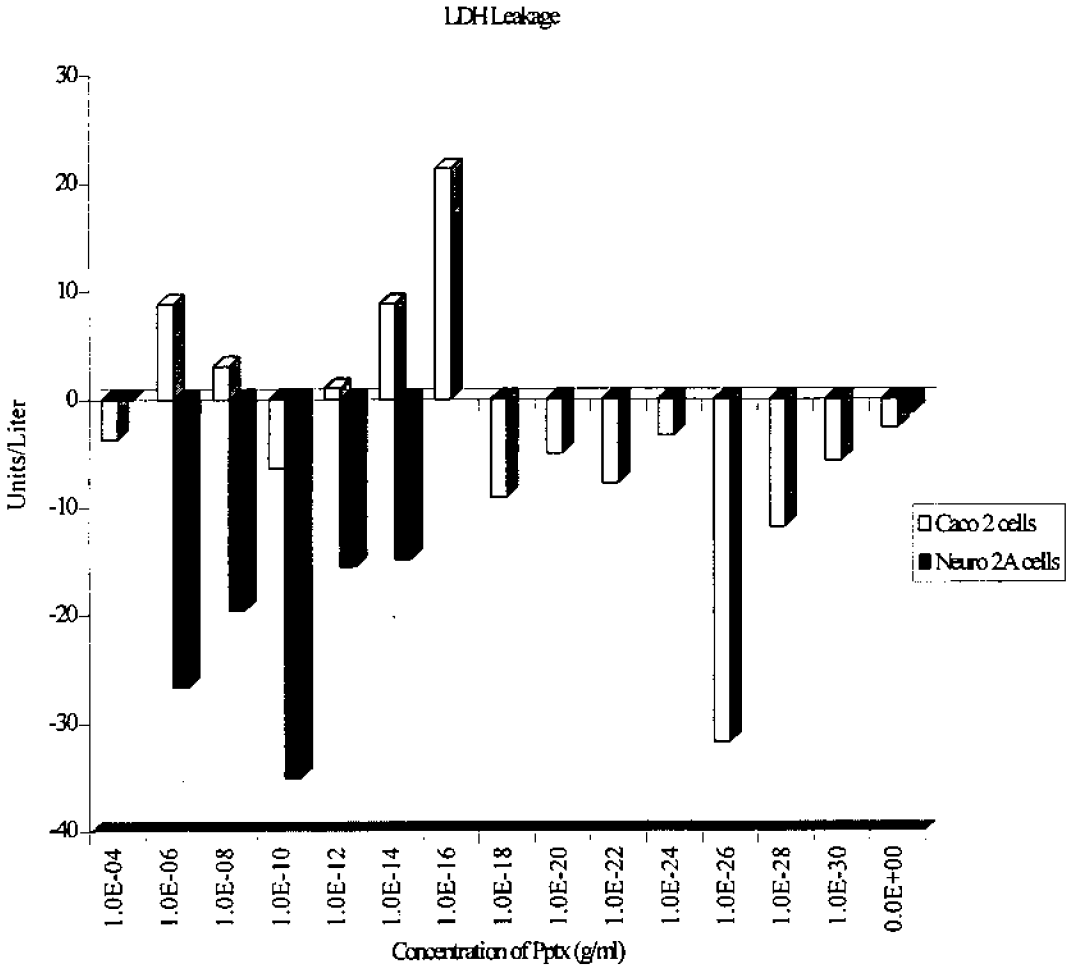
Portions of the lateral muscle were extracted as described above and utilized in our *in vitro* cell bioassay. As seen in Figure 7, a total of eight species were analyzed for the presence of toxin.

Figure 5. ATP content of Pptx dosed Neuro 2A and Caco 2 cells. Cells were dosed with increasing concentrations of crude Pptx and allowed to incubate for 24 hours. At the end of the exposure, the cells were removed and their cellular ATP concentration determined as described. Concentration is the mean of three assays.



Species of fish sampled include Spot (*Leiostomus xanthurus*), Striped mullet (*Mugil cephalus*), Croaker (*Micropognius undulatus*), Spanish mackeral *(Scombermouus maculatus*), Silver perch (*Bairdiella chrysaura*), Pinfish (*Lagodon rhomboides*), Atlantic menhaden (*Brevoorita tyrannus*) and a blind internal negative control, Southern Flounder (*Paralichthys lethostigma*). The samples were numbered from 1-33 and the individual performing the assays was not informed of the

Figure 6. Lactate dehydrogenase activity of Pptx dosed Neuro 2A and Caco 2 cells. Cells were dosed with increasing concentrations of crude Pptx and allowed to incubate for 24 hours. At the end of the exposure, the media was removed and the level of LDH activity determined as described.



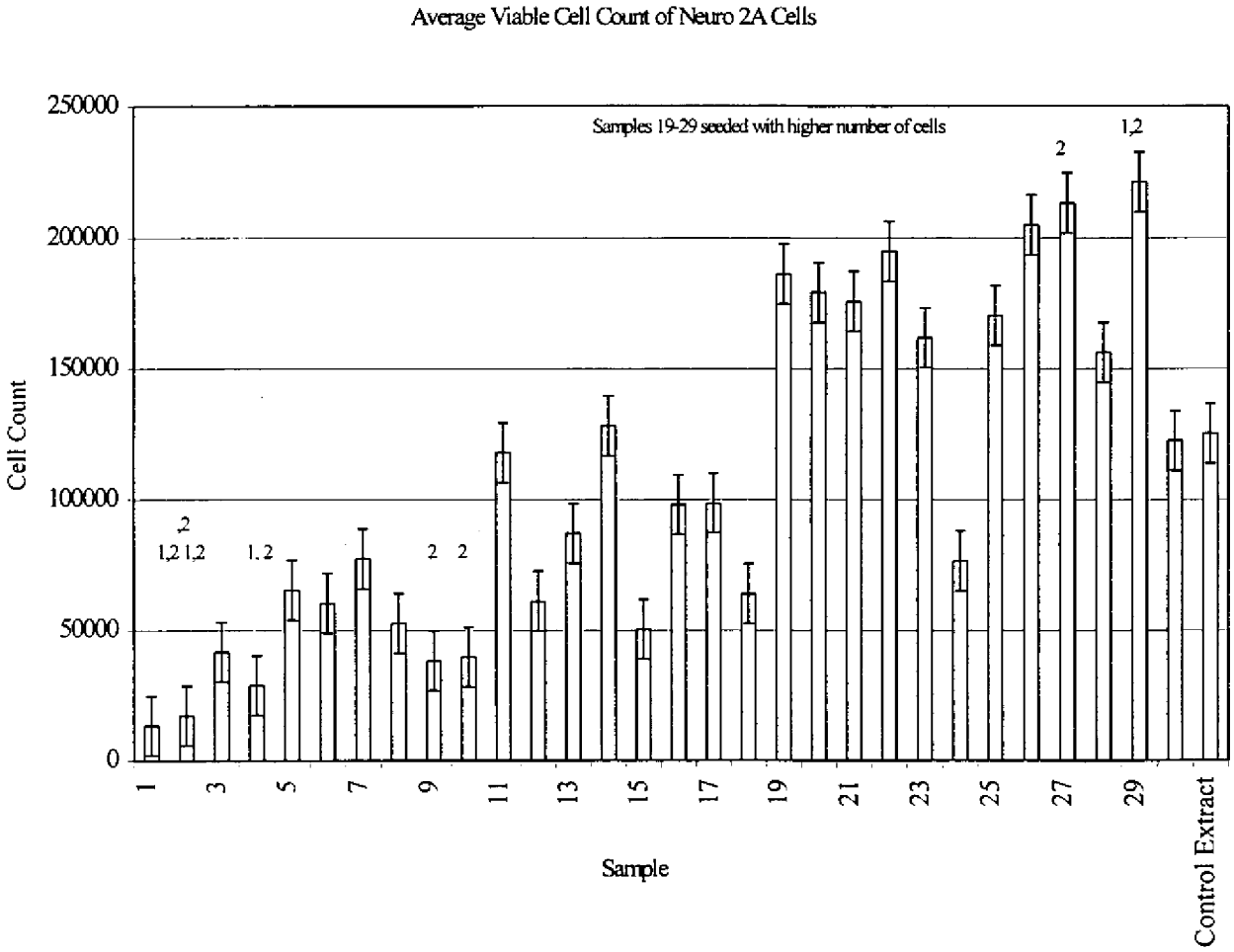
specific species until the completion of the assays.

The majority of samples analyzed showed no effect on the viability of Neuro 2A cells (Figure 7). However, samples 1, 2, 4, 9 and 10 exhibited a significant decrease in the number of live cells present after 24 hours as compared to the DMSO or Control Fish Tissue extracts. Control fish tissues examined in this assay included southern flounder, croaker and pinfish. All

controls behaved in a similar manner and are therefore depicted by a single bar on each graph.

Samples 27 and 29 exhibited a significant increase in the number of live cells present after a 24hour exposure.

Figure 7. Average Cell Count. Neuro 2A Cells were plated at approximately 100,000 cells per well. Cells were treated with 10 µl DMSO, control fish tissue extract or experimental fish tissue extracts (2.5g tissue ml DMSO). Samples were incubated for 24 hours at 37°C and analyzed as previously described. The total cell count was predicted using a linear regression equation obtained from the standard curve. Values equal mean ± standard error. Significance determined by Student's t-test.
[1] represents a significant difference between experimental sample and DMSO ($p \geq 0.05$)
[2] represent a significant difference between experimental sample and control extract ($p \geq 0.05$)



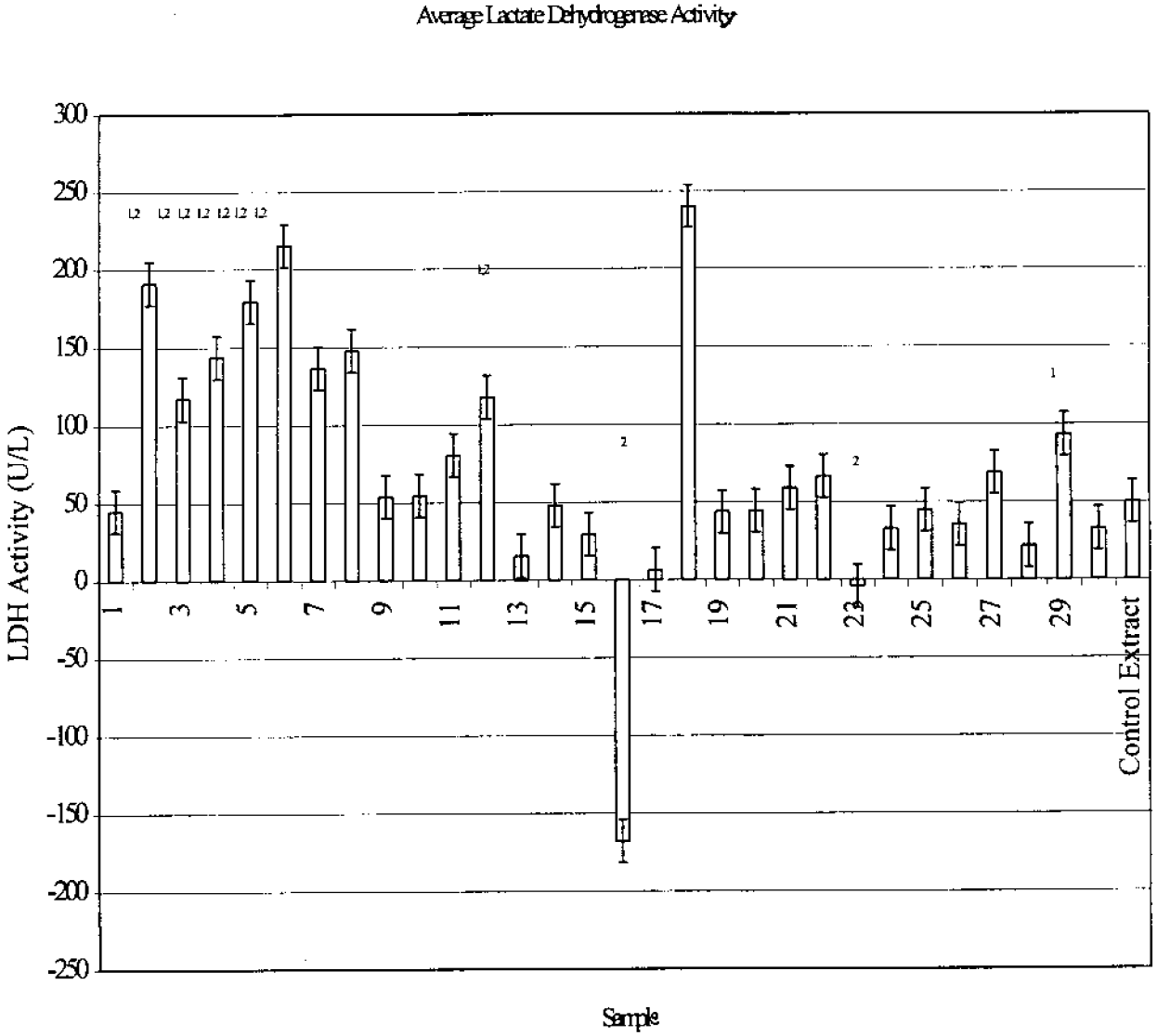


Figure 8. Average Lactate Dehydrogenase Activity. Following the incubation period of 24 hours, 50 µl media was combined with 1 ml reconstituted Lactate Dehydrogenase Reagent (Sigma). Conversion of NADH to NAD was used to estimate the quantity of enzyme leakage into the media. Values equal mean ± standard error. Significance determined by Student's t-test.
[1] represents a significant difference between experimental sample and DMSO ($p \geq 0.05$)
[2] represent a significant difference between experimental sample and control extract ($p \geq 0.05$)

In addition to the viability assays, both LDH activity and ATP content of the cells was examined.

As seen in Figures 8 and 9 several samples produced results that were significantly different than

the control samples. However, neither LDH activity nor ATP content could be correlated with a particular species or to the behavior of the extracts in the cell viability bioassay.

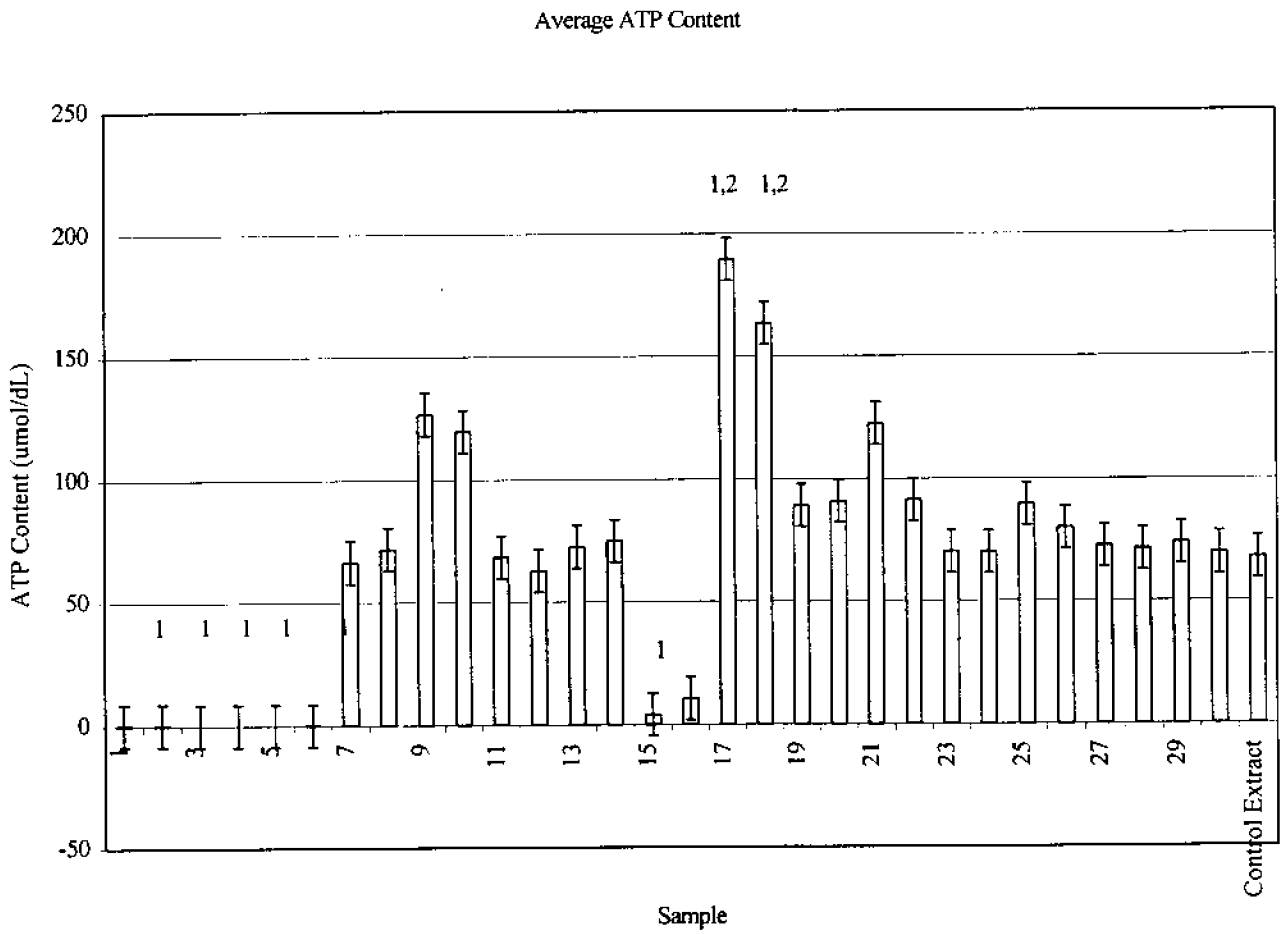


Figure 9. Average ATP Content. The amount of ATP present in the cells was calculated based on the conversion of NADH to NAD for an aliquot of cell suspension using glyceraldehyde phosphate dehydrogenase (GAPD) and phosphoglycerate phosphokinase. Values equal mean ± standard error. Significance determined by Student's t-test.

[1] represents a significant difference between experimental sample and DMSO ($p \geq 0.05$)

[2] represent a significant difference between experimental sample and control extract ($p \geq 0.05$)

An examination of the cell viability influenced by exposure to the fish tissue extracts revealed that only one species, Atlantic menhaden, consistently produced a significant reduction in

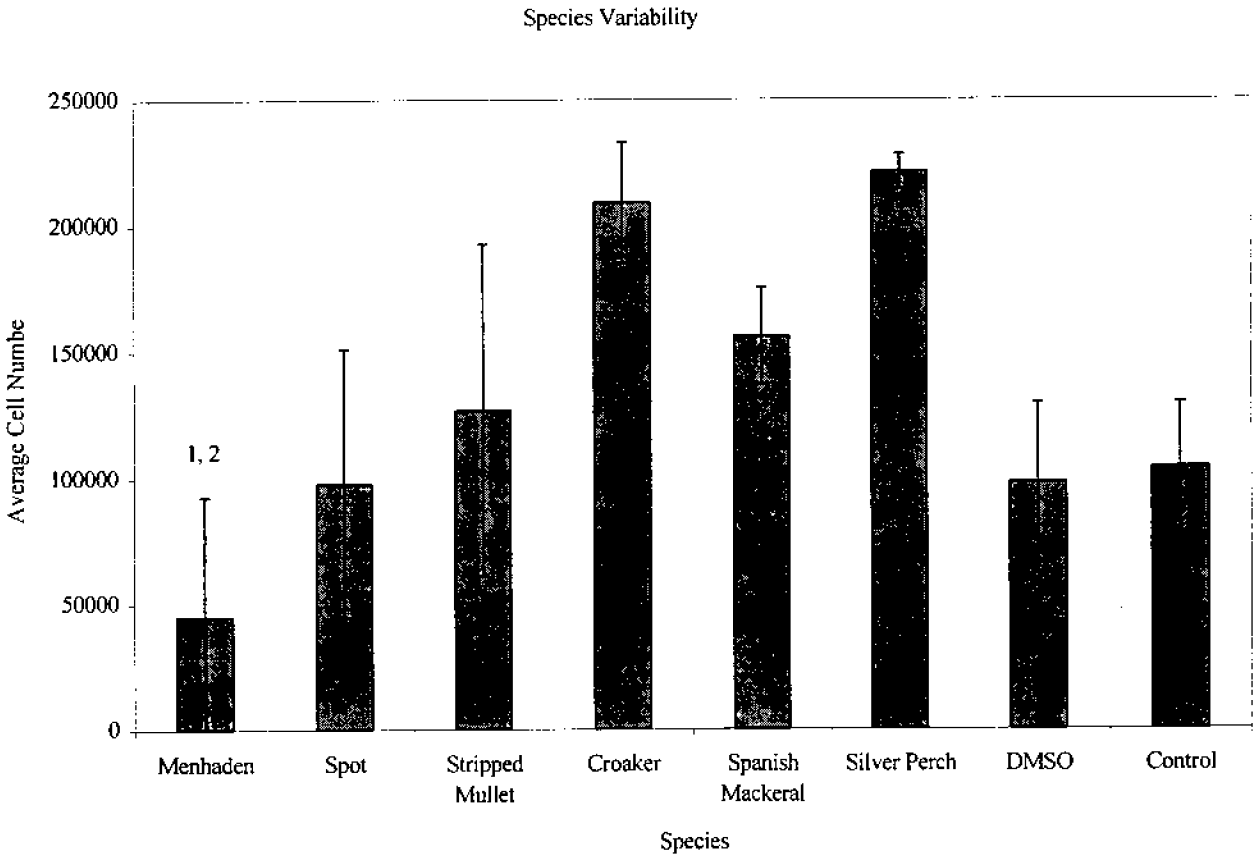


Figure 10. Species Variability. The average total cell count was compared between each species and it's control as described. Values equal mean ± standard deviation. Significance determined by Student's t-test. [1] represents a significant difference between experimental sample and DMSO ($p \geq 0.05$). [2] represents a significant difference between experimental sample and control extract ($p \geq 0.05$)

the number of live cells (see Figure 10). This reduction in cell number occurred in 5 of the 8 individuals analyzed (Samples 1, 2, 4, 9 and 10 as shown in Figure 7). The other menhaden samples (Samples 3, 11 and 12) did not significantly differ from the control fish tissue extracts. It

is possible a species-specific effect is responsible for the decreased viability of cells exposed to the menhaden extracts. Although some menhaden extracts did not affect the viability of the cells in culture (see Figure 7) this aspect should be further investigated by preparing tissue extracts from control menhaden tissues. Although the extraction protocols used in this assay were previously employed by Quilliam *et. al.*, (1993) for the identification of diarrhetic shellfish poison, okadaic acid and dinophysistoxin, it is possible these methods were insufficient to detect all toxins present in the tissues. Future studies should employ a step-wise extraction process that will maximize the recovery of Pptx from naturally and artificially dosed tissues.

Other samples, such as Samples 27 and 29, Croaker and Silver Perch respectively, appear to produce an increase in the number of cells present at the end of the exposure period. However, closer examination of the data indicates that the controls used in the analysis of these latter samples possessed a significantly higher number of cells than the other assays (DMSO=206,866 cells, Control Fish Tissue = 172,233 cells, Sample 27 = 213,200 cells and Sample 29 = 221,166 cells).

Conclusions: In summary, *Pfiesteria piscicida* produces an extremely potent biotoxin(s) having an apparent LC_{50} of 1×10^{-16} g/ml to 1×10^{-17} g/ml. This concentration was estimated by measuring the weight of the residue present following extraction of the lipophilic portion. It is highly probable that more than one bioactive compound was present in this residue. Previous studies using other marine biotoxins have resulted in LC_{50}s of 1×10^{-12} for ciguatoxin to 2×10^{-9} for saxitoxins and brevetoxins (Manger *et. al.*, 1993). At this time, the LC_{50} for our crude

preparation of Pptx(s) should only be considered an estimate. This value will have to be reassessed once pure compounds are obtained.

Morphologically cells exposed to Pptx exhibit characteristics similar to those experienced with other marine toxins. These include rounding, membrane blebbing, graininess, and lysis. Visual comparison of cells exposed to both tetrodotoxin and *Pfiesteria* toxin reveal a more rapid and severe progression of the symptoms in the *Pfiesteria* dosed samples. The greatest difference is in the rate and degree of membrane blebbing. The mechanism responsible for this reaction by the cells is unknown. Many marine toxins, such as the brevetoxins enhance the action of the sodium channel causing an alteration in the morphology of exposed cells. Other marine toxins, such as saxitoxins, act to block the sodium channel thereby resulting in an altered cellular morphology. Thus far it is unknown whether the *Pfiesteria* toxin functions as a sodium channel-specific toxin or whether it carries out its activity through some other mechanism.

Use of our analysis system with a second marine toxin, tetrodotoxin, tended to validate our assay. Other cell-based bioassays using TTX have relied on the sodium channel blocking ability of this compound to antagonize the combined effects of veratridine and ouabain. The method used in this study relies on the ability to detect live cells based on their absorbance at 800nm. Mohler et. al. (1996) hypothesized that the red-infrared absorbance of live cells in suspension was due to the size and structure of intact cells and not to any chemical component of the cells themselves. These phenomena proved very useful for the detection of cell viability in the Pptx-treated cells as the mechanism of action of this toxin is currently unknown. Preliminary studies (Burkholder and Glasgow, 1997) on a water-soluble compound isolated from *Pfiesteria* have demonstrated cytotoxic activity in GH4C1 rat pituitary cells. This system has been used to identify toxins that

influence the ser/thr protein phosphatases and may ultimately provide some insight into the mechanism of action of Pptx.

The analysis of environmentally exposed samples revealed that only one species, Atlantic menhaden, possessed a compound that was biologically active. These fish along with Spot, Croaker, Stripped Mullet, and Spanish Mackeral, were collected during an active fish kill. However, because no biomarker has been identified for the presence of *Pfiesteria* and the chemical structures of its toxins have not been identified we cannot conclusively state that *Pfiesteria* was responsible for this cytotoxic effect. At the time of the fish kill two dinoflagellates were reported to be blooming in the river. These included *Gyrodinium aureolum,* a red tide organism, and *Pfiesteria piscicida.* Both dinoflagellates produce toxins and although our assay cannot distinguish between the two it can serve as a useful first screen in the labor intensive and expensive process of toxin screening. If a toxic compound were present in the other species it is anticipated that our *in vitro* cell bioassay would be able to detect its presence.

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Environmental Control of *Pfiesteria* Outbreaks:

The Role of Anthropogenic Nutrient Loading

Final Report of the Project

October 30, 1997

Revised January 16, 1998

Hans W. Paerl and James L. Pinckney

ABSTRACT

The recently described toxic dinoflagellate *Pfiesteria piscicida* Steidinger et Burkholder and *Pfiesteria*-like dinoflagellates have become a major water quality issue with possible linkages to fish mortality and human health impacts. Reports have suggested that nutrient enrichment of estuarine waters may support *P. piscicida* growth and outbreaks. The linkages between accelerated nutrient loading, eutrophication, and environmental conditions on the emergence and growth of this dinoflagellate under *natural* conditions, however, are not well established. As a group, *Pfiesteria*-like dinoflagellates are non-photosynthetic (heterotrophic) and require suspended microalgae (phytoplankton) prey as a primary food source. The suggested link between nutrient enhancement and *P. piscicida* growth may be either direct or indirect by enhancing the availability of phytoplankton prey. We examined the possibility that, as the chief supply of readily-utilizable organic matter, phytoplankton primary production is the key link between nutrient inputs and potential outbreaks of *Pfiesteria*-like dinoflagellate zoospores in the Neuse River Estuary, NC. **The primary research goals were to: 1) determine the potential regulatory roles of inorganic nutrient enrichment, sediments, and water column mixing on phytoplankton community dynamics, including *Pfiesteria*-like cells, in nature and 2) Clarify implications for nutrient input reductions proposed for the Neuse River Estuary.** Using mesocosm bioassays, coupled with biweekly environmental and biological sampling in the Neuse River where fish kills attributed to *P. piscicida* have been reported, we examined the impacts of dominant anthropogenic nutrient (NO_3^-- nitrogen and PO_4^{3-}- phosphorus) supply rates, sediment-water column exchange, water column mixing, and natural phytoplankton community (including prey) on the abundance of *Pfiesteria*-like non-toxic biflagellated zoospores. Mesocosm experiments and techniques for assessing phytoplankton community responses to nutrient loading scenarios were designed to examine and evaluate these factors seasonally in the Neuse Estuary during 1996 and 1997.

Phytoplankton community responses to the manipulated variables indicated that biomass and productivity were consistently N-limited throughout the study period. Dominant phytoplankton taxa, including chlorophytes, diatoms, and cyanobacteria, exhibited significant increases in biomass in response to N (as NO_3^-) additions and indicated that phytoplankton growth was N-limited. Phosphate (as PO_4^{3-}) additions did not additionally influence the relative growth and abundance of individual algal groups, suggesting P sufficiency. The mesocosm bioassay array

provided a range of suitable physical-chemical conditions and prey species for growth of *Pfiesteria*-like dinoflagellates. However, there was an absence of a significant increase in the number of *Pfiesteria*-like zoospores in response to mixing, sediment, and nutrient additions. Nitrate (NO_3^-) and phosphate (PO_4^{3-}) enrichment similar to that encountered in nature did not directly increase the abundance of these dinoflagellates. *Pfiesteria*-like zoospores constituted a small proportion of the total number of planktonic cells. On a seasonal basis, the number of *Pfiesteria*-like biflagellated zoospores was positively correlated with phytoplankton biomass and productivity. The abundance of *Pfiesteria*-like zoospores followed general trends in phytoplankton production, indicating the source of organic nutrition supporting growth is likely phytoplankton-based. Proposed nutrient reduction strategies for the Neuse Basin that target phytoplankton taxa dominating primary production will assist in the amelioration of the unwanted consequences of eutrophication such as chronic oxygen depletion, toxic algal blooms, fish mortality, and the abundance of *Pfiesteria*-like dinoflagellates in the Neuse River Estuary. Given current N limited conditions and N loading dynamics, such reductions should yield the desired impact of reducing growth potentials of dominant phytoplankton genera central to the eutrophication process. We conclude that reduction of phytoplankton growth and bloom potentials will translate into broad-based water quality improvement, including a declining trend in the frequency, spatial extent, and magnitudes of nuisance algal blooms, O_2 depletion, and associated fish and shellfish mortality.

INTRODUCTION

The recent description of *Pfiesteria piscicida* Steidinger et Burkholder (Steidinger et al. 1996) and "look-alike" dinoflagellates (Landsberg et al. 1995, Steidinger et al. 1997, Appendix I) capable of killing fish in laboratory experiments (Burkholder et al. 1992, 1993, 1995b, Burkholder & Glasgow 1995, Glasgow et al. 1995, Lewitus et al. 1995, Noga et al. 1996) has heightened concerns about potentially adverse environmental and human health impacts (Glasgow et al. 1995). So far, *Pfiesteria*-like dinoflagellates have been detected in estuarine waters ranging from Florida to Maryland along the Eastern US Atlantic Coast (Burkholder et al. 1995b, Burkholder & Glasgow 1997b, Lewitus et al. 1995, Steidinger et al. 1997). In the laboratory, non-toxic stages exposed to fish and fish excreta undergo transformation to a toxic form, kill fish, and within hours disappear from the water column (encyst and settle to the sediment) (Burkholder et al. 1995a, Burkholder & Glasgow 1995, Lewitus et al. 1995). Research efforts have been concentrated on dinoflagellate-fish interactions in waters experiencing fish kills and the potential detrimental impacts on fish in nature (Burkholder et al. 1993, 1995b, Glasgow et al. 1995, Lewitus et al. 1995). While these interactions are clearly the most visible and potentially harmful from the public perspective, widespread fish kills and diseased fish may result from several causes. The toxic biflagellated vegetative stages are considered transitory, while the non-toxic zoospore stage appears to be the commonly-encountered planktonic form in nature (Burkholder & Glasgow 1995, Steidinger et al. 1996a, Steidinger & Tangen 1997). Effective abatement strategies can be predicated on regulating any part of the life cycle susceptible to controlling measures (e.g., abundance of non-toxic stages). Although most of the previous research and attention has focused on the toxic stages, the factors controlling the population dynamics of non-toxic zoospore stages in nature may be most important in terms of understanding environmental regulation of the overall abundance of *Pfiesteria*-like dinoflagellates.

Pfiesteria piscicida belongs to a group of heterotrophic dinoflagellates that do not synthesize photopigments (i.e., chlorophylls and carotenoids) and rely on external food sources. Nearly half of all known species of dinoflagellates are heterotrophic predators of phytoplankton (Steidinger & Tangen 1997). Like many non-photosynthetic dinoflagellates, *P. piscicida* may supplement its nutritional requirements using photosynthate produced by chloroplasts captured from algal prey and sequestered in vacuoles (cleptochloroplasty) (Fields & Rhodes 1991, Burkholder & Glasgow 1995, 1997a,b, Steidinger et al. 1996a). The primary food source for the non-toxic biflagellated zoospore stage is phytoplankton (Burkholder & Glasgow 1995, Burkholder et al. 1995b) but we know little

about the trophic linkage between the zoospore and its diet in natural settings. Factors that directly affect phytoplankton biomass and species composition (nutrients, mixing, light, etc.) may therefore indirectly affect (through trophic interactions) the relative abundance of *P. piscicida.* In this regard, it has been suggested that nutrients (nitrate and phosphate) supporting phytoplankton growth may play a role in the growth and proliferation of *P. piscicida* (Burkholder et al. 1992, 1993, Glasgow et al. 1995, Burkholder & Glasgow 1995). This possibility merits consideration because a strong link between nutrient enrichment and accelerated phytoplankton production (eutrophication) has been established in many of the estuaries in which *Pfiesteria*-like cells have been reported. As part of our ongoing seasonal monitoring and experimental studies on naturally-occurring nutrient-phytoplankton growth and bloom dynamics in the Neuse River Estuary, NC (Paerl 1983, 1987; Paerl et al. 1995; Pinckney et al. 1996, 1997, 1998), we examined parallel impacts of environmental factors that control phytoplankton growth such as nutrient enrichment, sediments (as a seed source), and water column mixing (stratification) on natural populations of the non-toxic zoospore stage of *P. piscicida.* A field and experimental team was assembled to undertake tasks related to water quality monitoring, experimental assessments phytoplankton responses, and taxonomy of Neuse River phytoplankton and zoospores of *Pfiesteria*-like dinoflagellates. We specifically evaluated the possibility that the abundance of *Pfiesteria*-like biflagellated zoospores was controlled directly by phytoplankton prey abundance and indirectly by environmental factors that control phytoplankton biomass and community structure in nature.

Research Goals, Objectives, and Products

The primary research goals were to determine the potential regulatory roles of inorganic nutrient enrichment, sediments, and water column mixing on the dynamics of natural phytoplankton communities (including *Pfiesteria*-like biflagellated zoospores) and clarify the implications of these findings for proposed nutrient management strategies aimed at minimizing potential negative impacts of eutrophication on the Neuse River Estuary.

1) Determine if growth and abundance of the non-toxic biflagellated zoospore stage of *Pfiesteria*-like dinoflagellates are linked to natural phytoplankton prey abundance using manipulative mesocosm bioassays of Neuse River water collected from a region where several large fish kills have occurred.
2) Examine the effects of water column mixing and sediments (as a "seed bank" for dinoflagellate amoebae and cysts) on the growth of non-toxic stages of *Pfiesteria*-like dinoflagellates.

3) Determine if emergence, growth, and proliferation of naturally-occurring *Pfiesteria*-like dinoflagellate zoospores is directly and/or indirectly (via direct effects on phytoplankton) controlled by enhanced inorganic nutrient loading.

4) Assess proposed nutrient input management strategies as potential controls of the abundance of *Pfiesteria*-like zoospores in estuarine waters.

Rationale for Examining Inorganic Nutrient Controls of *Pfiesteria*

Existing information on nutrient-phytoplankton interactions and water quality in estuarine tributaries of North Carolina's Albemarle-Pamlico Sound System indicate that productivity is generally moderate to high and nitrogen (N)-limited throughout much of the year (Chowan River: Witherspoon et al. 1979, Kuenzler et al. 1982, Paerl 1982) (Pamlico River: Hobbie 1970, Hobbie & Harrison 1972, Kuenzler et al. 1979, Stanley 1988) (Neuse River: Tedder et al. 1980, Paerl 1983, 1987, Stanley 1983, Christian et al. 1986, Paerl et al. 1995). Phosphorus (P), at times, may play a secondary role as a co-limiting nutrient but only after nitrogen enrichment (Rudek et al. 1991, Paerl et al. 1995). Limitations by other nutrients (Si, Fe, trace metals) have not been observed in the Chowan, Neuse, and Pamlico Rivers (Paerl 1983, Rudek et al. 1991). N limitation dominates in large part because P, Si and trace metals are supplied readily from natural sources (i.e., watershed weathering processes, marine sediments) and effectively cycled between the sediments and water column (Kuenzler et al. 1979, 1982). Marine sources of N are chronically low and a fraction (thus far undetermined) of the N input is denitrified and lost to the atmosphere as biologically-inactive dinitrogen gas (N_2) (Nowicki 1994, Rysgaard et al. 1995).

In all the abovementioned estuarine tributaries, excessive **inorganic** N loading (NO_2^-/NO_3^-, NH_4^+) has been linked to enhanced primary productivity, sometimes culminating in nuisance algal blooms (i.e., cyanobacteria, cryptomonads, and dinoflagellates) (Witherspoon et al. 1979, Paerl 1982, 1983, 1987, Christian et al. 1986). Phytoplankton blooms are a major source of organic matter triggering bottom water hypoxia (oxygen levels < 4 mg/L) and anoxia (no detectable oxygen). Chronic oxygen depletion is a major cause of finfish and shellfish mortality in North Carolina estuaries (Matson et al. 1983, Paerl & Pinckney 1996). Blooms are most frequent and problematic during spring through summer, following nutrient-laden (especially NO_3^--N) runoff events impacting estuarine waters (Rudek et al. 1991, Boyer et al. 1993, Paerl et al. 1995). Periodic and ephemeral salinity stratification (i.e., salt wedges) results in dense, non-mixed bottom waters that

trap decaying phytoplankton blooms, further aggravating hypoxic and anoxic conditions (Matson et al. 1983, Paerl & Pinckney 1996).

Advanced symptoms of eutrophication, including nuisance and toxic phytoplankton blooms, hypoxia, and anoxia, have been linked to enhanced anthropogenic N loading to the estuary from runoff, atmospheric, or groundwater sources (Paerl 1988, Copeland and Gray 1991, Nixon 1995). In addition, sediment inputs, dispersal, and burial play integrative roles in mediating the bioavailability, cycling, and fate of N and other nutrients, including other inorganic N compounds (ammonium, NH_4^+), organic nitrogen, inorganic phosphorus (PO_4^{3-}) and organic phosphorus. Nitrate (NO_3^-) and PO_4^{3-} are the nutrients of choice for mesocosm experiments because these forms are the most abundant biologically-available, anthropogenically-derived nutrients impacting phytoplankton production and bloom dynamics in most estuaries.

In current Sea Grant-supported research we are evaluating ecosystem-level responses to acute vs. chronic N and P loading events using a replicated array of mesocosms. Effective and reliable methods for assessing relative responses among phytoplankton groups in natural communities in mesocosms have been developed and validated (Paerl et al. 1990, Rudek et al. 1991, Paerl et al. 1995, Pinckney et al. 1997). Outdoor mesocosms most closely approximate natural irradiance and temperature conditions, critical for evaluating natural phytoplankton community responses to nutrient enrichment. The mesocosm array is also well-suited for measuring differential community responses to varying nutrient loading rates. Evidence suggests that *Pfiesteria*-like dinoflagellates may respond to one or several such scenarios (Burkholder et al. 1993, 1995b, Burkholder & Glasgow 1995, 1997a,b). Therefore, we extended our work to include a better understanding of the ecological requirements of this group of planktonic organisms. Ongoing *in situ* mesocosm bioassays and year-round environmental monitoring of the lower Neuse River were used to examine the impacts of specific nutrient (NO_3^- and PO_4^{3-}) loading on phytoplankton community structure, growth, and bloom potentials, with special emphasis on the dynamics and environmental controls of *Pfiesteria*-like dinoflagellate zoospores.

MATERIALS AND METHODS

Water Collection Bulk water for all mesocosm bioassays was collected at a depth of 1-m along the southwestern shore (35.08° N, 77.00° W) of the Neuse River between Cherry Point and New Bern, NC (Fig. 1). This estuary experienced large fish kills in 1991, 1995, 1996, and 1997 which were reportedly associated with relatively high densities of *Pfiesteria*-like dinoflagellates

(Burkholder et al. 1995b, Burkholder & Glasgow 1997b). Water was pumped into a pre-cleaned (flushed with river water) trailer-mounted 4500 liter (inert polyethylene) tank and transported to the Institute of Marine Sciences (IMS). A large volume diaphragm pump, which is non-destructive to phytoplankton and zooplankton (Miller & Judkins 1991), was used to fill the trailer tank. Bulk water from the trailer tank was administered (within 2 h of collection) to 36 translucent (85% PAR transmittance) fiberglass tanks (55 liters) arranged in a concrete pond at IMS. The pond was filled with seawater from the adjacent Bogue Sound for temperature and light control during the incubation period. Each treatment was assigned a specific water sampler to minimize cross-contamination between tanks. Glassware and plasticware were acid-washed (0.1 N HCl) and rinsed with deionized water before sample collection.

Mesocosm Experimental Design The purpose of these experiments was to provide a range of potential phytoplankton prey species, biomass, and environmental conditions to determine their effects on the abundance of *Pfiesteria*-like zoospores. Tanks were assigned to 12 replicated treatment groups using a random number table. Treatment groups, factors, and factor levels are summarized in Table 1. Mixing was achieved by a gentle air stream flowing from a small pipe in the bottom of the tank. Sediment additions consisted of surface sediments collected from a water depth of 1.5 m at the water collection site in the Neuse River Estuary. Nutrient enrichments [10 μM NO_3^- (140 mg N m^{-3}) and 3 μM PO_4^{3-} (93 mg P m^{-3}), final concentrations], reflecting concentrations commonly encountered in the estuary, were administered to the respective treatments in the early morning (0800) on specified days (Table 2).

For statistical analyses, responses (productivity, photopigments, etc.) to the manipulated factors were analyzed using a General Linear Model (GLM) Repeated Measures analysis of variance (ANOVA) with three fixed factors (mixing, sediment, nutrients) (Neter et al. 1985). Each tank was treated as a single case with repeated measures at fixed time intervals. Time intervals for repeated measures consisted of days 0, 1, and 3 for primary productivity; days 0, 1, 3, and 6 for photopigments; and days 0 and 3 or 6 for *Pfiesteria*-like cell counts. All data were ln-transformed before analysis to satisfy the normality assumption. Equality of error variances was checked using Levene's Test (Neter et al. 1985). Type IV sums of squares method was used because of missing data for some time points. The Games-Howell test ($\alpha = 0.05$) was used for *post hoc* multiple comparisons of means for factors without significant interaction terms (Neter et al. 1985).

Physical Measurements Salinity, specific conductivity, temperature, pH, and dissolved oxygen measurements were obtained near noontime at the surface (0.25 m below) and bottom (0.25 m above) of each tank using a Hydrolab H20 water quality monitor. Biweekly to weekly surveys of

water column physical/chemical/biological characteristics were obtained at a fixed site in the Neuse River (navigation marker 15; 35.014° N, 76.960° W) near the mesocosm water collection site (Fig. 1). Vertical profiles of conductivity, salinity, temperature, dissolved O_2, and pH at 0.5 m intervals throughout the water column were obtained with the Hydrolab water quality monitor. Water samples were collected near the water surface (0.5 m depth) and at depth (0.5 m from the bottom), transferred to acid-cleaned 20 L carboys, and kept cool and shaded during transport to the laboratory. Subsamples for nutrient and photopigment analyses were removed from bulk water samples within 3 h of collection.

Nutrient Analyses Water samples (50 - 100 ml) were filtered through pre-combusted (500°C, 16 h) 25 mm Whatman GF/F glass-fiber filters before chemical analyses. Nitrite + nitrate (NO_2^- + NO_3^-), ammonium (NH_4^+), and dissolved inorganic phosphate (PO_4^{-3}) were quantified with a Lachat AutoAnalyzer (Quikchem 8000) using standard protocols (Lachat Quikchem methods 31-107-04-1-C, 31-107-06-1-A, 31-115-01-3-C, respectively).

Phytoplankton Photopigments Chlorophylls and carotenoids were identified and quantified using high performance liquid chromatography (HPLC) (Millie et al. 1993, Tester et al. 1995, Jeffrey et al. 1997). Aliquots (0.3 to 1 L) of water were filtered under a gentle vacuum (<50 kPa) onto 4.7 cm dia. glass fiber filters (Whatman GF/F), immediately frozen, and stored at -80 °C. Frozen filters were placed in 100% acetone (3 ml), sonicated, and extracted at -20 °C for 12 - 20 h. Filtered extracts (200 µl) were injected into a Spectra-Physics HPLC equipped with a single monomeric (Rainin Microsorb-MV, 0.46 x 10 cm, 3 mm) and two polymeric (Vydac 201TP, 0.46 x 25 cm, 5 mm) reverse-phase C_{18} columns in series. This column configuration was devised to enhance the separation of similar photopigments and degradation products. Monomeric columns provide strong retention and high efficiency, while polymeric columns select for similar compounds with minor differences in molecular structure and shape (Van Heukelem et al 1994, Jeffrey et al. 1997). A nonlinear binary gradient, adapted from Van Heukelem et al. (1994), was used for pigment separations (for details, see Pinckney et al. 1996). Solvent A consisted of 80% methanol : 20% ammonium acetate (0.5 M adjusted to pH 7.2) and solvent B was composed of 80% methanol : 20% acetone. Absorption spectra and chromatograms (440 nm) were acquired using a Shimadzu SPD-M10av photodiode array detector. Pigment peaks were identified by comparison of retention times and absorption spectra with pure crystalline standards, including chlorophylls *a*, *b*, β-carotene (Sigma Chemical Company), fucoxanthin, and zeaxanthin (Hoffman-LaRoche and Company). Other pigments were identified by comparison to extracts from phytoplankton cultures (Wright et al. 1991)

and quantified using the appropriate extinction coefficients (Mantoura & Llewellyn 1983, Rowan 1989, Jeffrey et al. 1997).

Phytoplankton Productivity A single subsample (150 ml) of water was collected from mid-depth of each mesocosm tank and dispensed in clear polycarbonate bottles for phytoplankton primary productivity measurements. In addition, subsamples from 12 tanks were randomly selected for determination of dark uptake rates. Samples were injected with $NaH^{14}CO_3$ (185 - 260 kBq ml^{-1} final activity) and incubated in respective mesocosm tanks. Productivity incubations of 3 to 4 h (centered around local noon) were completed for each tank. After incubation, phytoplankton were filtered onto 25 mm GF/F filters, air dried, and fumed with concentrated HCl to remove unincorporated ^{14}C. Filters were then placed in vials containing scintillation cocktail (Ecolume, ICN, Inc.) and the counts per minute (CPM) enumerated with a Beckman model LS5000TD liquid scintillation counter. CPM were converted to disintegrations per minute (DPM) using quench curves constructed from a calibrated ^{14}C-toluene standard. Dissolved inorganic carbon in water samples was determined by infrared gas analysis (Beckman model 864 IRGA) (Paerl 1987).

***Pfiesteria* Taxonomy** Several species of small armored dinoflagellates (5-20 μm) are known to co-occur at fish kill sites (see Steidinger et al. 1997, Appendix I). Some of these dinoflagellates are heterotrophic and polymorphic with thin thecal plates (lightly armored) and may be ichthyotoxic. The nature of the toxins and toxicity of these "look-alikes" is unclear at this time (Burkholder & Glasgow 1997b, Steidinger et al. 1997). Although these small dinoflagellates resemble *Pfiesteria piscicida*, they cannot be distinguished from *P. piscicida* using a light microscope (Landsberg et al. 1995, Steidinger et al. 1996a). Currently, the only method available to definitively identify these small dinoflagellates and distinguish them from *P. piscicida* is a time-consuming, labor intensive and costly chemical fixation followed by examination of photographic images obtained by scanning electron microscopy (SEM) (Steidinger et al. 1996a, 1996b, Truby 1997). In this report, biflagellated zoospores of heterotrophic dinoflagellates that could not be distinguished from *Pfiesteria piscicida* using light microscopy were counted and identified as *Pfiesteria*-like cells. Therefore the cell counts reported in the present study are likely upper estimates of the actual abundance of *P. piscicida* zoospores.

Culturing Enrichment protocols used were developed in the laboratory (E. Haugen & P. Tester, pers. comm.) to establish monocultures of *Pfiesteria*-like zoospores. Water collected from the Neuse River mesocosms was prefiltered through 20 μm nitex mesh and enriched with an algal prey mixture. Optimal growth was achieved using a mixture of *Isochrysis* (a 3-4 μm prymnesiophyte) and a cryptomonad (10 μm phytoflagellate). *Pfiesteria*-like zoospores exhibited typical growth rates of 1

division day^{-1} under culture conditions. Seven cultures, obtained from representative mesocosm bioassay dates, were used for scanning electron microscope (SEM) analyses and species identification. Direct SEM analyses of natural water samples was difficult because of low *Pfiesteria*-like cell numbers, heavy accumulations of suspended sediment, and large numbers of other microalgal species.

Cell Counts Water samples from each mesocosm tank were collected in opaque polyethylene bottles, preserved with an acetate-buffered Lugol's solution (Utermöhl 1958) and stored at 4°C until counting in the laboratory. This slightly alkaline (pH ≈ 7.5) Lugol's recipe is an excellent preservative for delicate dinoflagellates such as *Gymnodinium breve* (P.Tester, pers. comm.) and has been tested on cultured *Pfiesteria*-like dinoflagellates and amoebae in the lab. Enumerations were undertaken for 24 of the 36 tanks in each experiment and included 2 replicates for each of the experimental manipulations. Subsamples of 5 - 25 ml were settled in 25 ml Hydrobios settling chambers for a minimum of 12 hours to quantify *Pfiesteria*-like dinoflagellates using the Utermöhl method (Utermöhl 1931). Maximum sample size was restricted by the amount of sediment and plankton biomass in the water. A minimum of one half of each chamber was examined on an ausJena Sedival inverted microscope (300 x) to quantify cells. Standard light optics assisted in the location and identification of the colorless dinoflagellate in the iodine-staining preservative. Cells were not readily distinguishable for counting purposes with phase-contrast optics (P. Tester, pers. comm.). *Pfiesteria*-like cell counts were based on enumerations of the dinoflagellate biflagellated zoospore stage that appeared consistent with those reported for *Pfiesteria piscicida* at the light microscope level. *Pfiesteria*-like cells were always present in numbers typical for heterotrophic cells of this size range (6-10 μm) in the Neuse estuary.

SEM Preparations and Species Identification Culture isolates of *Pfiesteria*-like biflagellated zoospores from each of the 7 mesocosm bioassays were sent to Dr. Richard G. Zingmark (University of South Carolina, Columbia, SC) for species identification using SEM. Samples were prepared for SEM using the protocols outlined by Truby (1997). Briefly, the technique involves removing the outer membrane (amphiesma) using an ethanol dehydration series to expose the cell plates (theca). Samples were then fixed with a glutaraldehyde and osmium tetroxide fixative adjusted to the proper osmolality and critical-point-dried. SEM micrographs were obtained at 4,000 x to 10,000 x and plate counts determined after examining several cells in different orientations. The plate counts for all mesocosm isolates were identical, but did not match the published plate counts for *Pfiesteria piscicida* (Steidinger et al. 1996). Dr. Karen Steidinger (Florida Institute of Marine Research, St. Petersburg, FL) has examined the *Pfiesteria*-like cell found in our

samples (referred to as cell "H"in Appendix I) and compared our isolate with other "look-alike" dinoflagellates (Landsberg et al. 1995). For the purpose of this report, the *Pfiesteria*-like cells enumerated in the current study are a "peridiniopsoid" to be placed in a new genus (Steidinger et al. 1997). This peridiniopsoid dinoflagellate (cell "H") has 5 apical, 6 precingular, and no anterior intercalary plates (Po, cp, X, 5', 0a, 6", 6?c, 4s, 5"',0p, and 2"") differing from the tabulation for *Pfiesteria piscicida* (Po, cp, X, 4', 1a, 5", 6c, 4s, 5"', 0p, and 2"") (Steidinger et. al. 1996a, 1997). Cell "H" has been isolated from known fish kill sites in the Pamlico and Neuse Rivers and has been identified from fish kill areas in Maryland and from a fish lesion area in northeast Florida (Steidinger et al. 1997).

RESULTS

Incubation Conditions

Mesocosm bioassay experiments were conducted on 7 occasions from March 1996 to August 1997 (Table 2). The salinity of water in the mesocosm tanks ranged from 0.1 to 5.2 psu during the experimental period and mean salinities varied depending on incubation dates (Table 3). Salinities were similar (± 1 SD < 0.9) between tanks for each bioassay. The pH ranged from 7.42 to 9.66 but was generally between 8 and 9 for all experiments. Total dissolved inorganic carbon (DIC) fluctuated between tanks and experiments, reflecting inorganic carbon uptake and productivity of phytoplankton. These environmental conditions were similar to *in situ* conditions in the Neuse River during respective experiments (see Fig. 5).

Dissolved oxygen (O_2) concentrations were near saturation in all tanks throughout the experimental period (Table 4). Mean surface (0.25 m below surface) values ranged from 7.37 to 11.44 and bottom values (0.25 m above bottom) ranged from 7.13 to 11.63 mg O_2 liter^{-1}. Surface and bottom O_2 concentrations in the static (non-mixed) mesocosms were compared using a paired samples t-test to determine if mixing state affected O_2 distributions. In the absence of mixing, bottom water O_2 concentrations were significantly higher than near surface values for both sediment ($N = 265$, $p<0.01$) and no sediment treatments ($N = 268$, $p<0.01$). However, the mean difference between surface and bottom O_2 was <0.5 mg O_2 liter^{-1} for all static tanks. Checks of dissolved O_2 in the early morning (just after sunrise) indicated that tanks were not hypoxic/anoxic at night.

Nutrients

For the first 3 experiments, nutrients were administered as a single dose at the initiation of the experiment (Table 2). Rapid uptake by phytoplankton within the first 48 h of the experiment

resulted in depletion of NO_3^- (Figs. 2a, 2b). Both the addition frequency and total amounts of NO_3^- and PO_4^{3-} were increased for subsequent experiments (Table 2). Phosphate (PO_4^{3-}) concentrations in amended treatments were maintained at levels higher than the control or nitrate (NO_3^-) amended treatments for all experiments (Figs. 3a, 3b). The concentrations of ammonium (NH_4^+), an additional source of N for phytoplankton, increased in the October 1996 mesocosm experiment, possibly indicating high zooplankton grazing rates (Figs. 4a, 4b). The range of nutrient and salinity exposure levels in the mesocosm tanks were representative of *in situ* conditions in the Neuse River during respective incubation periods (Fig. 5). The mesocosm tanks effectively simulated a realistic range of potential chemical/physical conditions likely encountered by phytoplankton communities in this region of the Neuse River during the study period.

Phytoplankton Biomass and Productivity

Phytoplankton responses to the manipulated factors (mixing, sediment, nutrients) are summarized in Table 5. A repeated measures ANOVA analysis was used to compare the responses of individual tanks (cases or subjects) to the three factors (mixing, sediments, nutrients) for the duration of the incubation for all seven bioassays combined. Although two-way and three-way interaction terms were computed, no significant interactions were detected ($p < 0.05$). Significant effects for the nutrient treatments (control, NO_3^- addition, and $NO_3^- + PO_4^{3-}$ addition) were further analyzed using the Games-Howell procedure to determine significant differences between treatment means (Table 5).

The graphical results for phytoplankton biomass (chlorophyll *a*, Chl *a*) (Fig. 6a, 6b) and primary productivity (Fig. 7a, 7b) at the initiation of the incubation and two other time intervals are shown for each experiment. In general, both biomass and productivity peaked within 2 days of the last nutrient addition and declined for the remainder of the incubation period. Phytoplankton biomass (Chl *a*) increases were significantly higher in mixed tanks in comparison with static (un-mixed) treatments (Table 5, Fig. 6a, 6b). The NO_3^- and $NO_3^- + PO_4^{3-}$ additions resulted in significantly higher phytoplankton biomass than unamended control treatments. However, the $NO_3^- + PO_4^{3-}$ additions were not significantly different from the NO_3^- additions. These results indicate that N was consistently the limiting nutrient for phytoplankton growth. Phosphorus was not limiting or co-limiting for any of the experiments. Phytoplankton primary productivity was higher in the mixed tanks than the static tanks (Table 5). No significant differences in productivity were detected for the sediment vs. no sediment treatments. Nitrate-amended tanks had a higher productivity than non-amended control tanks. Similar to the biomass response, primary

productivities of phosphate amended tanks were not significantly different from the nitrate amended tanks, demonstrating that P was not limiting.

Microalgal Group-Specific Responses

Chemosystematic photopigments (chlorophylls and carotenoids) characteristic for different microalgal taxa were used to assess the relative responses of groups to experimental manipulations. Qualitative microscopic examinations of mesocosm samples indicated that chlorophytes, diatoms, cryptomonads, and cyanobacteria were the numerically-abundant algal groups present in the phytoplankton community. The relative abundance of chlorophytes, as indicated by the photopigment chlorophyll *b*, was highest in the July 1996, October 1996, and March 1997 experiments (Fig. 8a, 8b). Mixed conditions and NO_3^- additions promoted the highest chlorophyte biomass (Table 5). Diatom (fucoxanthin) abundance was higher in the sediment amended tanks and mixed tanks, possibly because of the added contribution of benthic diatoms associated with the sediment (Fig. 9a, 9b; Table 5). Diatom response to NO_3^- additions was rapid and persistent for the duration of the incubation period. This response was most clearly reflected in the March 1997 bioassay (Fig. 9b). Cryptomonads (alloxanthin) were present at moderate abundances for all mesocosm assays and reached peak values in the March 1997 experiment (Fig. 10a, 10b). The mixed tanks produced significantly higher cryptomonad biomass than the static tanks. In contrast to the other algal groups, cryptomonad biomass in the nutrient amended treatments was not significantly higher than the control treatments. Cyanobacterial abundance (zeaxanthin) was highest in the summer (July 1996, May 1997, August 1997) (Fig. 11a, 11b). Nitrate amended tanks produced significantly higher cyanobacterial biomass than control (non-amended) tanks (Table 5). For all algal groups, the PO_4^{3-} additions did not elicit responses distinguishable from those of NO_3^- additions alone.

Pfiesteria-like Zoospore Responses

The biflagellated zoospore stage of *Pfiesteria*-like cells was present in all mesocosm experiments (Fig. 12a, 12b). Although samples from the May 1997 experiment were examined for the presence of *Pfiesteria*-like cells, quantitative enumerations were not undertaken due to low cell abundances. *Pfiesteria*-like cell counts were highest (50 - 100 cells ml^{-1}) in the July 1996 and August 1997 experiments and at densities less than 6 cells ml^{-1} for the other incubations. Cell counts for similar-sized phytoplankton and dinoflagellate species ranged from 1000 - 10,000 cells ml^{-1}. *Pfiesteria*-like zoospores therefore constituted a small proportion of the total number of planktonic

cells. In some experiments and treatments, cell numbers increased or remained constant for the length of the incubation period (Fig. 12a, 12b). *Pfiesteria*-like cells did not show a significant response to nutrient, sediment, or mixing treatments in any of the experiments (Table 5).

The abundance of *Pfiesteria*-like cells was positively correlated with phytoplankton biomass and productivity (Fig. 13). Spearman rank correlation coefficients (a nonparametric measure of the strength of the relationship between two variables) were calculated for *Pfiesteria*-like cell counts and phytoplankton group-specific pigment concentrations. The abundance of *Pfiesteria*-like cells was positively correlated with primary productivity, total phytoplankton biomass (Chl *a*), cryptomonads (alloxanthin), cyanobacteria (zeaxanthin), and chlorophytes (Chl *b*) ($N = 278$, $p < 0.001$). *Pfiesteria*-like cell abundance reflected the relative abundance of potential phytoplankton prey species. No significant correlations ($p < 0.01$) were detected for *Pfiesteria*-like cells and nutrient concentrations (NO_3^-, NH_4^+, PO_4^{3-}), salinity, pH, or diatom biomass (fucoxanthin).

DISCUSSION

The physical, chemical, and environmental conditions in the mesocosms reflected the range of *in situ* water column properties in the Neuse River during the incubation period. This segment of the Neuse River has experienced large fish kills in past years (1991, 1995, 1996, 1997) that have been associated with the presence of *Pfiesteria*-like cells (Burkholder et al. 1995b, Burkholder & Glasgow 1997b). The water collection site for the mesocosm incubations was selected because of repeated instances and persistence of reported *Pfiesteria* fish kills in this area. Under natural conditions, a major factor that may regulate the abundance of heterotrophic *Pfiesteria*-like dinoflagellates is the availability of phytoplankton prey (Burkholder & Glasgow 1995, Burkholder et al. 1995b). Elevated nutrient concentrations have also been reported to stimulate growth of *Pfiesteria piscicida* (Burkholder et al. 1992, 1993, Glasgow et al. 1995, Burkholder & Glasgow 1995). The mesocosm bioassays were designed to provide a range of currently encountered physical, nutrient, and phytoplankton conditions to determine the potential role of key nutritive factors in regulating the abundance of the non-toxic biflagellated zoospore stage of *Pfiesteria*-like cells.

The experimental approach for this project emphasized the use of natural water samples collected from the Neuse River Estuary during an 18-month period. Since environmental conditions (weather, rainfall, salinity, etc.) in the Neuse River Estuary could not be manipulated *a priori*, the strategy for sampling was based on the characterization of *Pfiesteria*-like zoospore responses at a site where the negative impacts of this organism have been reported. Therefore, the

mesocosm bioassays assessed *Pfiesteria*-like zoospore responses within the constraints of the natural environmental conditions experienced in the Neuse River Estuary during 1996 - 1997. Although salinities were relatively low (< 3 psu) during some experiments, growth and toxin production of *P. piscicida* and *Pfiesteria*-like zoospores has been observed in 0 psu salinity waters (Burkholder & Glasgow 1997b). Major alterations in environmental conditions (higher salinity, warmer temperatures, calm weather, algal blooms) within the estuary could conceivably create conditions more conducive for the growth of heterotrophic dinoflagellates. However, the characterization of growth responses of *Pfiesteria*-like zoospores to all possible combinations of regulating factors and variables was not the objective of this project. Instead, the experimental approach relied on the manipulation of three selected variables (mixing, sediments, and inorganic nutrients) which were constrained and dictated by the environmental conditions within the Neuse River Estuary during the study period.

There is no evidence that water collection methods employed in this study damaged fragile plankton species or had a significant negative impact on abundance. Qualitative microscopic examinations of pre- and post-collection water samples did not reveal any obvious differences in species composition. The abundance of *Pfiesteria*-like zoospores in the mesocosm tanks at the beginning of the incubations was similar to *in situ* abundances at the site where water was collected (E. Haugen & P. Tester, pers. comm.). At the start of the experiments, other small dinoflagellates (*Katodinium* sp.) were also present in mesocosm tanks at concentrations similar to those found in the estuary (E. Haugen, pers. comm.). Comparisons of HPLC-derived photopigment concentrations showed that the overall phytoplankton community composition was not significantly altered by pumping and transportation. Burkholder & Glasgow (1997b) report that turbulence slows the growth of *Pfiesteria*-like zoospores. The experimental manipulations in the present study included both turbulent (mixed) and non-turbulent (static) treatments to examine the role of turbulence as a regulator of zoospore abundance. The absence of a detectable difference between static and mixed treatments suggests that turbulence did not affect the abundance of *Pfiesteria*-like zoospores.

Previous experiments (including the current study) indicated that six days was sufficient to quantify and characterize Neuse River Estuary phytoplankton community responses to manipulative experiments (Paerl & Bowles 1987, Rudek et al. 1991). The microalgal community in mesocosm tanks showed rapid (1 - 3 days) increases in biomass, productivity, and changes in taxonomic composition following nutrient additions. The range of phytoplankton responses in the different treatments presented an abundant and diverse food source for *Pfiesteria*-like zoospores. Changes in the phytoplankton community within the mesocosms closely simulated natural bloom events that

occur in the Neuse River Estuary following nutrient inputs from rainfall events and subsequent discharge (Christian et al. 1991, Rudek et al. 1991, Paerl et al. 1995, Pinckney et al. 1997, 1998). Phytoplankton community responses in the mesocosm bioassays conducted in this project were consistent with field observations in long-term studies of the Neuse River (Paerl et al. 1995, Pinckney et al. 1997). Incubations were limited to six days because the utility of mesocosm-based experiments is compromised by atypical conditions (i.e., algal growth on tank walls, nutrient depletion, overgrazing, pH, [DIC], etc.) in the tanks after this period.

The duration of the mesocosm incubations (6 days) should have allowed sufficient time to evaluate the growth responses of *Pfiesteria*-like zoospores. Heterotrophic dinoflagellate zoospores similar in size and feeding rate to *P. piscicida* typically have growth rates (μ) of 0.5 to 1.0 d^{-1} (Strom 1991, Hansen 1992, Strom & Buskey 1993, Jakobsen & Hansen 1997). In addition, previous studies of *P. piscicida* cultures in nutrient enrichment experiments showed significant (2 to 10 fold) increases in zoospore counts within 4 to 7 days (Burkholder et al. 1993, Glasgow et al. 1995, Burkholder & Glasgow 1997b). Under culture conditions with a suitable diet of phytoplankton prey, the growth rates of *Pfiesteria*-like biflagellated zoospores obtained from the present study were ca. 0.8 - 1.0 d^{-1} (Tester & Haugen, pers. comm.). Increases in the abundance of *Pfiesteria*-like zoospores in some experimental bioassays (March, May, and October 1996 & March 1997) clearly indicate that the duration of the mesocosm experiments was adequate for assessing responses of zoospores to experimental conditions. The differences in responses between culture and mesocosm conditions suggests that factors other than nutrients or short-term increases in phytoplankton prey species may regulate the natural abundance of *Pfiesteria*-like biflagellated zoospores.

The phytoplankton community responses to the manipulated variables indicated that, in general, biomass and productivity were consistently N-limited in all experiments. The inability to demonstrate different responses for the NO_3^- and the $NO_3^- + PO_4^{3}$ treatments suggested that P was not limiting for phytoplankton growth. These results support previous nutrient bioassay and uptake kinetics results for Neuse River phytoplankton (Paerl 1987, Stanley 1988, Rudek et al. 1991, Boyer et al. 1994, Paerl et al. 1995). The duration of the mesocosm bioassay incubations (6 days) was sufficient to allow at least a 3 fold increase in phytoplankton biomass. Phytoplankton communities in some of the mesocosm tanks bloomed following nutrient additions and subsequently "crashed" when nutrients became limiting. The increase in ammonium (NH_4^+) concentrations in some of the experiments may have indirectly reflected the response of zooplankton and microheterotroph grazers, which excrete NH_4^+, to enhanced availability of phytoplankton prey.

Chlorophytes, diatoms, and cyanobacteria exhibited significant increases in biomass in response to NO_3^- additions. Phosphate (PO_4^{3-}) alone did not seem to influence the abundance of any single microalgal group. Diatoms responded rapidly (with 1 day) to NO_3^- additions and biomass was consistently highest in the mixed tanks. The mixed tanks also promoted higher chlorophyte and cryptomonad biomass. The sediment addition treatments supported higher diatom biomass but did not have a significant effect on other algal groups, total biomass (Chl *a*) or primary productivity. Collectively, these data suggest that the phytoplankton community exhibits higher growth under mixed conditions and NO_3^--enhanced concentrations.

Benthic sediments collected from the Neuse River were added to half the mesocosm tanks to provide a potential source for *Pfiesteria*-like zoospore precursor stages (cysts, amoebae). The abundance of *Pfiesteria*-like flagellated zoospores in the sediment-treated tanks did not differ from tanks without sediment additions. These results suggest that the presence of sediments from a fish kill area had no significant impact on the abundance of *Pfiesteria*-like zoospores. In contrast, diatom biomass was significantly higher in the mesocosms that received sediment additions. Diatoms form a major component of benthic microalgae in shallow Neuse River sediments (Rizzo et al. 1992, Pinckney & Zingmark 1993). Resuspension of benthic or deposited diatoms and subsequent growth resulting from the sediment additions could explain the higher diatom biomass in the tanks receiving sediments.

The mesocosm bioassay array provided a range of suitable physical-chemical conditions and prey species for *Pfiesteria*-like cells. Heterotrophic dinoflagellates, including *Pfiesteria*-like zoospores, graze on a variety of phytoflagellates (chlorophytes, cryptomonads, prymnesiophytes, other dinoflagellates) and coccoid cyanobacteria (Fields & Rhodes 1991, Hansen 1991, Burkholder & Glasgow 1995, 1997b, Mallin et al. 1995). Taxa for all of these algal groups were present in a range of concentrations, providing a rich and diverse food source for *Pfiesteria*-like zoospores. In some experiments, there were detectable increases in the number of *Pfiesteria*-like cells, indicating that the mesocosms were capable of supporting and enhancing the growth of heterotrophic dinoflagellates. Zooplankton and ciliate grazers, which readily consume *Pfiesteria*-like zoospores (Burkholder & Glasgow 1995, Mallin et al. 1995), may play a major role in regulating the abundance of heterotrophic dinoflagellates and could explain the high mortality/low cell counts observed in some experiments (Hansen 1991, Jakobsen & Hansen 1997). Among the three manipulated factors in the experiment, there was no significant positive or negative effect on the abundance of *Pfiesteria*-like biflagellated zoospores. The absence of a significant *Pfiesteria*-like cell response to the nutrient

treatments suggests that NO_3^- and PO_4^{3-} concentrations similar to those encountered *in situ* in natural environments do not increase the abundance of *Pfiesteria*-like biflagellated zoospores.

Although cell numbers were low throughout the experimental period, the abundance of *Pfiesteria*-like flagellated zoospores was positively correlated with phytoplankton biomass and productivity. Comparisons with the relative abundance of other microalgal groups suggest that *Pfiesteria*-like zoospore counts tracked phytoflagellates (chlorophytes and cryptomonads) and cyanobacteria, which are known prey items for heterotrophic dinoflagellates. However, there was no significant correlation between *Pfiesteria*-like zoospores and diatom biomass. The zoospore stage of *Pfiesteria piscicida* feeds on algal cells using a peduncle to ingest cellular contents (Spero 1981, Steidinger et al. 1996a, Burkholder & Glasgow 1997b). Diatoms, which have a silica frustule, may be protected from this mode of grazing. The Neuse River Estuary experiences large blooms of phytoflagellates and cyanobacteria that closely follow discharge-related nutrient pulsing events in the spring and summer (Mallin et al. 1993, Mallin 1994, Paerl et al. 1995). These blooms provide a periodic, abundant food source that supports the growth and abundance of *Pfiesteria*-like dinoflagellates. Carbon loading by phytoplankton production followed by bloom senescence promotes oxygen depletion (Paerl & Pinckney 1996). Therefore it is reasonable to suggest that the co-occurrence of high abundances of *Pfiesteria*-like dinoflagellates and hypoxia/anoxia could be explained based on nutrient-driven phytoplankton bloom dynamics in the Neuse River Estuary.

The absence of significant responses of *Pfiesteria*-like zoospores to the manipulated variables in the mesocosm bioassays (Table 5) and the significant non-parametric correlations between *Pfiesteria*-like zoospore counts and phytoplankton groups (Fig. 13) may seem contradictory. However, these results provide valuable insights into other factors that may regulate the abundance of *Pfiesteria*-like zoospores in this estuary. The mesocosm bioassays simulated short-term bloom events that frequently occur in the Neuse River Estuary in response pulsed nutrient inputs (Pinckney et al. 1997). This estuary also experiences more chronic high abundances of some phytoplankton groups (cryptomonads, cyanobacteria, chlorophytes) during summer months (Pinckney et al. 1997, 1998). The correlations between the abundance of *Pfiesteria*-like zoospores and different phytoplankton groups can be attributed to measurements obtained at the start (time 0) of the mesocosm bioassays. Therefore, these correlations may reflect longer-term (months) changes in the seasonal abundance of *Pfiesteria*-like zoospores that closely track seasonal changes in phytoplankton abundance and composition. Another, equally plausible explanation is that the abundances of phytoplankton and *Pfiesteria*-like zoospores are autocorrelated. For example, secondary factors

(meteorological conditions, temperature, salinity, microheterotroph grazers, etc.) may regulate the seasonal abundance of both phytoplankton and heterotrophic dinoflagellates.

Implications for Nutrient Management

Results indicate that a consistently low density (<100 cells ml^{-1}) of *Pfiesteria*-like cells were present throughout the 18 month sampling and mesocosm bioassay period in a location of the Neuse River estuary which, over the past decade, has exhibited symptoms of accelerating eutrophication; including increased frequencies, magnitudes, and duration of phytoplankton blooms, hypoxia (<4 mg O_2 $liter^{-1}$), anoxia (no detectable O_2), and fish kills. Neuse estuary phytoplankton production and bloom dynamics are dominated by several species of photosynthetic dinoflagellates (e.g., *Heterocapsa triquetra*, *Gymnodinium* spp.), cryptomonads (*Cryptomonas* spp.) and coccoid cyanobacteria (*Synechococcus* spp., *Synechocystis* spp.), with diatoms and chlorophytes present in non-bloom proportions (Mallin 1994). These taxa uniformly showed a strong positive response to nitrogen (as NO_3^-) enrichment approximating current loading events, while being relatively insensitive to P (PO_4^{3-}) enrichment, indicating persistent N limitation and P sufficiency. These responses are similar to earlier findings of Paerl (1987), Rudek et al. (1991), and Boyer et al. (1993, 1994), confirming at least a two decade long history of N limitation in this eutrophying system (Hobbie & Smith 1975, Stanley 1983, Paerl 1983, 1987, Christian et al. 1991, Paerl et al. 1995). These taxa typically proliferate in cell numbers exceeding 10,000 cells ml^{-1} and even higher amounts during active blooms. In some years (c.f. 1991-1994; Paerl et al. 1995) as much as 50% of the annual productivity can be attributed to such bloom events. Therefore, these taxa represent the dominant autochthonous source of biologically available carbon supporting heterotrophic *Pfiesteria*-like (and possibly other non-photosynthetic dinoflagellates), and other heterotrophic microalgae, bacteria, and fungi responsible for O_2 consumption associated with periods of hypoxia and anoxia. While *Pfiesteria*-like cells did not show direct or indirect responses to nutrient enrichment, they did "track" general trends in phytoplankton production in this estuary, indicating the source of organic nutrition supporting *Pfiesteria*-like cells is likely phytoplankton-based.

Clearly, nutrient input reduction is the only manageable option for stemming and potentially reversing water quality degradation of the Neuse as well as neighboring estuarine tributaries (Tar-Pamlico, Roanoke, Chowan) of the greater Albemarle-Pamlico Sound system. It seems logical, if not imperative, to target nutrient reductions at those phytoplankton taxa dominating primary production and the eutrophication process, since these taxa are the key source of organic matter supporting and exacerbating the unwanted consequences of eutrophication (i.e., hypoxia, toxic algal

species, fish kills, etc.), including *Pfiesteria*-like cells. Since *Pfiesteria*-like zoospores showed insignificant direct and indirect responses to enrichment of nutrients closely associated with human activities and sources in the Neuse Basin (inorganic N and P), while the main bloom-forming phytoplankton did, it seems prudent, and potentially most effective that nutrient input constraints focus on the main "players" driving the eutrophication process. In all likelihood, nutrient-reduction controlled growth and bloom constraints on taxa dominating the production process will translate into improved water quality conditions throughout the food web, starting at the microbial level.

A 30% reduction in N loading, accompanied by a nutrient loading cap (set at 1995 loading levels) has been recommended as an initial target for long-term reduction of eutrophication and achieving perceptible improvement of water quality in the Neuse estuary by consensus of the scientific and management community (North Carolina State Senate 1996). This reduction level may need further refinement pending ongoing research, modeling, projected water use patterns, hydrological modifications, and land-based nutrient management changes. Mesocosm-based results presented here indicate that, given current N limited conditions and N loading scenarios, such reductions will yield the desired impact of reducing growth potentials of dominant phytoplankton genera central to the eutrophication process. We conclude that reduction of growth and bloom potentials of these genera will translate into broad-based water quality improvement, including a declining trend in the frequency, spatial extent and magnitudes of nuisance algal blooms, O_2 depletion, and associated fish and shellfish mortality.

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Table 1. Experimental design for mesocosm bioassays.

Factor	Level	Description	Units
Mixing	Static	No mixing	18 Tanks
	Mixed	Bubbled Air	18 Tanks
Sediment	None		18 Tanks
	Addition	750 ml sediment per tank	18 Tanks
Nutrient	Control	No nutrients added	12 Tanks
	Nitrate	10 µM NO_3^- as KNO_3	12 Tanks
	Nitrate + Phosphate	10 µM NO_3^- as KNO_3 and 3 µM PO_4^{3-} as KH_2PO_4	12 Tanks

Table 2. Mesocosm bioassay dates, nutrient addition schedule, and total amount of nutrient added to each tank for respective treatment groups (see Table 1).

Date	Time Interval	Nutrient Addition Schedule	Total Nutrient Added
March 1996	5 - 11 March	day 0	560 µmol NO_3^- , 168 µmol PO_4^{3-}
May 1996	30 April - 6 May	day 0	560 µmol NO_3^- , 168 µmol PO_4^{3-}
July 1996	23 - 29 July	day 0	560 µmol NO_3^- , 168 µmol PO_4^{3-}
October 1996	22 - 28 October	day 0, 1, 2	1680 µmol NO_3^- , 504 µmol PO_4^{3-}
March 1997	11 - 17 March	day 0, 1, 2	1680 µmol NO_3^- , 504 µmol PO_4^{3-}
May 1997	28 May - 2 June	day 0, 1	1120 µmol NO_3^- , 336 µmol PO_4^{3-}
August 1997	19 - 25 August	day 0, 1, 2	1680 µmol NO_3^- , 504 µmol PO_4^{3-}

Table 3. Summary statistics for salinity, pH, and dissolved inorganic carbon (DIC) for each mesocosm experiment. Data are the pooled measurements for 36 mesocosm tanks over the 6 day incubation period.

	Salinity (psu)				pH				[DIC] (mg C liter^{-1})			
Date	***mean***	***±1 SD***	***min***	***max***	***mean***	***±1 SD***	***min***	***max***	***mean***	***±1 SD***	***min***	***max***
March 1996	3.4	0.9	2.8	5.2	8.10	0.65	7.41	9.32	8.21	1.04	6.10	9.57
May 1996	0.3	0.0	0.3	0.3	9.11	0.37	7.61	9.66	6.84	1.51	4.40	9.33
July 1996	0.7	0.0	0.7	0.7	8.15	0.26	7.52	8.59	10.67	0.53	9.95	11.21
October 1996	0.1	0.0	0.1	0.1	8.68	0.37	8.00	9.58	5.29	0.99	3.80	7.40
March 1997	0.1	0.0	0.1	0.4	8.94	0.39	8.32	9.63	6.89	0.73	4.30	8.90
May 1997	0.3	0.0	0.3	0.3	8.51	0.38	7.86	9.18	7.60	1.50	5.20	11.10
August 1997	4.2	0.1	4.1	4.3	8.55	0.31	7.49	9.20	9.71	0.97	7.80	12.70

Table 4. Summary statistics for surface (0.25 m below surface) and bottom (0.25 m above bottom) dissolved oxygen concentrations in the incubation tanks for each mesocosm experiment. Data are the pooled measurements for all 36 tanks over the 6 day incubation period.

	Surface O_2 mg liter^{-1}				Bottom O_2 mg liter^{-1}			
Date	***mean***	***±1 SD***	***min***	***max***	***mean***	***±1 SD***	***min***	***max***
March 1996	11.44	1.71	8.50	15.41	11.55	1.93	6.84	15.31
May 1996	9.28	1.51	6.08	13.23	9.72	1.89	5.83	13.46
July 1996	7.42	0.74	5.63	9.06	7.15	1.06	5.30	9.22
October 1996	8.75	0.66	7.13	9.92	9.79	0.41	8.34	10.73
March 1997	10.35	1.49	8.00	13.62	11.63	1.79	9.07	14.09
May 1997	8.79	0.62	7.79	10.37	8.59	0.67	7.20	9.97
August 1997	7.37	0.84	5.59	10.39	7.13	1.38	4.84	10.98

Table 5. Results of three factor repeated measures ANOVA for primary productivity, photosynthetic pigments (microalgal groups), and *Pfiesteria*-like biflagellate zoospore cell counts for the 7 mesocosm experiments. Samples sizes (cases) are indicated in the N column. Significant responses for the each of the three factors (mixing, sediment, nutrients) are denoted with the + symbols. Non-significant effects are indicated by the • symbol. The results of means comparisons for significant factor effects are given below each item and the highest level is indicated. For the nutrient treatment, the underline signifies homogeneous groups (not significantly different) and the group(s) with the highest mean(s) is listed first. Interaction terms were calculated for all analyses, but significant ($p < 0.10$) effects were not detected.

Variable	N	Mixing	Sediment	Nutrient
Primary Productivity	252	+	•	+ + +
means comparison		mixed		N NP C
Chlorophyll *a*	178	+ +	•	+ + +
means comparison		mixed		N NP C
Chlorophyll *b* (chlorophytes)	178	+ +	•	+
means comparison		mixed		N NP C
Fucoxanthin (diatoms)	178	+	+	+ + +
means comparison		mixed	sed added	N NP C
Alloxanthin (cryptomonads)	178	+ +	•	•
means comparison		mixed		
Zeaxanthin (cyanobacteria)	178	•	•	+
means comparison				N NP C
***Pfiesteria*-like Cell Counts**	135	•	•	•
means comparison				

+ = p < 0.10 + + = p < 0.01 + + + = p < 0.001

FIGURE LEGENDS

Figure 1. Map of the lower Neuse River showing the location of the water collection site and weekly/biweekly water quality monitoring site (navigation marker 15). The dashed line indicates the region of persistent fish kills since 1991.

Figure 2. Nitrite (NO_2^-) + Nitrate (NO_3^-) concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 3. Phosphate (PO_4^{3-}) concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 4. Ammonium (NH_4^+) concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 5. Salinity, nutrients, phytoplankton biomass (Chl *a*), and primary productivity at the water quality monitoring site (navigation marker 15) during the study period. Near surface values were obtained at 0.5 m below surface and near bottom values were 0.5 m above the bottom. The abbreviation NO_x^- signifies the sum of NO_3^- + NO_2^-.

Figure 6. Phytoplankton chlorophyll *a* concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 7. Phytoplankton primary productivity in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 8. Chlorophyte relative biomass (chlorophyll *b*) concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 9. Diatom relative biomass (fucoxanthin) concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 10. Cryptomonad relative biomass (alloxanthin) concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 11. Cyanobacteria relative biomass (zeaxanthin) concentrations in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 12. *Pfiesteria*-like flagellated zoospore cell counts in mesocosm bioassay tanks. Factor levels and treatments are detailed in Table 1. Values are mean ± 1 SD for triplicate samples.

Figure 13. Scatterplots of productivity, chlorophyll *a*, alloxanthin (cryptomonads), zeaxanthin (cyanobacteria), and chlorophyll *b* (chlorophytes) vs. *Pfiesteria*-like flagellated zoospore cell counts. Dashed lines indicate linear trends. Spearman rank correlation coefficients (ρ, $p<0.01$) are displayed for associations in each graph.

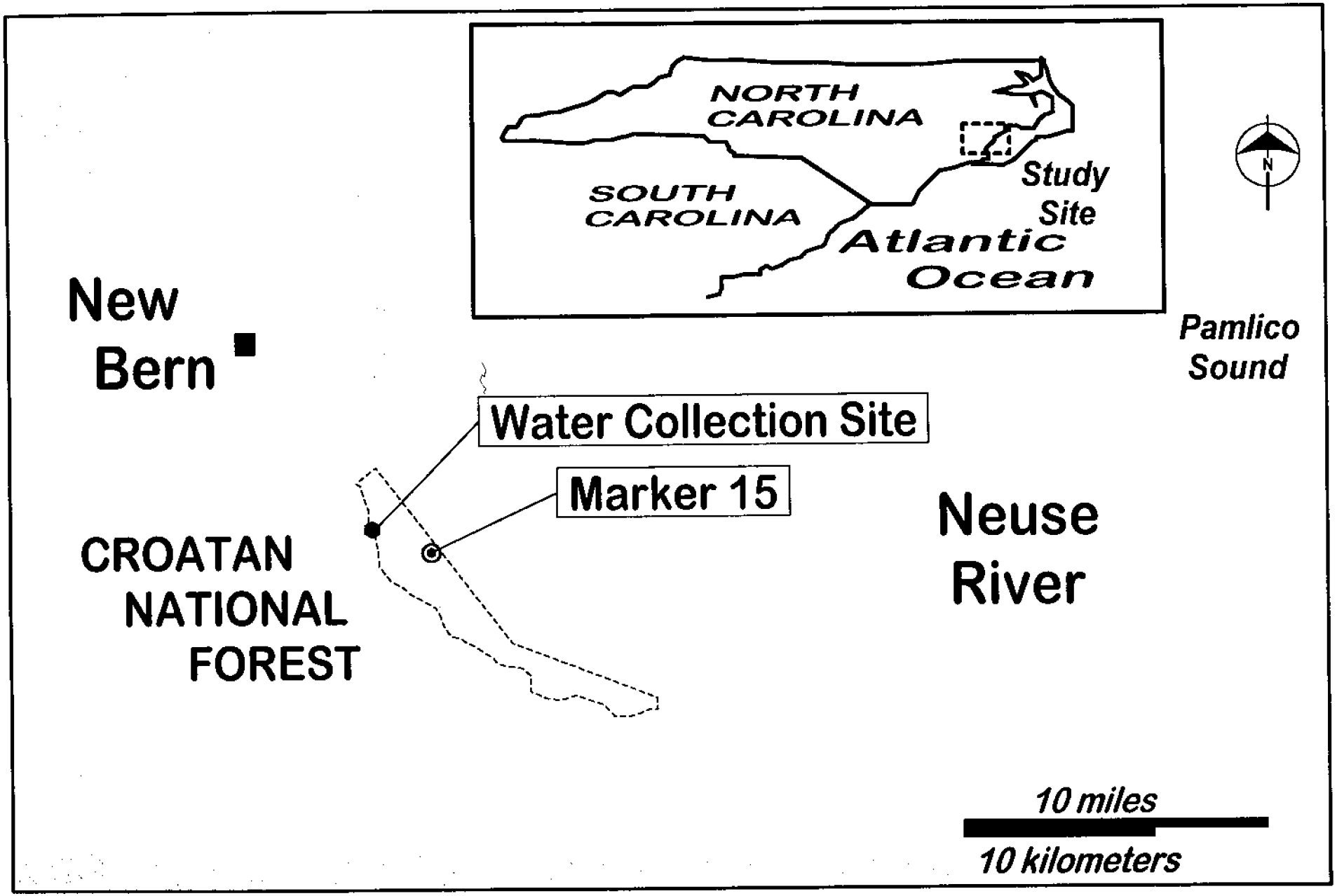

NORTH CAROLINA
SOUTH CAROLINA
Study Site
Atlantic Ocean
N
New Bern
Pamlico Sound
Water Collection Site
Marker 15
CROATAN NATIONAL FOREST
Neuse River
10 miles
10 kilometers

Figure 1

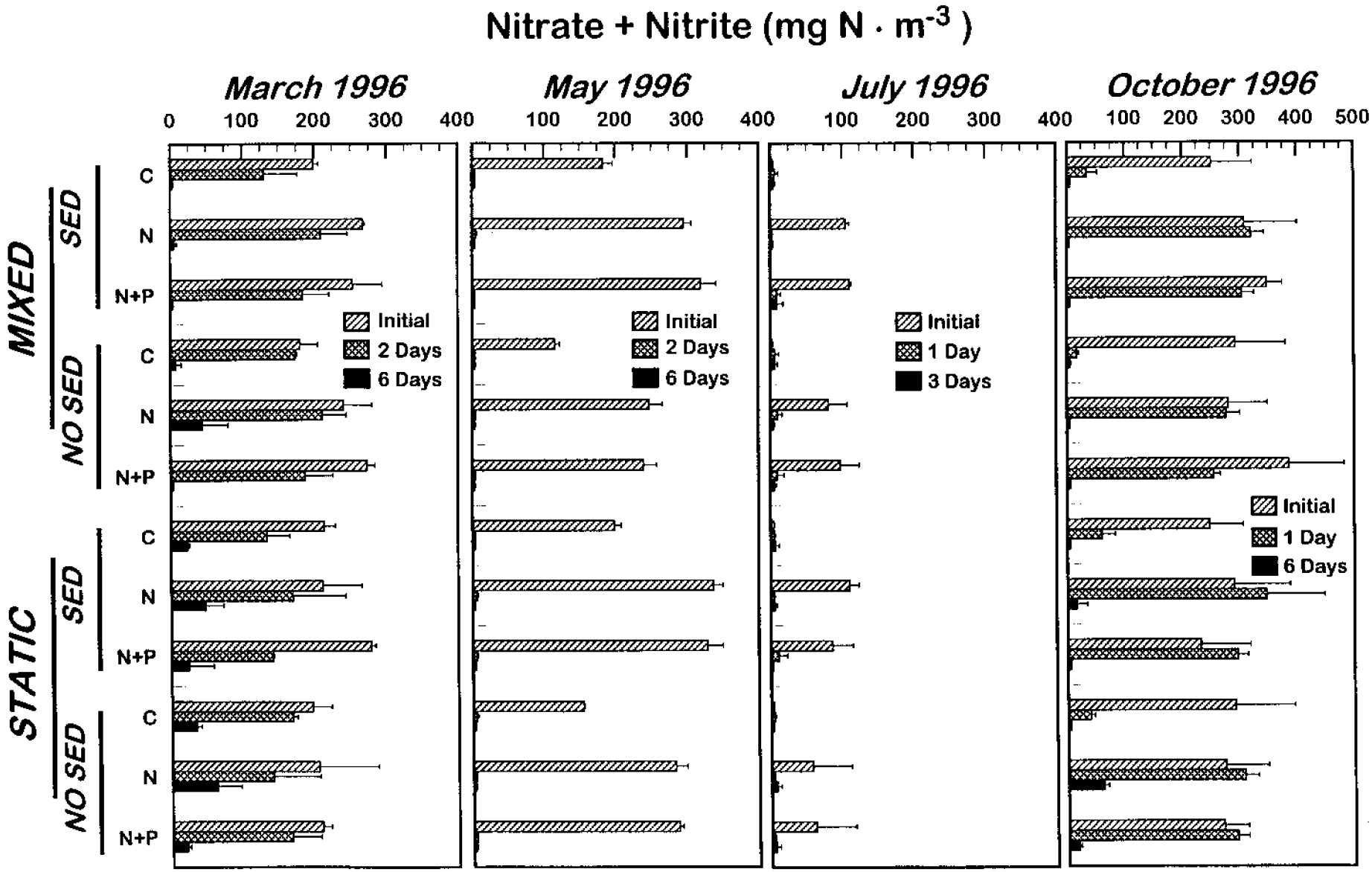
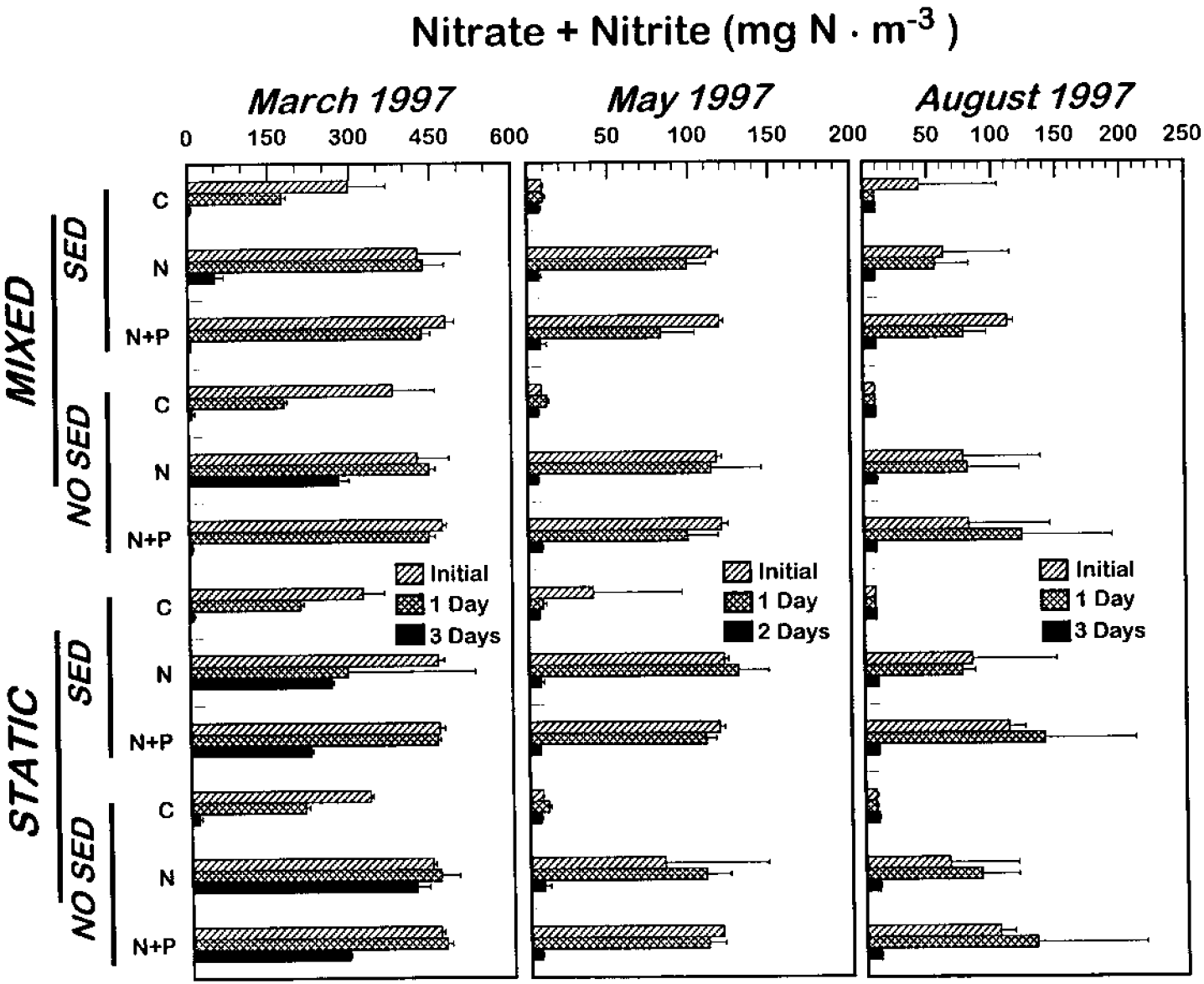

Nitrate + Nitrite (mg N · m-3)
March 1996
May 1996
July 1996
October 1996
0
100
200
300
400
500
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
2 Days
6 Days
1 Day
3 Days

Figure 2a


Nitrate + Nitrite (mg N · m-3)
March 1997
May 1997
August 1997
0 150 300 450 600
50 100 150 200
50 100 150 200 250
MIXED
STATIC
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C
N
N+P
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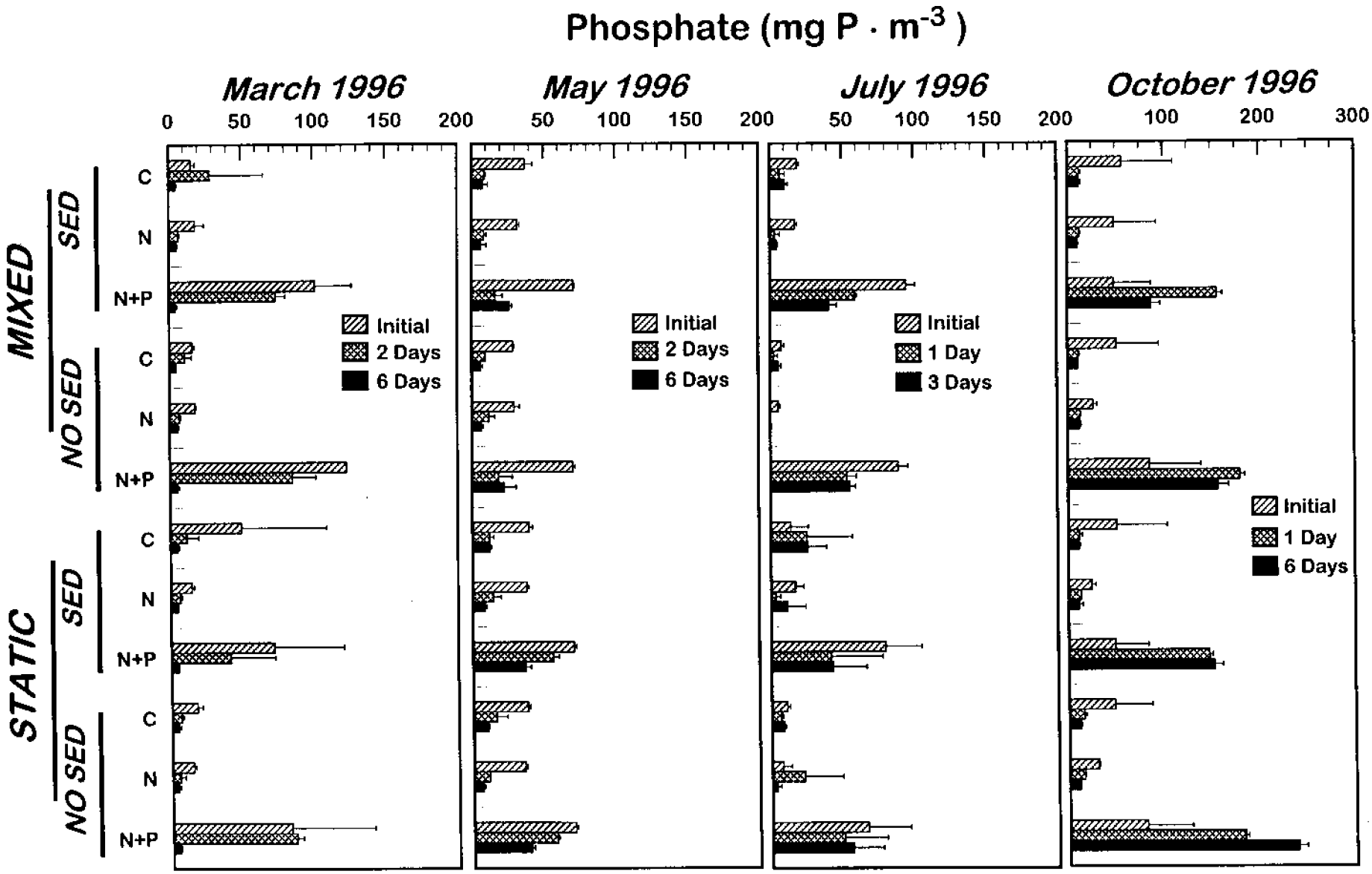
1 Day
3 Days
2 Days

Figure 2b


Phosphate (mg P · m-3)
March 1996
May 1996
July 1996
October 1996
0
50
100
150
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MIXED
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NO SED
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3 Days

Figure 3a

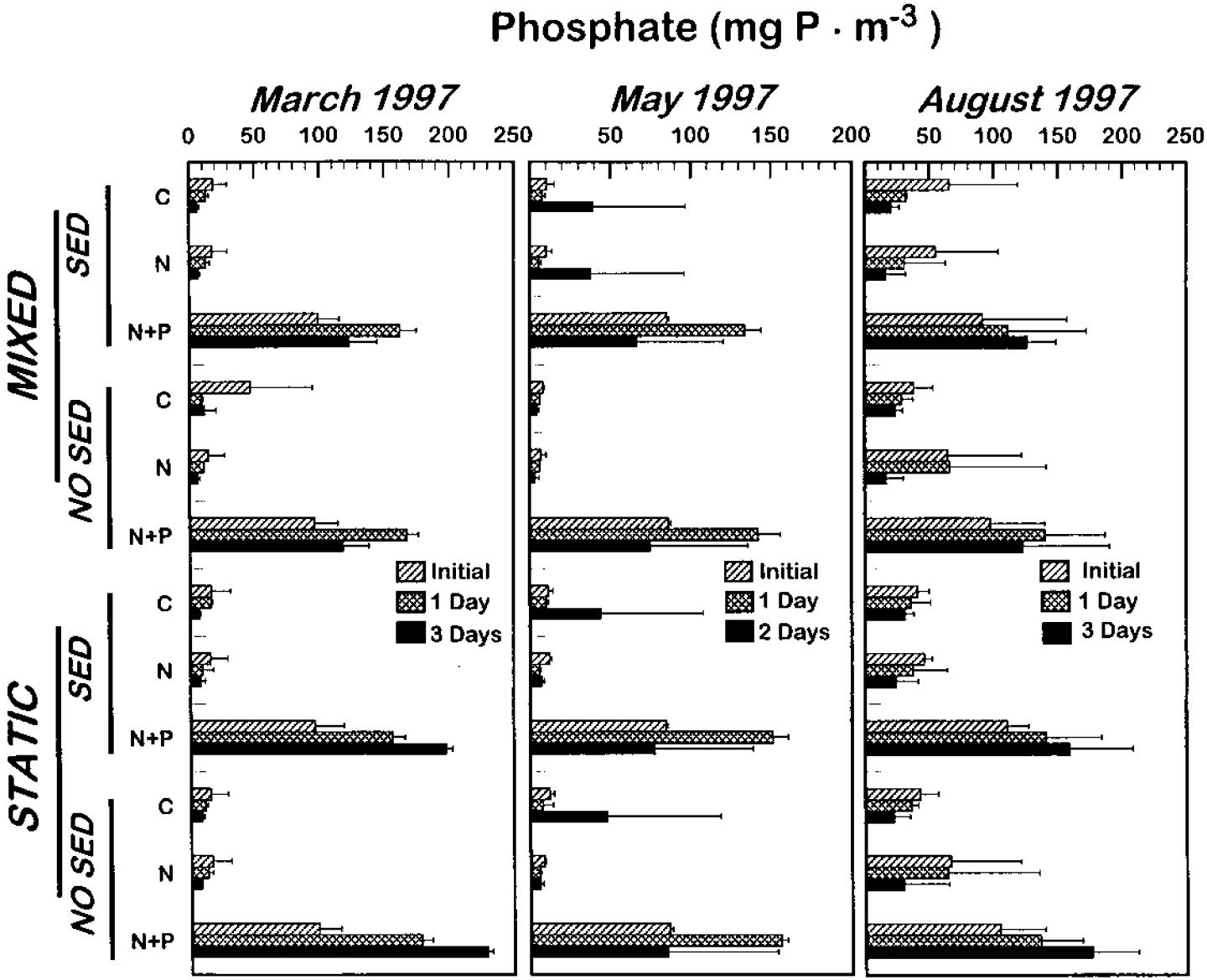

Phosphate (mg P · m-3)
March 1997
May 1997
August 1997
0
50
100
150
200
250
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
1 Day
2 Days
3 Days

Figure 3b

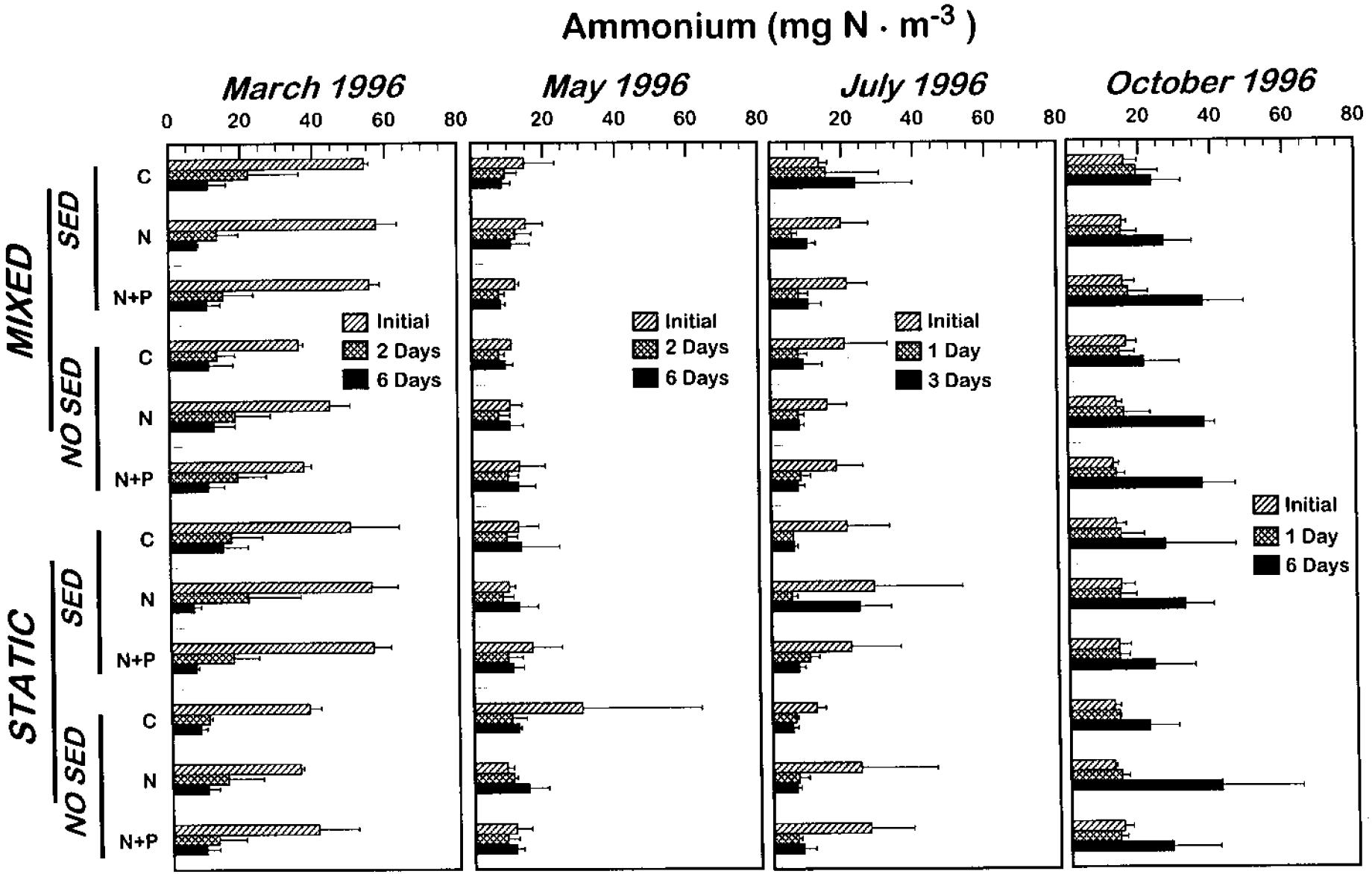
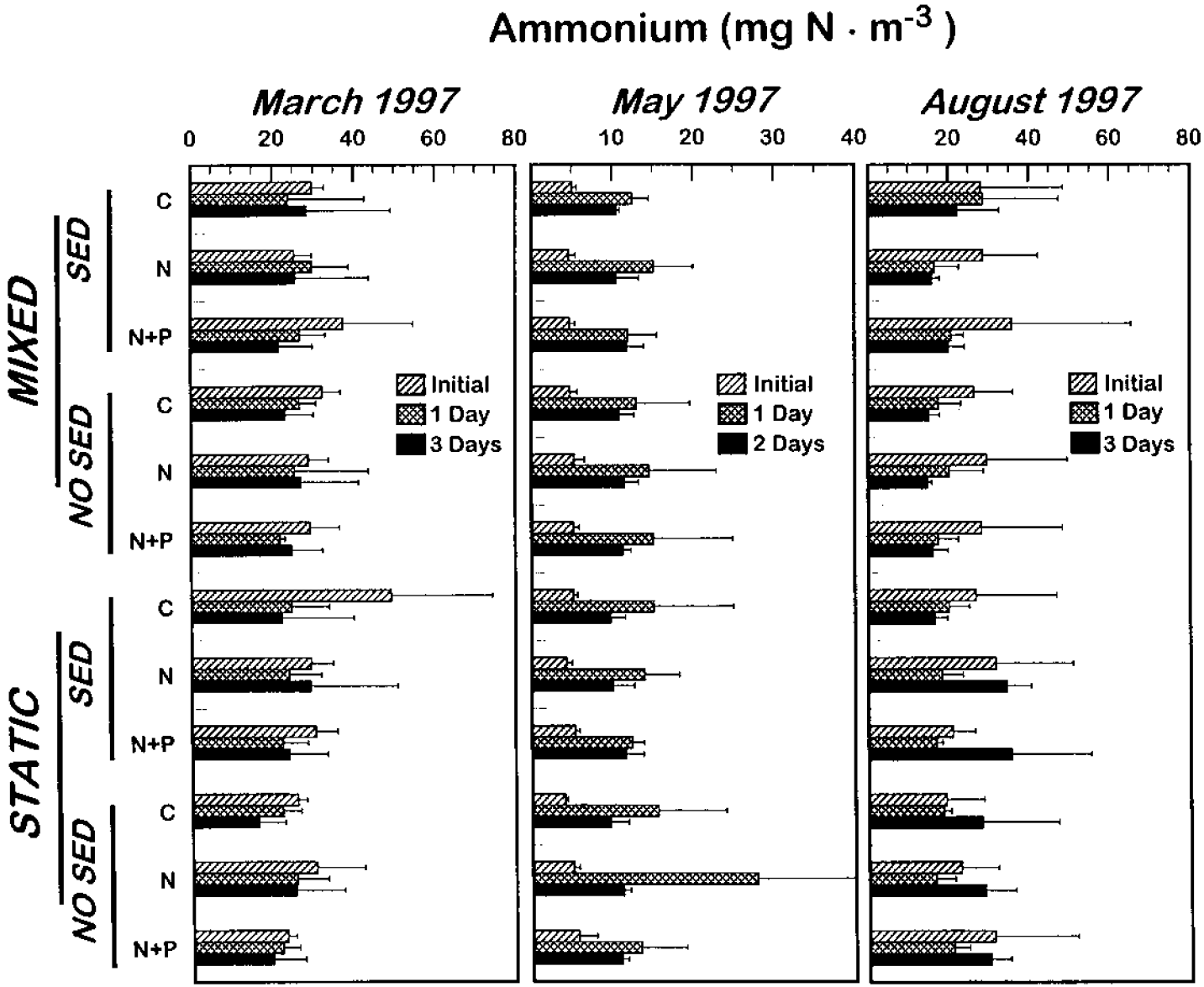

Ammonium (mg N · m-3)
March 1996
May 1996
July 1996
October 1996
0
20
40
60
80
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
2 Days
6 Days
1 Day
3 Days

Figure 4a


Ammonium (mg N · m-3)
March 1997
May 1997
August 1997
0
20
40
60
80
10
20
30
40
20
40
60
80
MIXED
STATIC
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SED
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Initial
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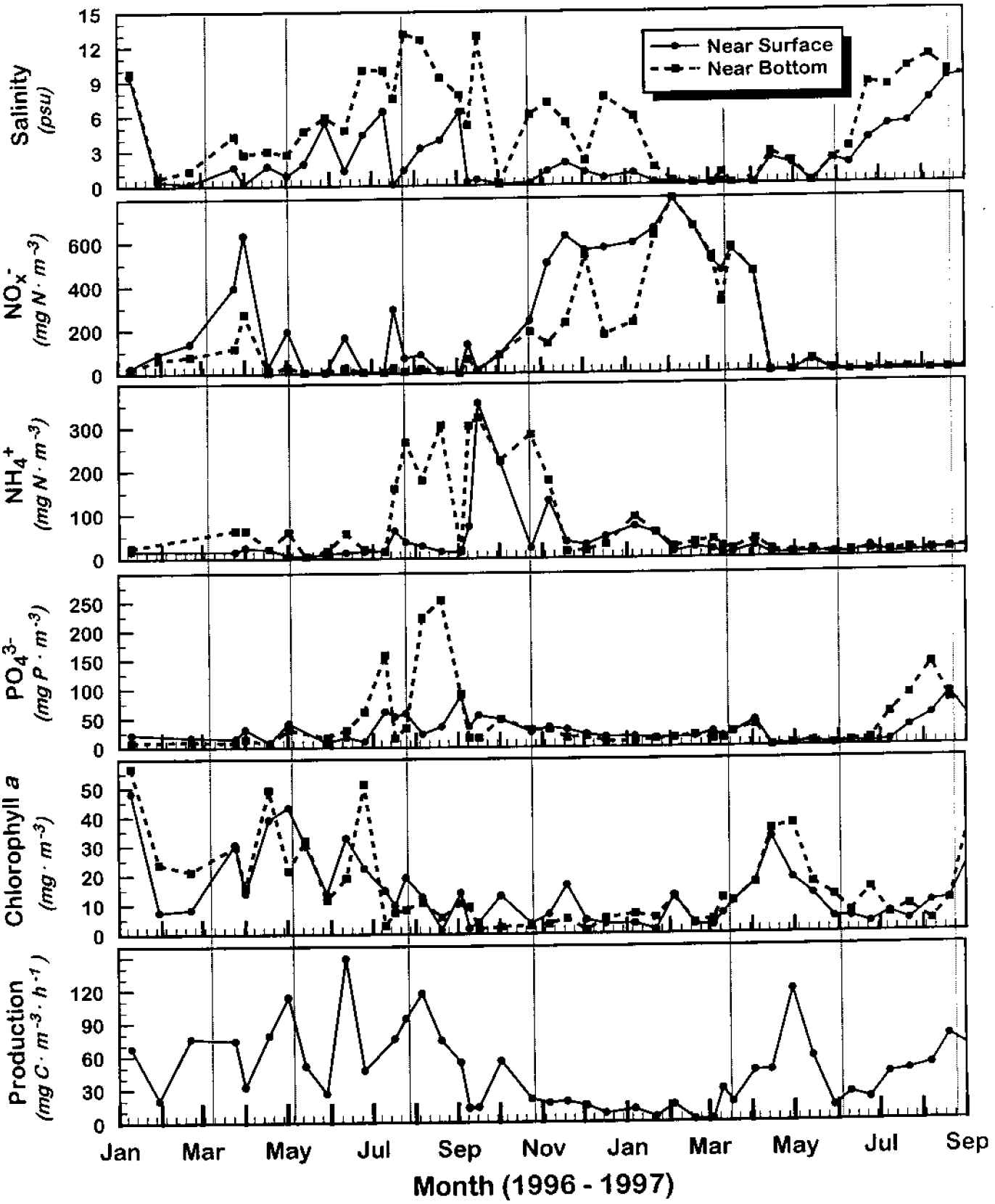
Initial
1 Day
2 Days
Initial
1 Day
3 Days

Figure 4b

Figure 5


Near Surface
Near Bottom
Salinity (psu)
NO_x^- (mg N · m^{-3})
NH_4^+ (mg N · m^{-3})
PO_4^{3-} (mg P · m^{-3})
Chlorophyll a (mg · m^{-3})
Production (mg C · m^{-3} · h^{-1})
Jan Mar May Jul Sep Nov Jan Mar May Jul Sep
Month (1996 - 1997)

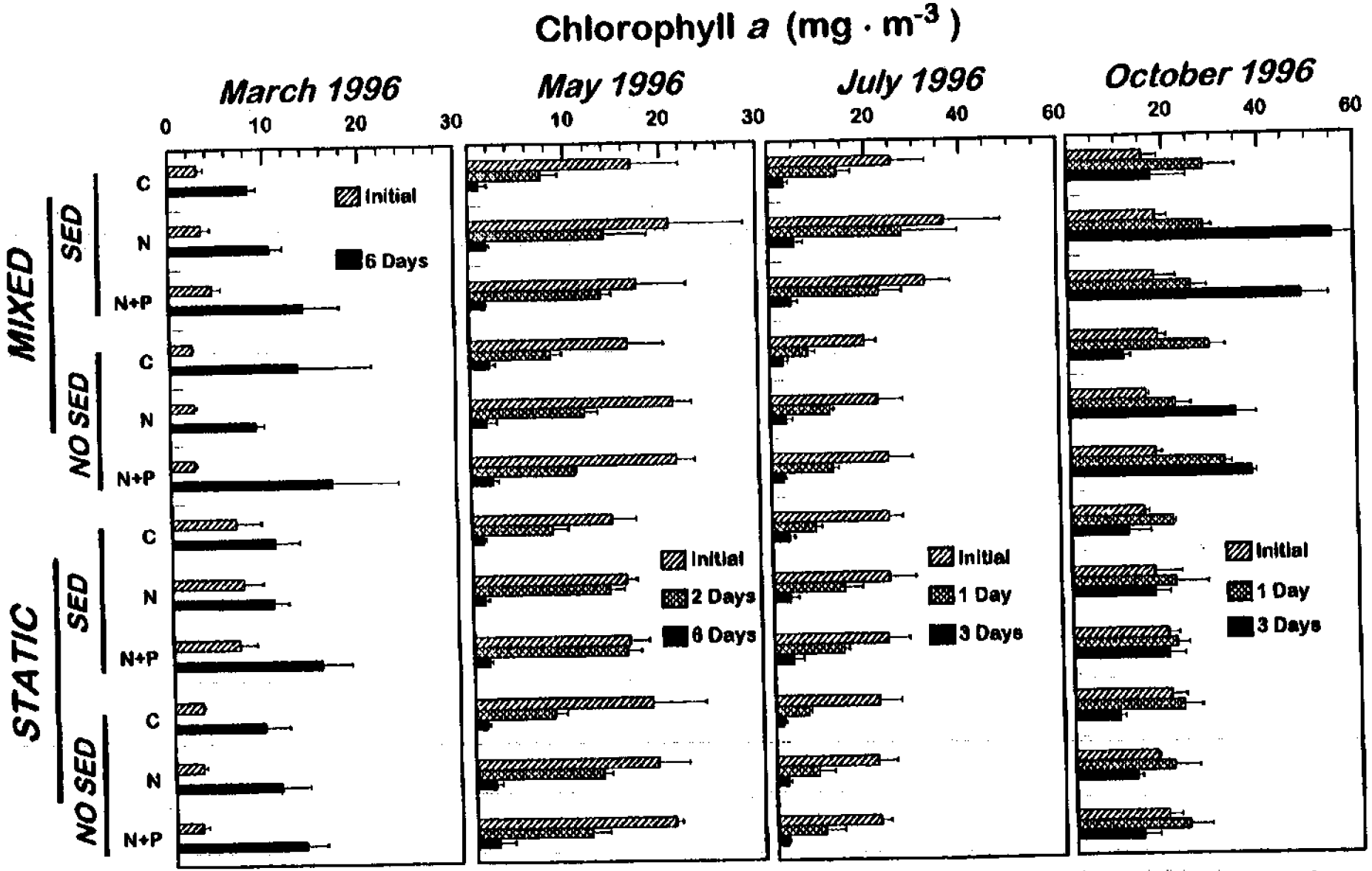

Chlorophyll a (mg · m-3)
March 1996
May 1996
July 1996
October 1996
0
10
20
30
10
20
30
20
40
60
20
40
60
MIXED
STATIC
SED
NO SED
SED
NO SED
C
N
N+P
C
N
N+P
C
N
N+P
C
N
N+P
Initial
6 Days
Initial
2 Days
6 Days
Initial
1 Day
3 Days
Initial
1 Day
3 Days

Figure 6a

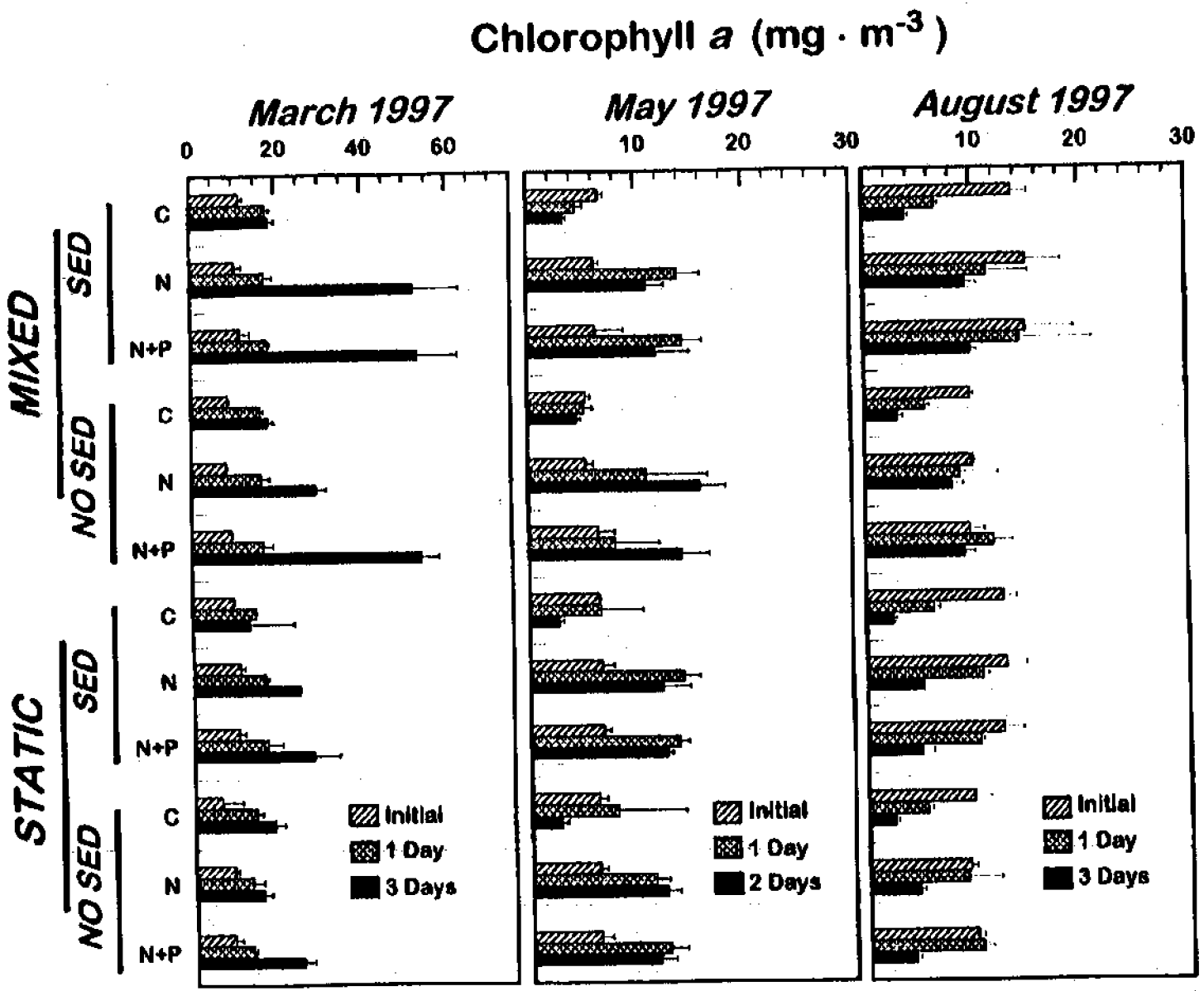

Chlorophyll a (mg · m-3)
March 1997
May 1997
August 1997
0
20
40
60
10
20
30
10
20
30
MIXED
STATIC
SED
NO SED
SED
NO SED
C
N
N+P
C
N
N+P
C
N
N+P
C
N
N+P
Initial
1 Day
3 Days
Initial
1 Day
2 Days
Initial
1 Day
3 Days

Figure 6b

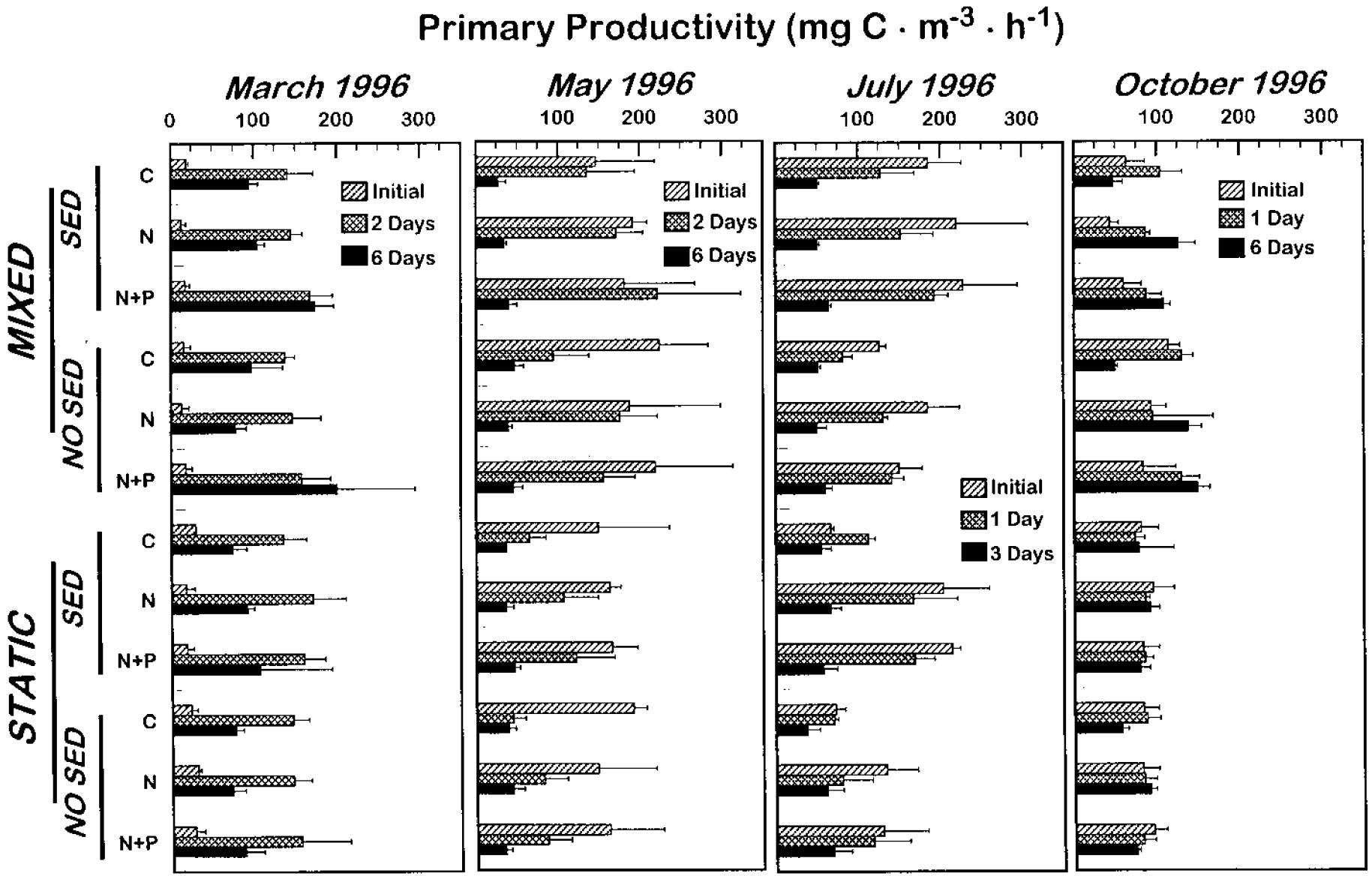

Primary Productivity (mg C · m-3 · h-1)
March 1996
May 1996
July 1996
October 1996
0
100
200
300
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
2 Days
6 Days
1 Day
3 Days

Figure 7a

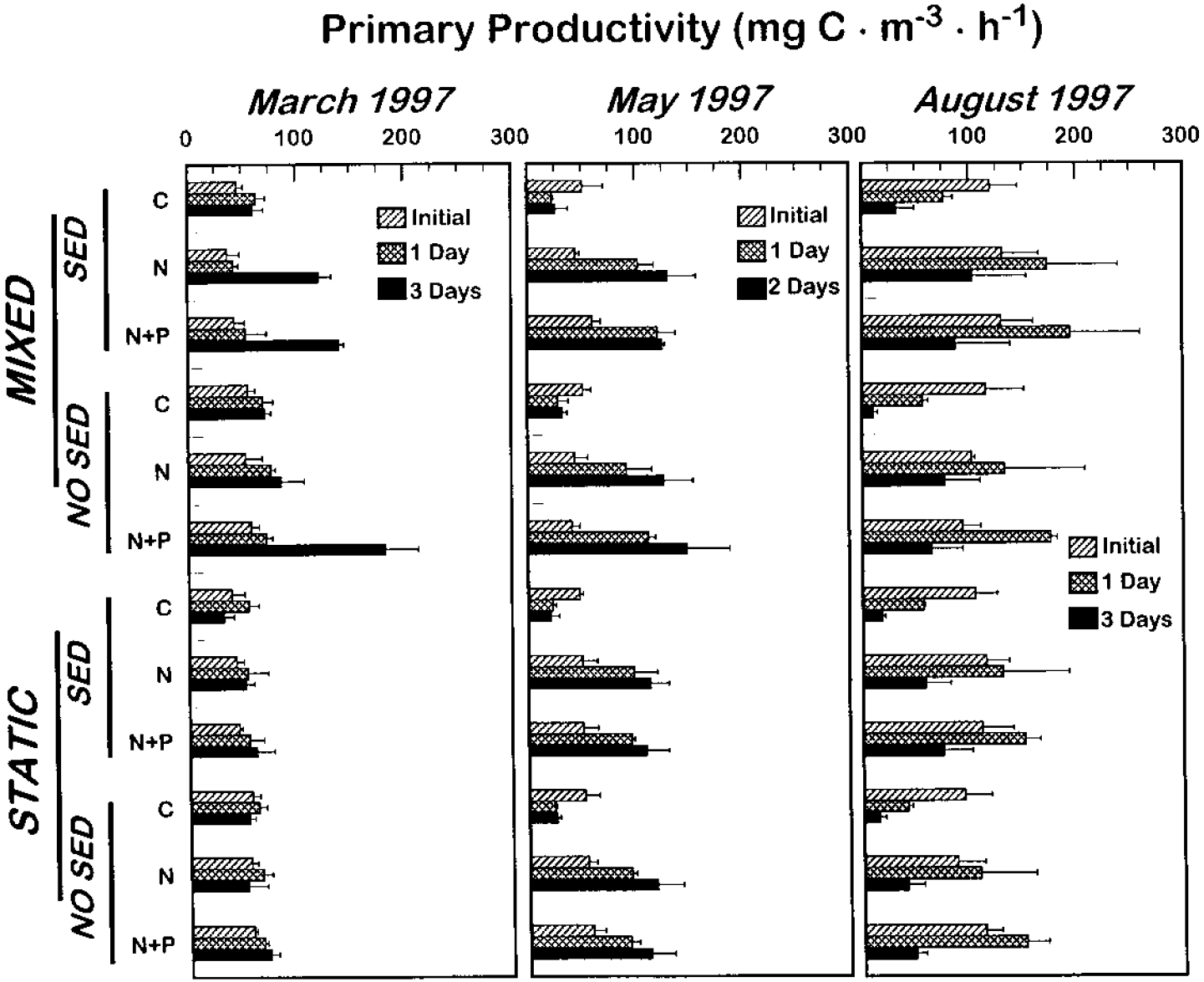

Primary Productivity (mg C · m-3 · h-1)
March 1997
May 1997
August 1997
0
100
200
300
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
1 Day
3 Days
2 Days

Figure 7b

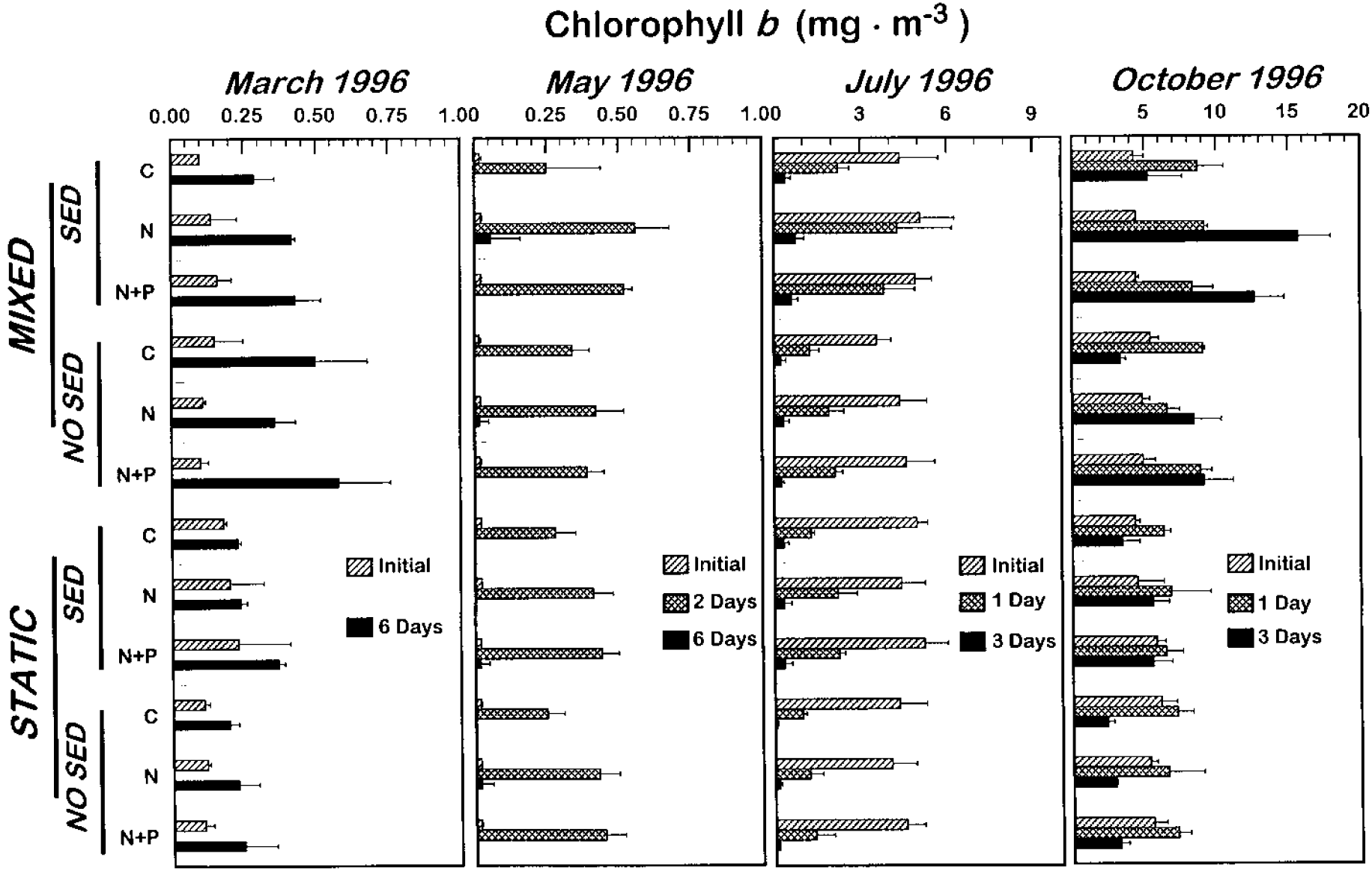

Chlorophyll b (mg · m-3)
March 1996
May 1996
July 1996
October 1996
0.00 0.25 0.50 0.75 1.00
0.25 0.50 0.75 1.00
3 6 9
5 10 15 20
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
6 Days
2 Days
1 Day
3 Days

Figure 8a

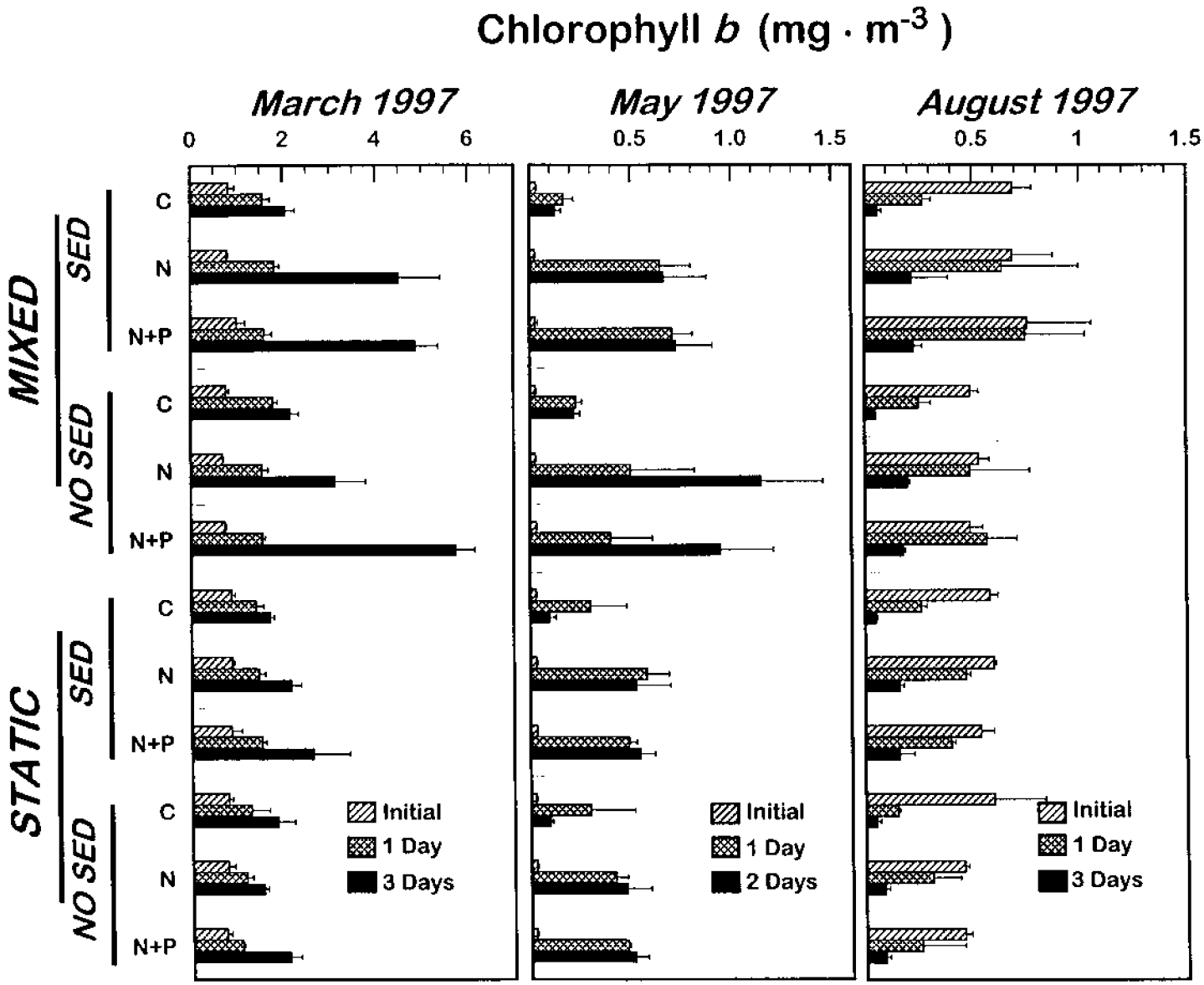

Chlorophyll b (mg · m-3)
March 1997
May 1997
August 1997
0
2
4
6
0.5
1.0
1.5
0.5
1
1.5
MIXED
STATIC
SED
NO SED
SED
NO SED
C
N
N+P
C
N
N+P
C
N
N+P
C
N
N+P
Initial
1 Day
3 Days
Initial
1 Day
2 Days
Initial
1 Day
3 Days

Figure 8b

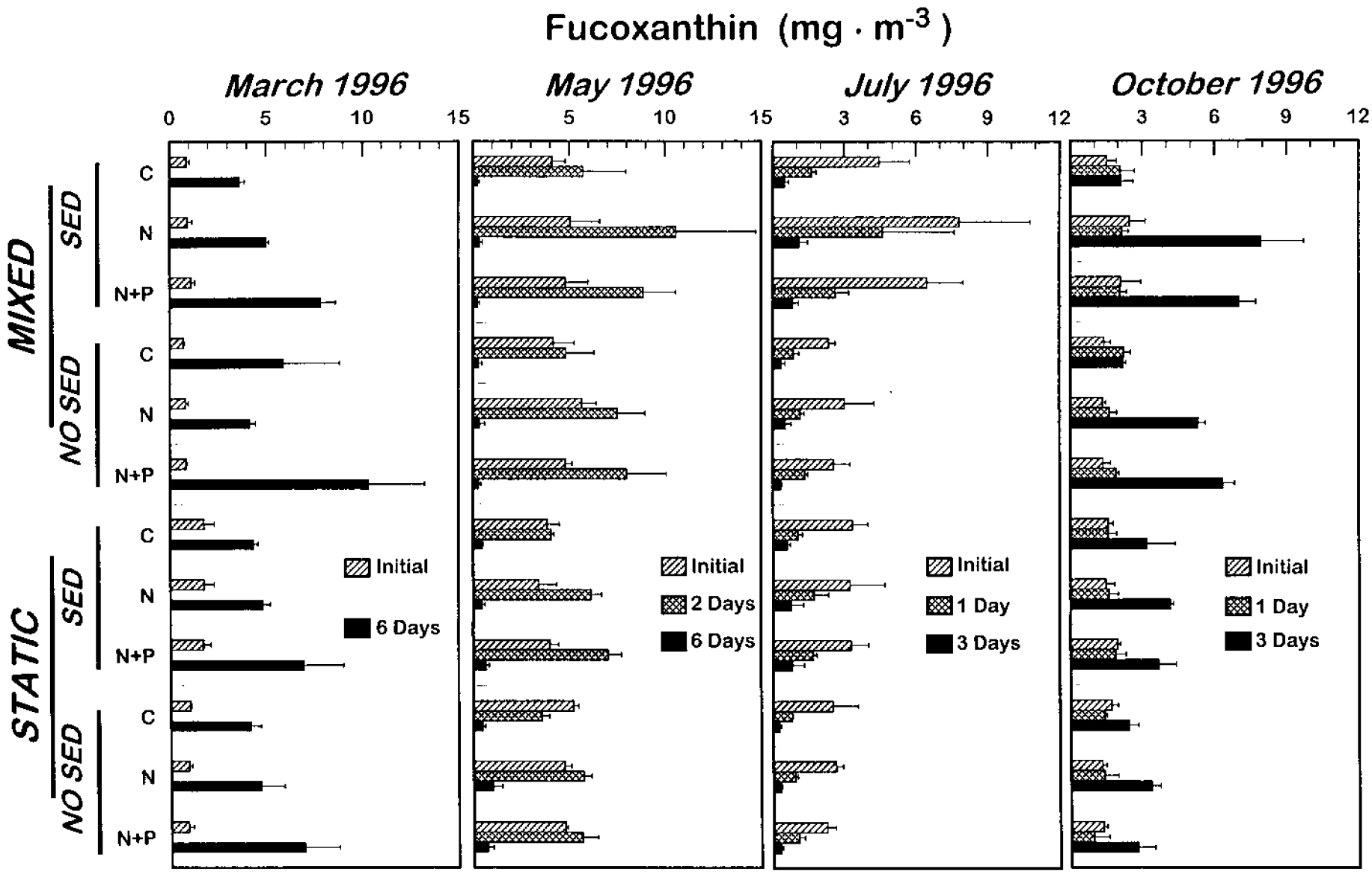

Fucoxanthin (mg · m-3)
March 1996
May 1996
July 1996
October 1996
0
5
10
15
3
6
9
12
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
2 Days
6 Days
1 Day
3 Days

Figure 9a

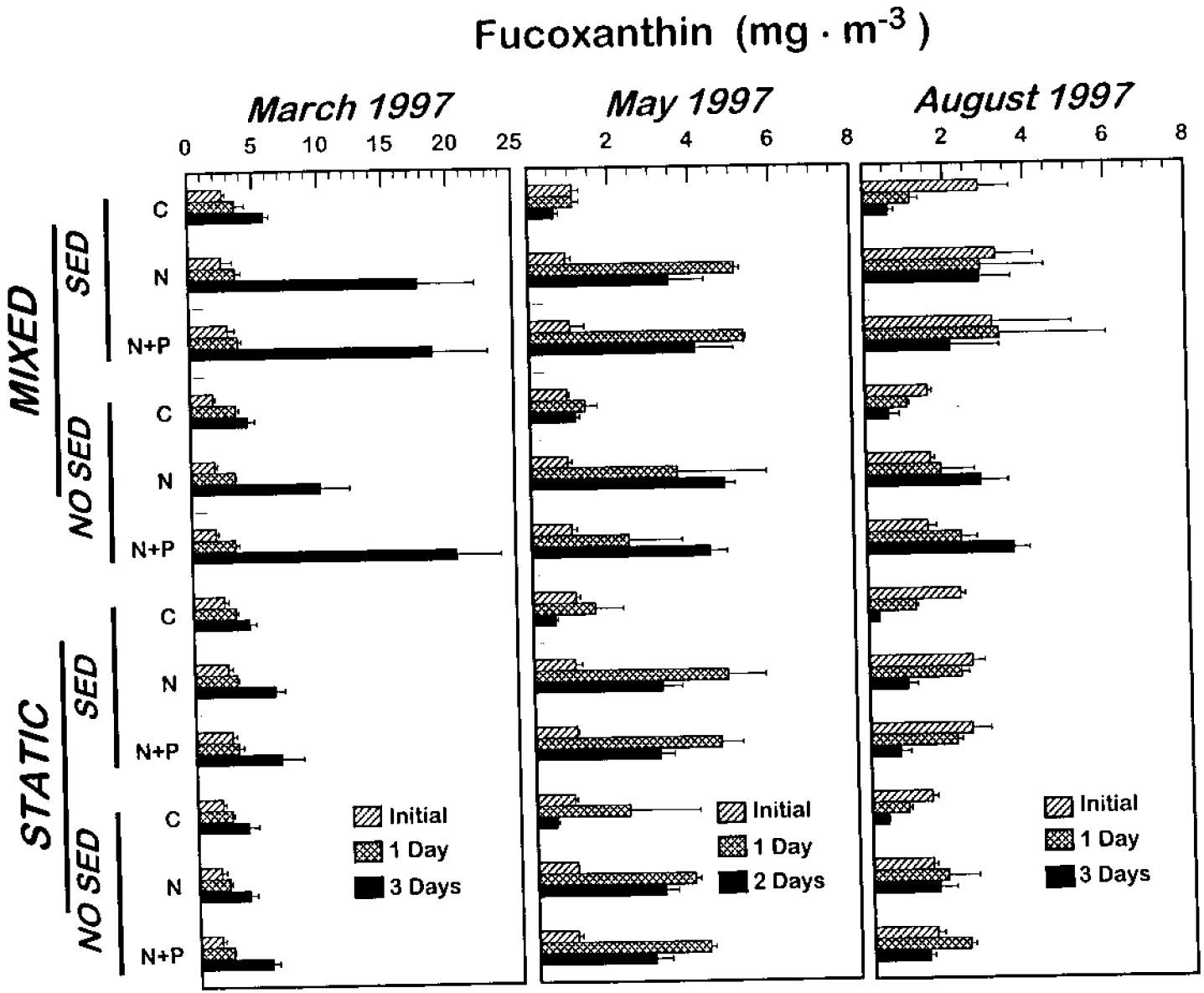

Fucoxanthin (mg · m-3)
March 1997
May 1997
August 1997
0 5 10 15 20 25
2 4 6 8
2 4 6 8
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
1 Day
2 Days
3 Days

Figure 9b

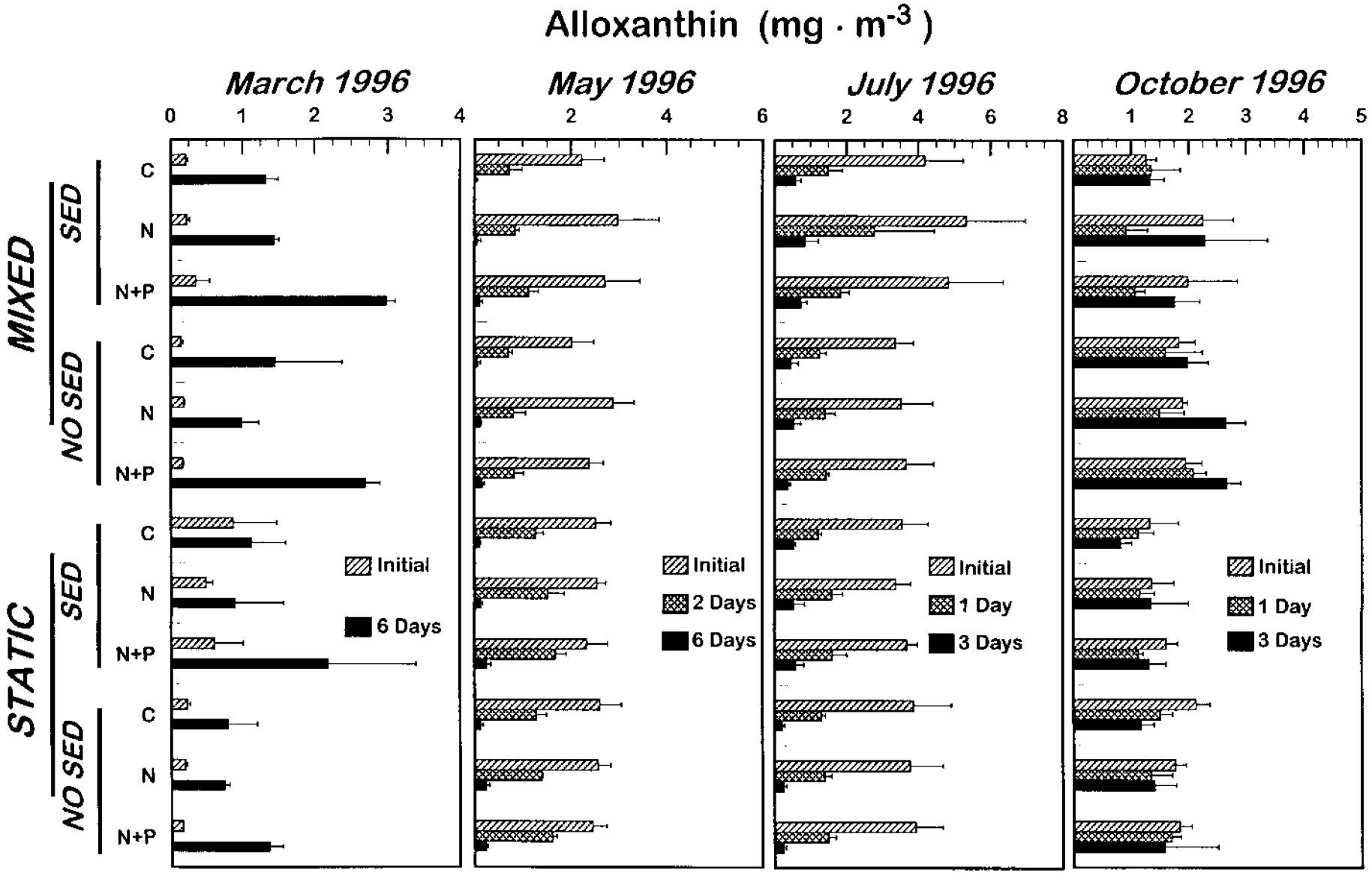

Alloxanthin (mg · m-3)
March 1996
May 1996
July 1996
October 1996
0 1 2 3 4
2 4 6
2 4 6 8
1 2 3 4 5
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
6 Days
2 Days
1 Day
3 Days

Figure 10a

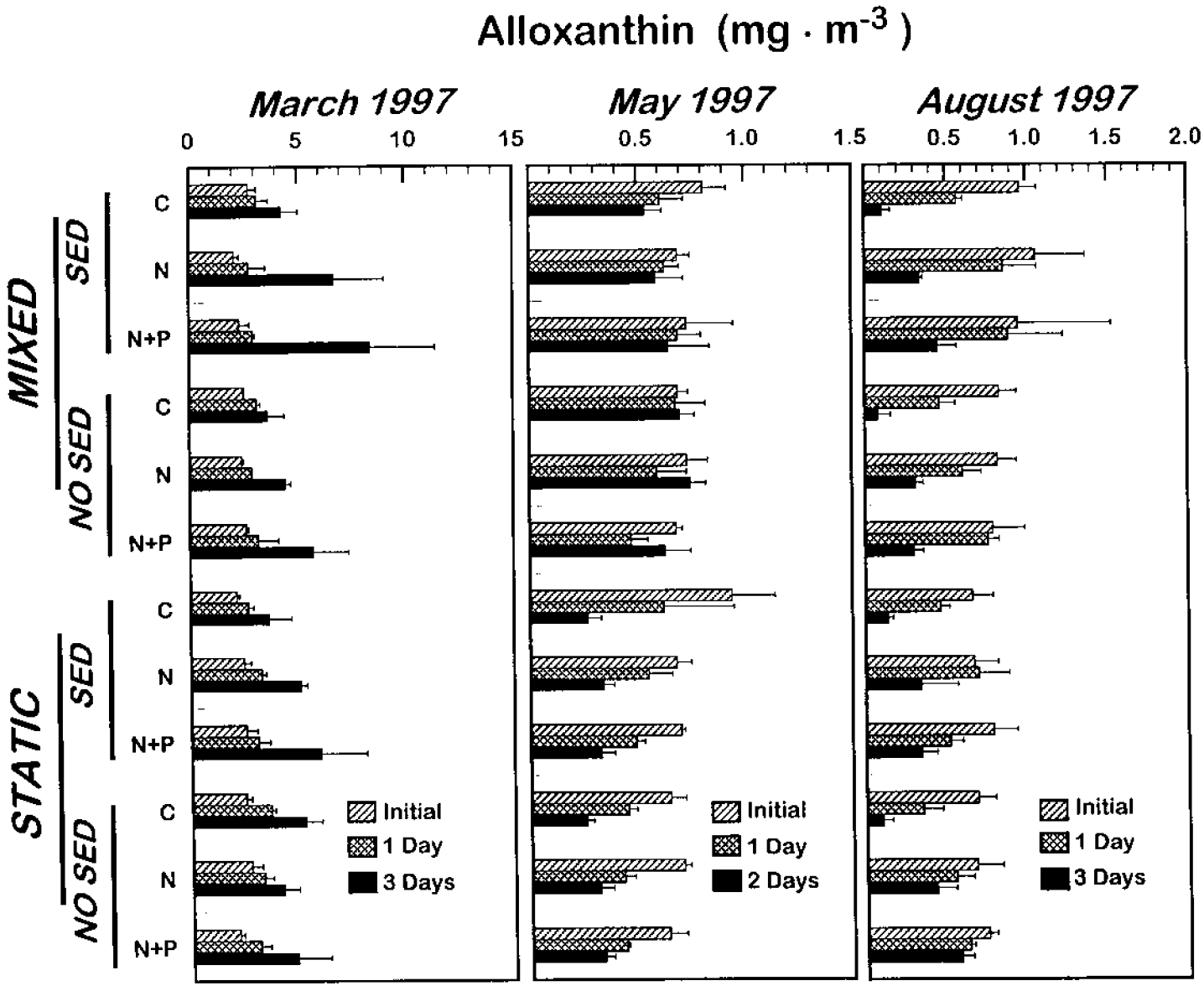

Alloxanthin (mg · m-3)
March 1997
May 1997
August 1997
0
5
10
15
0.5
1.0
1.5
0.5
1.0
1.5
2.0
MIXED
STATIC
SED
NO SED
SED
NO SED
C
N
N+P
C
N
N+P
C
N
N+P
C
N
N+P
Initial
1 Day
3 Days
Initial
1 Day
2 Days
Initial
1 Day
3 Days

Figure 10b

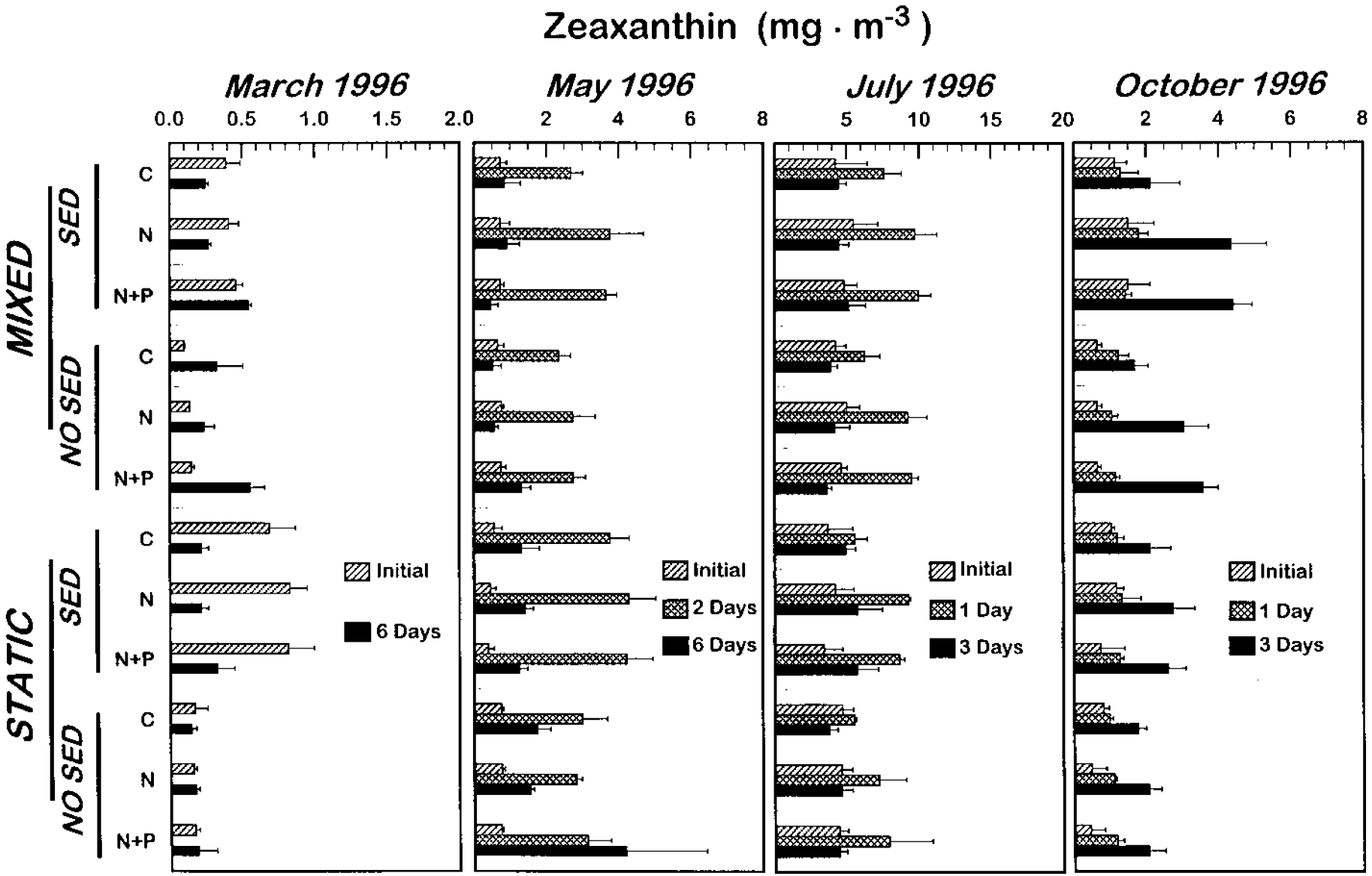

Zeaxanthin (mg · m-3)
March 1996
May 1996
July 1996
October 1996
0.0 0.5 1.0 1.5 2.0
2 4 6 8
5 10 15 20
2 4 6 8
MIXED
STATIC
SED
NO SED
SED
NO SED
C
N
N+P
C
N
N+P
C
N
N+P
C
N
N+P
Initial
6 Days
Initial
2 Days
6 Days
Initial
1 Day
3 Days
Initial
1 Day
3 Days

Figure 11a

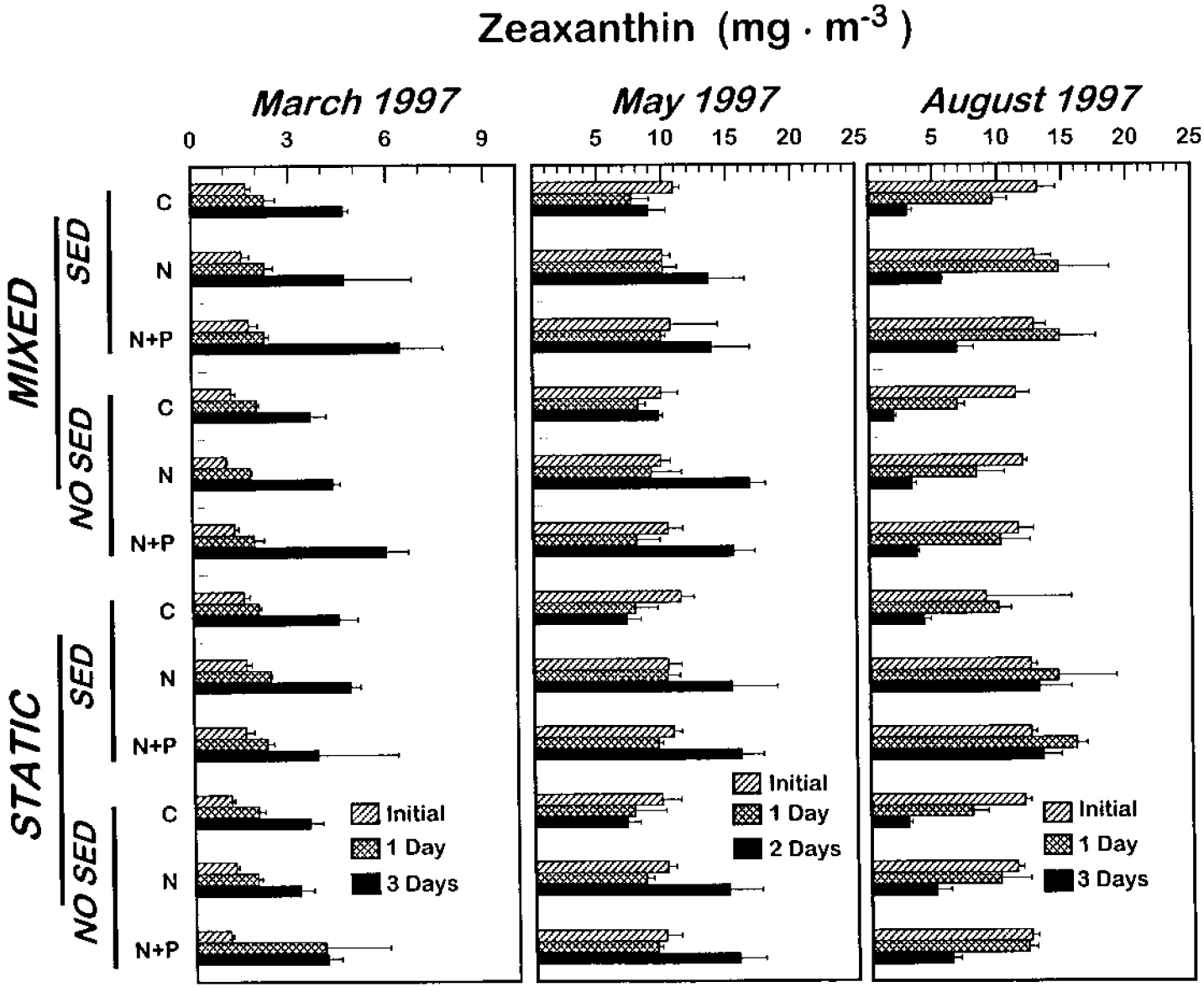

Zeaxanthin (mg · m-3)
March 1997
May 1997
August 1997
0
3
6
9
5
10
15
20
25
5
10
15
20
25
MIXED
SED
NO SED
STATIC
SED
NO SED
C
N
N+P
C
N
N+P
C
N
N+P
C
N
N+P
Initial
1 Day
3 Days
Initial
1 Day
2 Days
Initial
1 Day
3 Days

Figure 11b

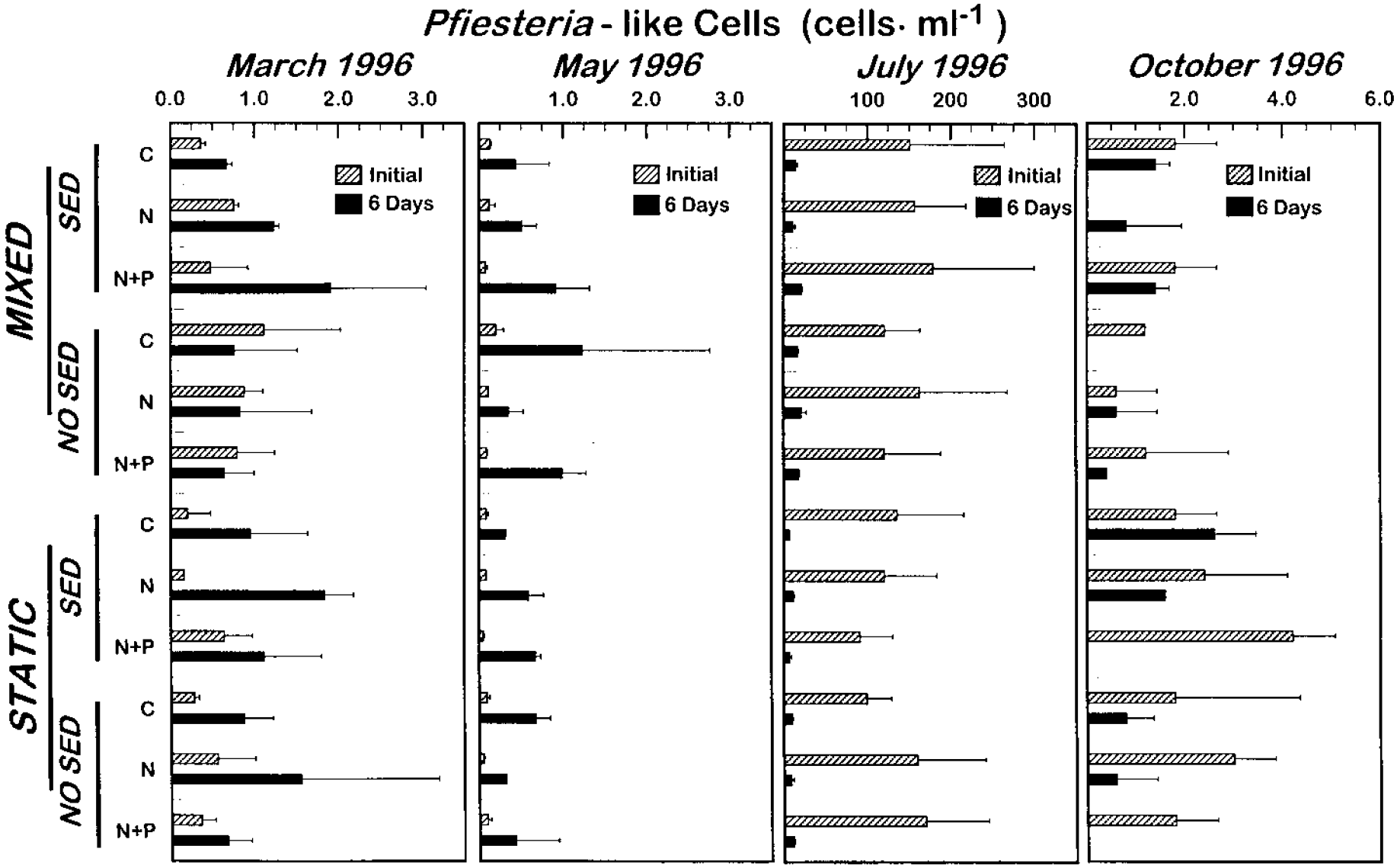

Pfiesteria - like Cells (cells· ml-1)
March 1996
May 1996
July 1996
October 1996
0.0
1.0
2.0
3.0
100
200
300
4.0
6.0
Initial
6 Days
MIXED
STATIC
SED
NO SED
C
N
N+P

Figure 12a

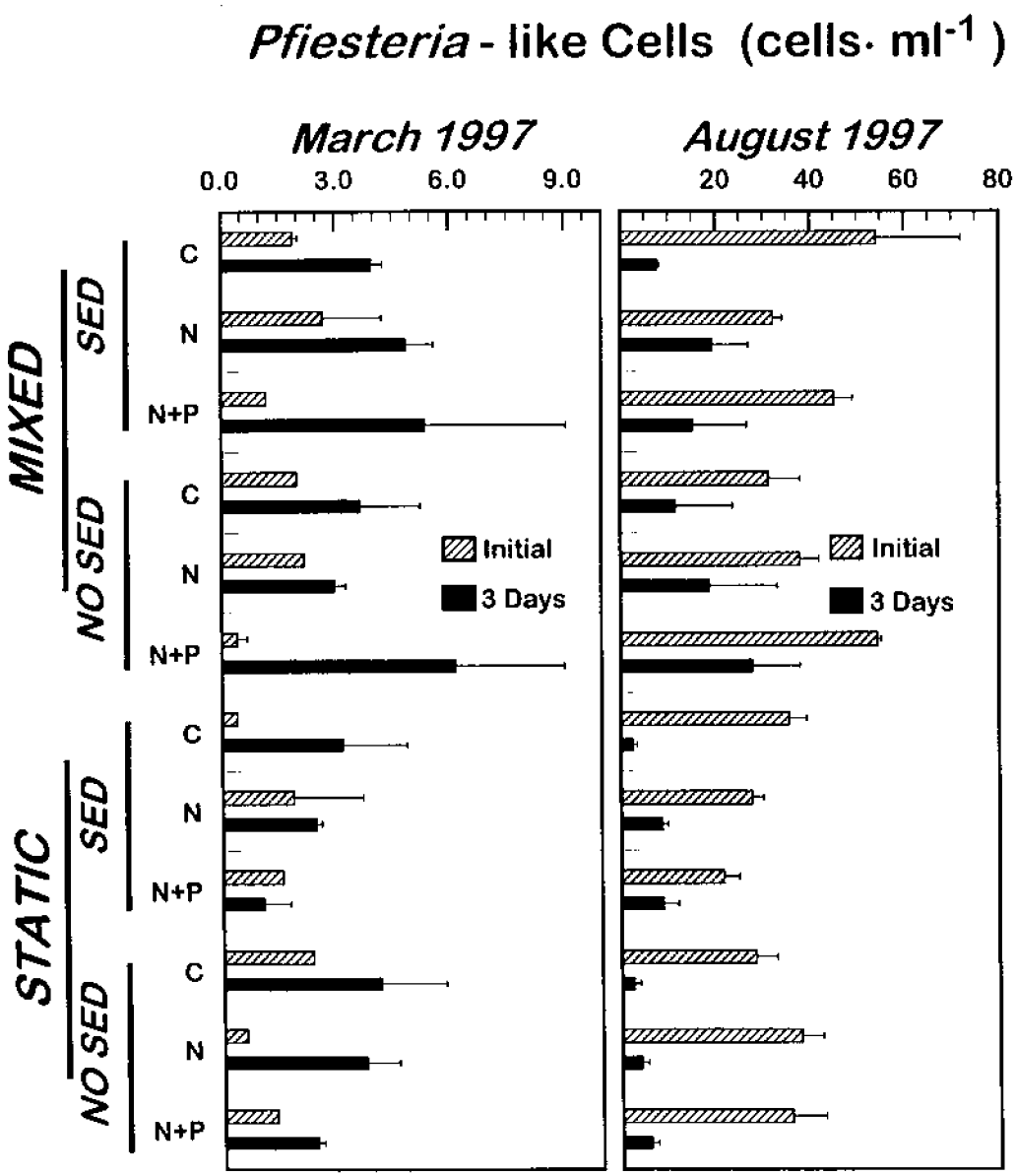
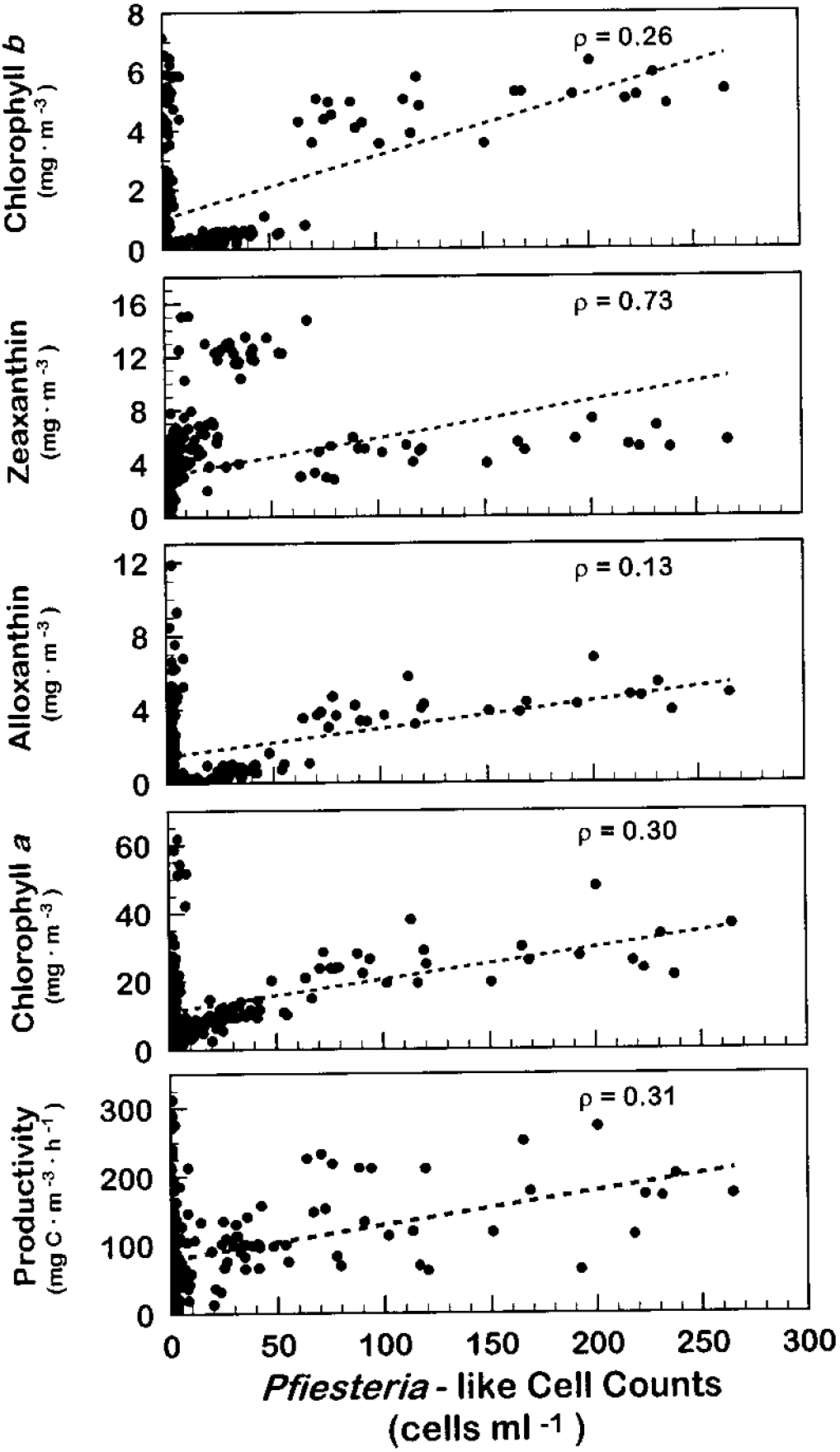

Pfiesteria - like Cells (cells· ml-1)
March 1997
August 1997
0.0
3.0
6.0
9.0
20
40
60
80
MIXED
STATIC
SED
NO SED
C
N
N+P
Initial
3 Days

Figure 12b

Figure 13


Chlorophyll b (mg · m-3)
ρ = 0.26
Zeaxanthin (mg · m-3)
ρ = 0.73
Alloxanthin (mg · m-3)
ρ = 0.13
Chlorophyll a (mg · m-3)
ρ = 0.30
Productivity (mg C · m-3 · h-1)
ρ = 0.31
0 50 100 150 200 250 300
Pfiesteria - like Cell Counts
(cells ml-1)

APPENDIX I

Steidinger, K., J. Lansberg, and E. Truby. 1997. *Pfiesteria piscicida*, other *Pfiesteria* species, and *Pfiesteria*-like species: A question of recognition and toxicity.

Informational Handout prepared on 31 July 1997 for the Pocomoke River Fish Health Technical Advisory Committee Meeting, August 1997

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Virginia B. Wetherell
Secretary

PFIESTERIA PISCICIDA, OTHER *PFIESTERIA* SPECIES, AND *PFIESTERIA*-LIKE SPECIES: A QUESTION OF RECOGNITION AND TOXICITY

[Informational handout prepared 7/31/97 by Karen A. Steidinger, Jan H. Landsberg, and Earnest W.Truby for the Pocomoke River Fish Health Technical Advisory Committee, August 1997]

WHO'S WHO

Pfiesteria piscicida was described in 1996 and is a known fish killer in North Carolina waters (Burkholder et al. 1992, Steidinger et al. 1996a). An undescribed *Pfiesteria* species, known to kill fish in tropical fish tanks, was characterized in 1995 (Landsberg et al. 1995). Are other small, heterotrophic dinoflagellates that look like *Pfiesteria*, but belong to different genera, toxic and do they cause fish kills or fish diseases? Why is this a problem? Because *Pfiesteria*-like species cannot be differentiated from *P. piscicida* using a light microscope and they can be counted as *P. piscicida.* We also do not know if these species are toxic and if so, what toxins are produced.

The standard SEM combination fix of GTA/OsO_4 at 4°C works well for unarmored dinoflagellate species and also is a good initial fix for the small gymnodinioid cells that may either be armored or unarmored (Truby, 1997). When cells are fixed in this manner, the cells typically appear to have a wrinkled surface (Figs. 1 and 2 Maryland, Figs. 1 to 3 North Carolina/Florida, Fig. 2 other *Pfiesteria*). In cases where species have such a wrinkled appearance due to the presence of an outer membrane, it is important to use alternative fixation techniques to strip off this membrane (Truby, 1997). This process will reveal if the species are lightly armored and will also help to identify their characteristic plate pattern. The plate pattern is critical for identification. *Pfiesteria piscicida* and other *Pfiesteria* look-alikes can only be identified by stripping off the outer membrane to reveal plates.

Small armored dinoflagellates (5-20 μm) often look like unarmored or "naked" dinoflagellates in the genera *Gymnodinium*, *Gyrodinium*, and *Katodinium* (Steidinger et al. 1996b). However, when these cells are processed for scanning electron microscopy (SEM) and the outer membrane of the theca is removed, thecal plates in identifiable patterns and sequences are revealed. The cells then can be identified by studying the number, position, and shape of the thecal plates. Numbering of plates follows the Kofoidian system. The SEM procedure is time-consuming, costly, and labor intensive but it is the only method available today to definitively identify *Pfiesteria* species and to distinguish them from other small dinoflagellates.

If *Pfiesteria* or *Pfiesteria*-like cells are found in water samples, they should be counted as "*Pfiesteria*-like" until their identity can be confirmed with SEM observations. A categorical count of "*Pfiesteria*-like" cells will then be relevant if these species are found to be toxic.

Figures 1 and 2 for the Maryland samples, Figures 1, 2, and 3 for North Carolina and Florida samples, and Figure 2 of other known *Pfiesteria* samples, show wrinkled outer membranes that obscure plates and make identification impossible. Compare Figure 1 of Maryland with Figure 1 of the North Carolina and Florida samples. Then compare Figure 3 of Maryland with Figure 5 of the samples from North Carolina and Florida. At the light microscope level the two species look the same except for a difference in cingular displacement. Even at the SEM level they are similar, but once the plates are seen it is apparent that they are two distinct species in two different genera.

WHERE WERE THESE *PFIESTERIA* LOOK-ALIKES ISOLATED FROM?

The North Carolina cultures came from a Pamlico isolate ("B"), a Neuse isolate ("H"), and a tank at North Carolina State University ("C"). The Florida isolate came from the St. John's River ("Jax"). All are the same species belonging to a new genus, possibly related to *Peridiniopsis*. This species may be toxic and could cause fish kills and/or fish disease.The Maryland isolate was from the Pocomoke ("5B"). This species may also be toxic and cause fish kills and/or fish disease. All cultures and isolates can be maintained on microalgal prey, either *Rhodomonas* alone or *Rhodomonas* and *Isochrysis*. We have looked at hundreds of specimens, and several separate incubations of all the strains; our results consistently show two different species - one from North Carolina/Florida and one from Maryland.

HAVE WE VERIFIED *PFIESTERIA PISCICIDA* IN MARYLAND, NORTH CAROLINA, AND FLORIDA WATERS?

Yes - in Maryland and North Carolina. No - in Florida. The original description of *Pfiesteria piscicida* was based on North Carolina samples provided by Dr. JoAnn Burkholder of North Carolina State University; these samples were incubated with live fish. In 1996, water samples from a Maryland fish farm where fish kills were occurring also revealed *P. piscicida* when the cells were processed with the SEM membrane-stripping technique. The Maryland water sample was incubated with *Rhodomonas* and *Isochrysis*. Since 1993, we have been analysing sediments and water samples from sites in the St. John's River, Florida where fish with ulcerations are known to occur. In this area, we have not yet identified *Pfiesteria piscicida* or the second *Pfiesteria* species, but other *Pfiesteria* look-alikes have been found (see above).

WHAT DO WE NEED TO DO NOW WITH THIS NEW INFORMATION ON *PFIESTERIA* LOOK-ALIKES?

1. Since there are samples available for North Carolina "B", "C", "H", Florida "Jax" and Maryland "5B" we need to determine if these species are toxic and if so, what toxins are produced. Since other researchers have isolates from the Pocomoke in Maryland, the Neuse and Pamlico in North Carolina, and Florida Bay in Florida, that are *Pfiesteria*-like, we need to verify the identity of these isolates so that experimental results can be compared. How do we proceed? We need to exchange cultures and split samples from natural waters to determine the identity of the *Pfiesteria*-like species and to assess their toxicity. This is the only way to determine potential threats to natural resources or to public health.

2. Currently SEM is the only way to resolve species identity. We need rapid and cheaper methods for identification. We need molecular probes that will identify and therefore differentiate species or their toxins. The probes must have a high level of specificity and a low level of cross- reactivity. And, they need to be rapid and simple. Could a technician, working with an inverted microscope (epifluorescence optics), use a probe with a fluorescent tag to count cells of toxic species and could they then calculate abundances so that water bodies can be surveyed?

Comparative plate tabulation of *Pfiesteria*, other *Pfiesteria*-like species, and other genera

	Epitheca								Hypotheca		
Species	Po	cp	X	'	a	"	c	s	""	p	""
Pfiesteria piscicida	+	+	+	4	1	5	6	4	5	0	2
Pfiesteria sp. (Florida)	+	+	+	4	1	5	6	4	5	0	2
Maryland Site 5b	+	+	+	4	2	7	≥ 6	5?	5	0	2
North Carolina/ Florida *"Peridiniopsis"*	+	+	+	5	0	6	6?	4	5	0	2
Peridiniopsis	+	0	+	3-5	0-1	6-7	6	3-5	5	0	2
Amyloodinium	+	+	+	4	1	7	6-8	?	5	0	2

August 4, 1997 Update: The *Peridiniopsis*-like species that has been found in three North Carolina cultures and one Florida isolate has now been identified from Maryland waters from Station 1 collected 6/17/97 near the Shelltown area. This means that this new species, yet to be officially described, occurs in Maryland, North Carolina, and Florida. Although cultures "B" and "C" from North Carolina are reported to cause fish kills in tanks, this needs to be confirmed.This species is not *Pfiesteria*, but it is a look-alike and can be easily confused with the notorious *Pfiesteria*. If it is verified to be toxic, then the toxins need to be characterized to determine whether the toxins are like *Pfiesteria* toxins or not. Also, more samples need to be collected at the original isolation site near Shelltown to confirm this new species presence in that area, and possibly surrounding areas. Again, the same is true for MD #5B, is it toxic, does it cause fish kills?

Attachments

5 pages of SEM micrographs illustrating two new species resembling *Pfiesteria*, one undescribed *Pfiesteria*, and one potential *Pfiesteria*
5 reprints
Letter to Dr. Leonard

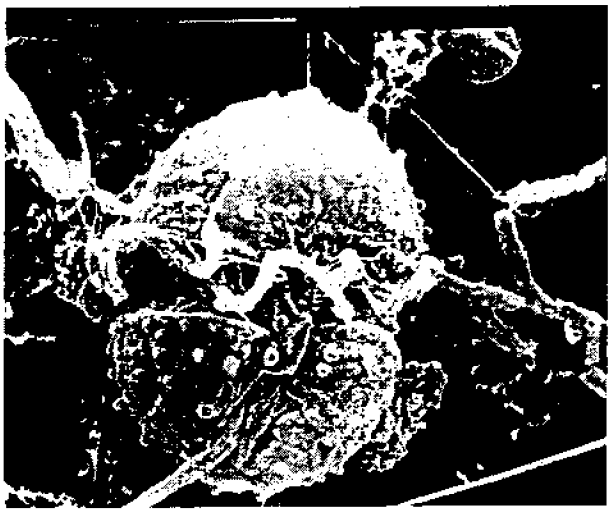


Fig.1

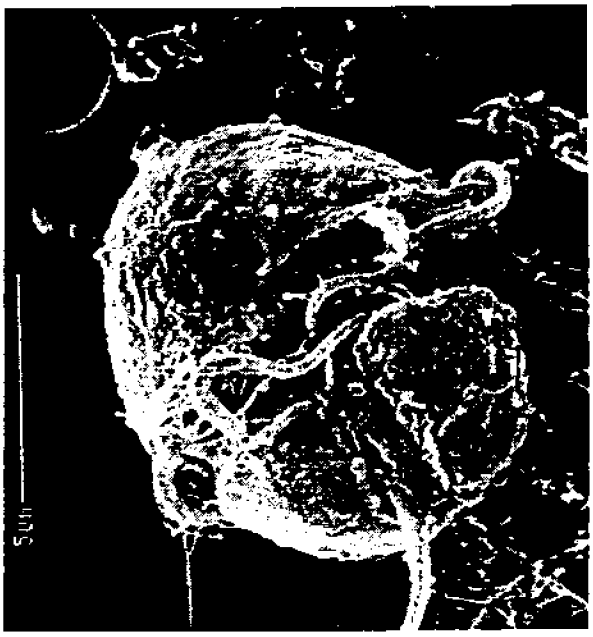


Fig.2

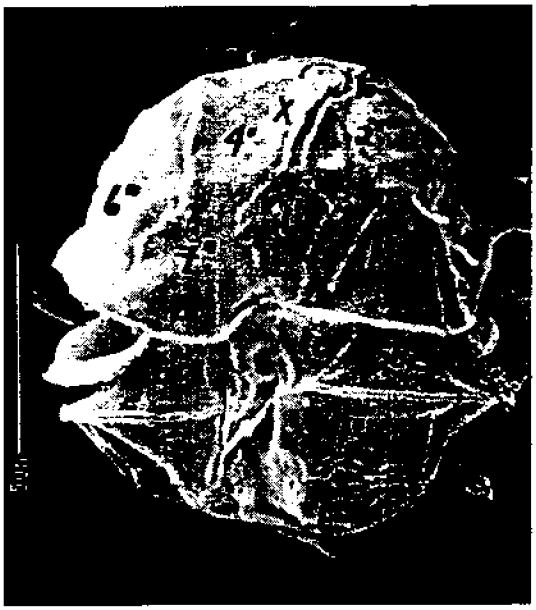


Fig.3

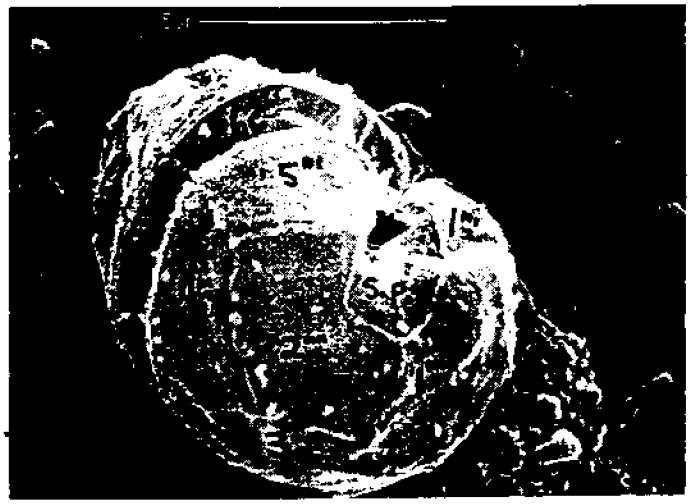


Fig.4

Maryland Samples

Figures 1-4. This is a new brackish water species isolated from the Pocomoke River (sample 5; May 7, 1997) after incubation with microalgal prey. It looks like *Pfiesteria piscicida* at the light microscope and scanning electron microscope levels of resolution until removing the outer membrane reveals thecal plates. It is a heterotroph with a peduncle-like structure and phagocytizes microalgae. Food vacuoles are often observed in the epitheca. Size and morphology are variable, but the plate tabulation and pattern are consistent in multiple growouts. The plate formula is Po, cp, X, 4', 2a, 7", >= 6c, 5?s, 5"', 0p, and 2"". The Apical Pore Complex (APC) of Po, cp and X is very characteristic it resembles a shepherd's crook; and has a long X plate. The cingulum is displaced 0.5 times like *Pfiesteria* species, however, the sulcus is not offset to the right. The s.a. plate is in line with the posterior sulcal plates (s.p.).

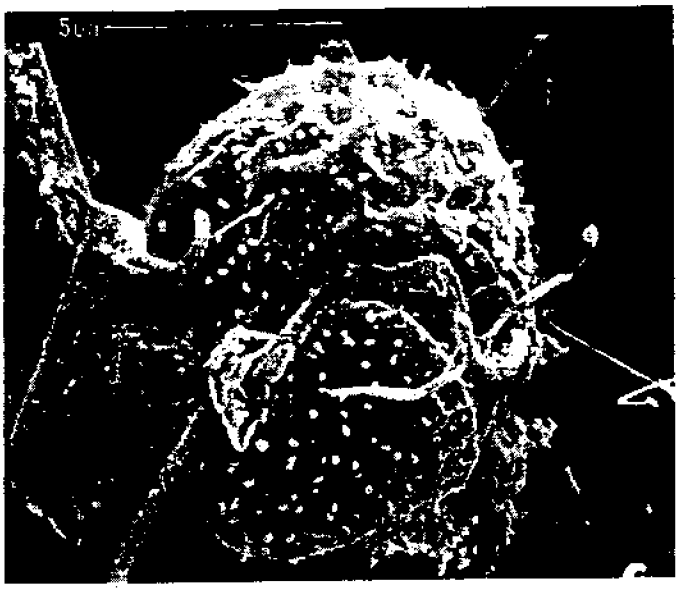


Fig.1

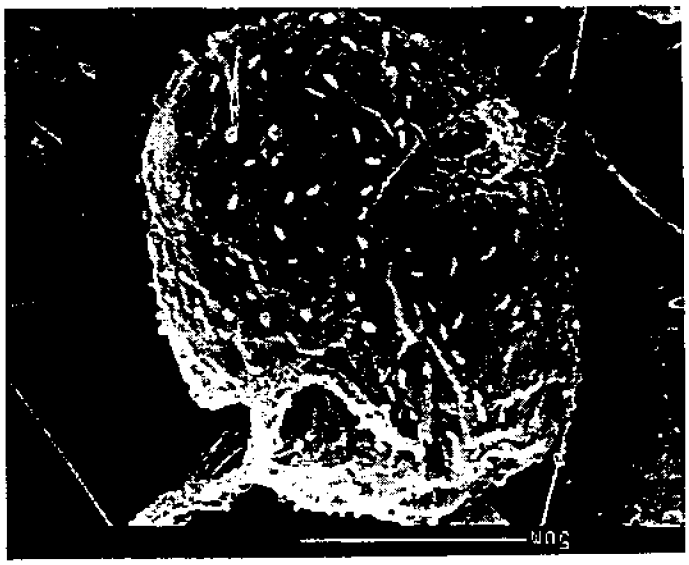


Fig.2

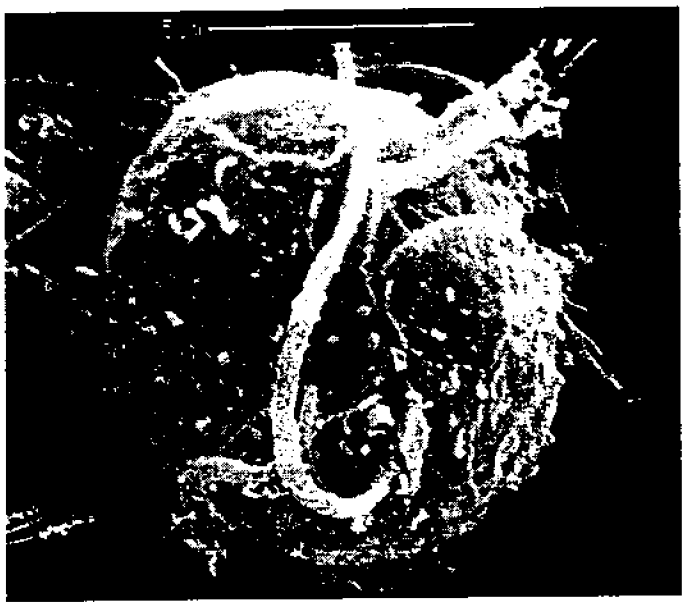


Fig.3

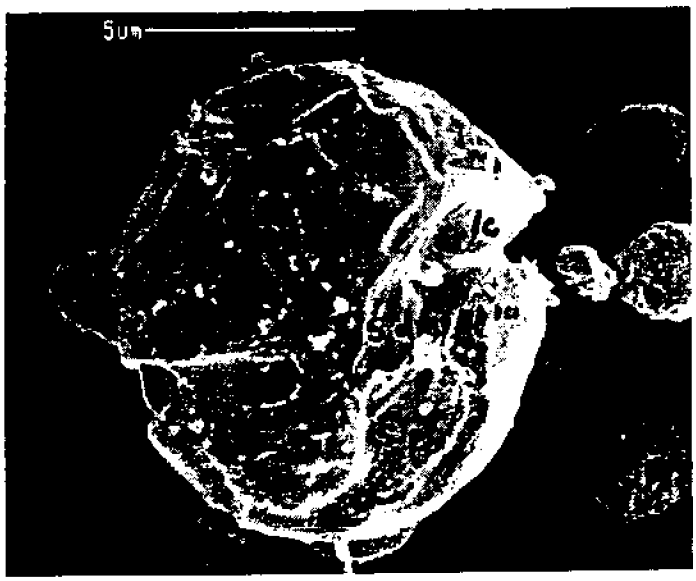


Fig.4

North Carolina and Florida samples

Figures 1-4. This is a new brackish water species that looks like *Pfiesteria piscicida* at both the light microscope and scanning electron microscope levels of resolution until thecal plates are revealed via removal of the outer membrane. It has a plate formula of Po, cp, X, 5', 0a, 6", 6?c, 4s, 5"', 0p, and 2"" which fits a *Peridiniopsis* tabulation. However, the plate pattern is different, and the species needs to be placed in a new genus. It is a heterotroph with a penduncle-like structure and phagocytizes microalgae. Food vacuoles are often observed in the anterior portion of the cell. Size and morphology are variable, but the plate tabulation and pattern are consistent among different isolates and within cultures. This species needs to be tested for toxicity because it has been isolated from areas having fish kills.

Fig.5

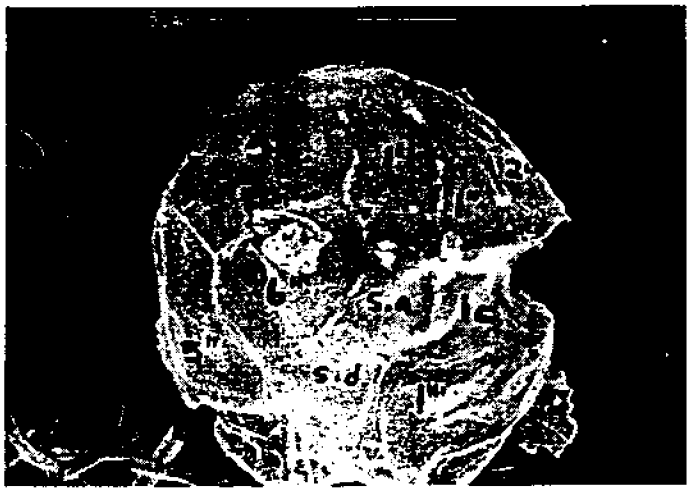


Fig.6

Fig.7

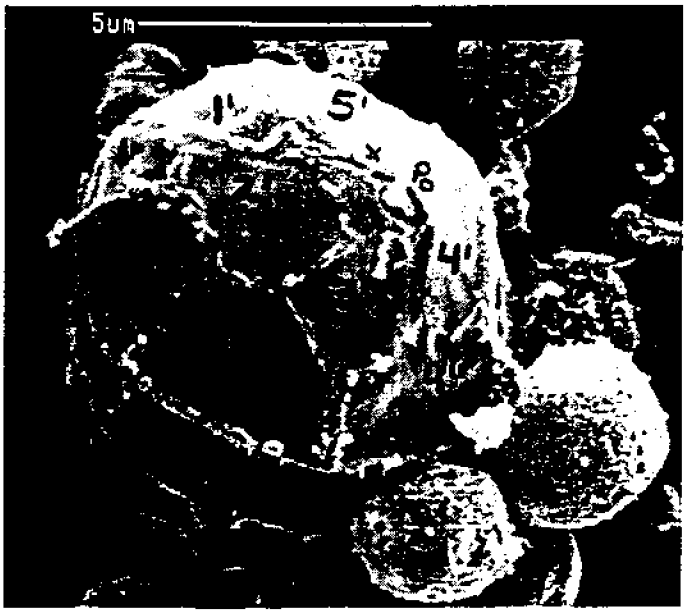


Fig.8

Figures 5 - 8. SEM of cells from North Carolina and Florida showing the plate arrangement. The anterior sulcal plate (s.a.) is under the 1' and above the right sulcal plate (s.d.). The longitudinal flagellum and penduncle-like structure emerge from under this flexible s.d. plate. The displacement of the cingulum is 1 to 1.5 times and the sulcus is offset to the right of the s.a. as in *Pfiesteria* species. The s.d. plate is directly under the 6". The pore platelet (Po) is on the dorsal surface and has a canopy or closing platelet.

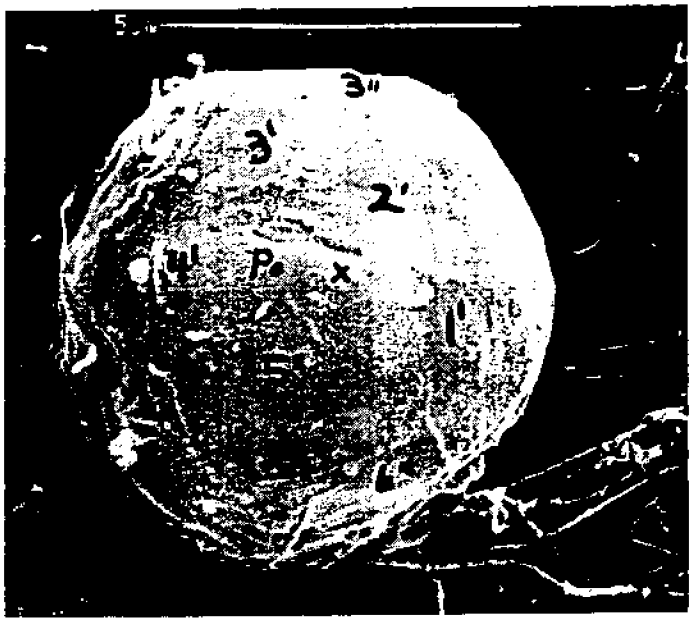


Fig.9

Fig.10

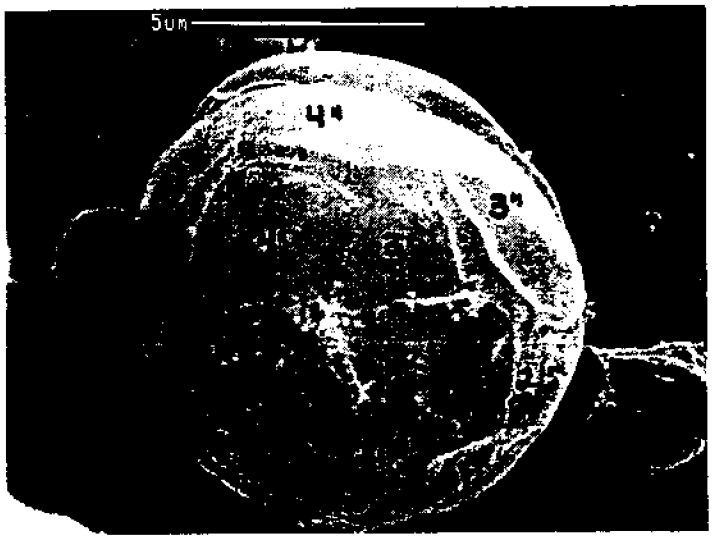
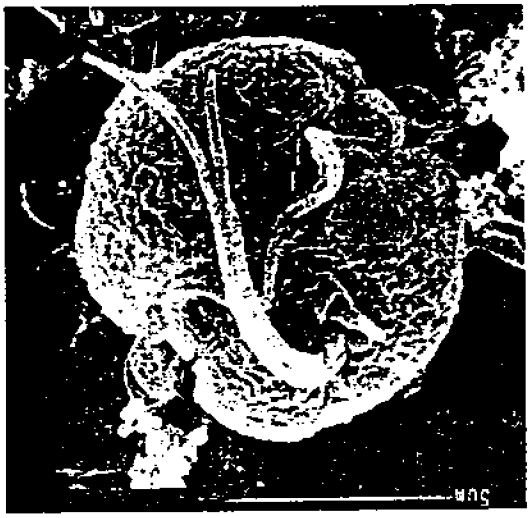


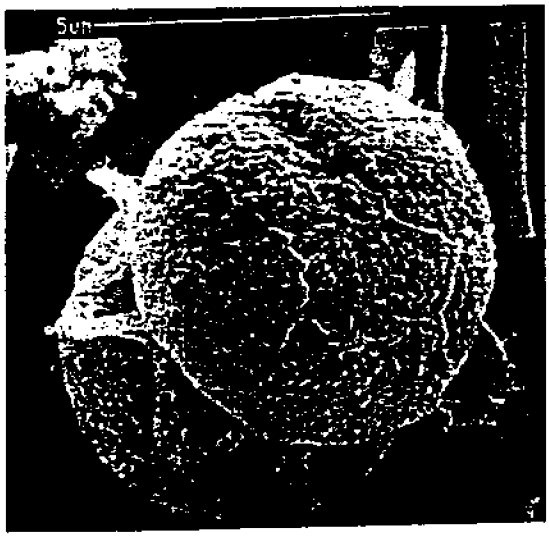
Fig.11

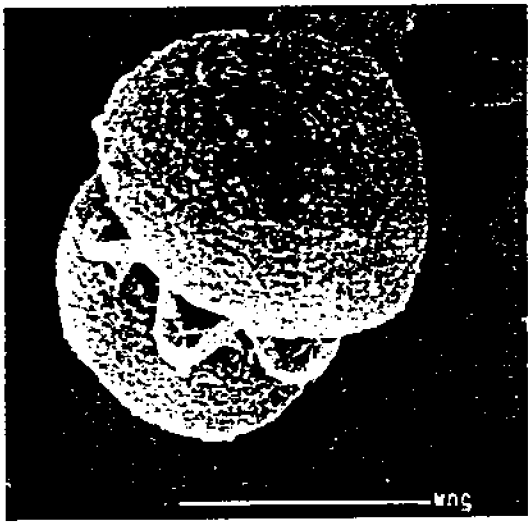
Fig.12

Figures 9-12. SEM of cells from North Carolina and Florida showing apical view and plate pattern. There are five apical plates ('); 2' and 5' are the largest and 3' is the smallest. The 2' plate is above the 1" and 2", while the 3' is above the 3" and 4". The 4' is above the 4" and 5", and the 5' is above the 5" and 6". There are no anterior intercalary plates (a).

Other Known *Pfiesteria*







Additional Possible *Pfiesteria* species