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RESEARCH NOTES

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INTERACTION OF PESTICIDES AND MARINE FUNGI

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Ever since the Chesapeake Bay region was first settled and land was cleared for agriculture, estuarine waters have been receiving increasing volumes of sediments, the run-off of topsoil that occurs during heavy rains. With the development of fertilizers and pesticides, complex chemicals have taken up residence in the soil; and now when sediments arrive in estuarine waters, they bring with them the residues of those chemicals which then can become pollutants. Atrazine, an extremely effective pesticide, is one of the most prevalent. Widely used on farms, it has taken up residence in the estuary and has been detected in surface waters, in the water column, in suspended particulates and bottom sediments. Questions arise: What happens to the atrazine in that environment? What are its effects on biota--microorganisms and plant and animal life? The work reported on here by Marilyn Speedie summarizes extensive laboratory experiments on the effects of atrazine on one important constituent of the ecosystem, marine fungi. Fungi play an important role in the estuary: Because they can concentrate pollutants through adsorption, uptake and degradation, they can potentially redistribute those pollutants as well. While the degradation of atrazine by terrestrial fungi, bacteria, higher plants and non-biological processes is well documented, there have been no published reports on indigenous marine fungi. To assess the effects on marine fungi, Dr. Speedie and her colleagues tested a number of fungal species for their interaction with atrazine. They found that different fungi reacted differently: for some atrazine was toxic; for others it had no effect; and still for others, atrazine stimulated growth. The implications for the ecosystem are double-edged: While fungi may prevent high levels of herbicide from reaching Bay waters, nevertheless, through lysis and desorption, they might also serve as a means for atrazine to be transported to other parts of the estuary. Thus, from an ecological perspective, any assessment of the implications of a pollutant such as atrazine must be evaluated on a species-specific case--interactions vary too much to generalize.

--The Editors

OBJECTIVES

The objectives of our research were to elucidate the interactions between pesticides and estuarine fungi. Part I dealt with the degradation and bioconcentration of atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) by the fungi and Part II dealt with the effect of herbicides, including atrazine, on the growth and cellulolytic activity of these fungi. All studies were performed with a collection of Ascomycetes and Deuteromycetes indigenous to Chesapeake Bay.

The specific objectives for Part I included:

1. Screening the collection of fungi to determine their ability to interact with atrazine, either by utilizing atrazine as a sole source of carbon and/or nitrogen for growth or by causing the loss of atrazine from its surrounding medium in the presence of other utilizable substrates (co-metabolism).

2. Conducting an in-depth study in pure culture of the rate, nature and extent of atrazine degradation by one or two selected species.

3. Determining the effect of atrazine on the growth and respiration of several species of fungi.

4. Evaluating the sorption (adsorption and bioaccumulation) mode of the interaction between fungi and atrazine.

The specific objectives of Part II included:

1. Determining the baseline parameters for cellulolytic enzymes produced by representative members of estuarine Deuteromycetes and Ascomycetes grown in shake culture.

2. Developing alternative methods for quantitatively determining if pesticides are affecting cellulase activity (a qualitative dye-release method had been tried previously).

EXPERIMENTAL APPROACH AND RESULTS

PART I

Screening of Fungi

Forty-seven isolates of estuarine Ascomycetes and Deuteromycetes were put through a two-part screen designed to (a) provide information about the ability of estuarine fungi, in general, to interact with atrazine and (b) to select one or two species for use in the in-depth study. The fungi were evaluated for their ability

1. To grow on defined solid medium with atrazine as a sole carbon source.

2. To grow on defined solid medium with atrazine as the sole nitrogen source.

3. To mediate the loss of atrazine from liquid shake culture using a medium containing both glucose and NH_4NO_3 (co-metabolic conditions).

In the utilization screen, 6 of 29 isolates showed increased growth when atrazine was present as sole carbon source; 8 of 29 showed increased growth when atrazine was present as the sole nitrogen source; and one species Nais inornata could utilize atrazine as a sole source of either carbon or nitrogen. In the co-metabolic study, 12 of the 42 isolates were able to cause the loss of 21-35% of 30 ppm atrazine; 14 of 42 were able to cause the loss of 6-20%; and 16 of 42 were unable to remove greater than 5% of the atrazine. The results of the two screens were used in a point scale to rate the organisms.

The ability of Nais inorata to utilize atrazine as a sole nutrient source was weighted most heavily since such a result necessarily implies some degradation of the

molecule, whereas loss of atrazine under co-metabolic conditions could be due to sorption as well as, or instead of, degradation. Moreover, the ability to utilize atrazine as a sole carbon source was given the greatest weight since approximately 40-50% of the biomass of fungi is carbon, while nitrogen content is much lower. Therefore, the ability to grow on atrazine as sole carbon source implies the breakdown of a substantial quantity of atrazine, at least to the point of dealkylation. Table 1 shows the scores for each isolate for which complete date was available. The remaining isolates either had a negative performance in one of the screens and were not tested further, or they did not grow on the medium used for one of the screens.

Table 1. Overall Scores of Fungi in the Preliminary Screens

Fungi	Overall Score
Nais inornata	21
Periconia prolifica	18
Trichocladium achrasporum	17
Zalerion maritimum	17
Trichocladium achrasporum	16
Halosphaeria mediosetigera	14
Periconia prolifica	13
Ceriosporopsis halima	13
Leptosphaeria oraemaris	11
Corollospora trifurcata	10
Flagellospora sp.	10
Zalerion varium	8
Monodictys pelagica	8
Trichocladiumachrasporum	6
Dendryphiella arenaria	6
Dendryphiella salina	4
Corollospora trifurcata	4
Buergenerula spartinae	0
Corollospora trifurcata	0
Leptosphaeria obiones	0
Leptosphaeria oraemaris	0
Zalerion maritimum	0

To select species for further study in the advanced screen, results of preliminary screens were evaluated together with experimental parameters (e.g., relative growth rate and biomass after three weeks incubation) and ecological considerations (e.g., frequency of isolation and growth substrates). Nais inornata had to be eliminated because of its inability to grow under advanced screen conditions; Lulworthia sp., obtained after completion of the preliminary utilization screen, was included in the advanced screen because of its ecological importance and its performance in the preliminary co-metabolic screen.

The ability of the eight selected fungi to utilize atrazine as sole carbon and/or sole nitrogen sources, and to mediate the loss of atrazine from a culture containing nutrients, was retested but with several differences. In summary, two organisms were able to utilize atrazine as a sole nutrient source: Leptosphaeria oraemaris grew with 500 ppm atrazine as the sole exogenous source of carbon or nitrogen, but not of both nutri-

ents at once, and Periconia prolifica had statistically significant growth compared to controls when atrazine was the sole exogenous nitrogen source. In addition to these two organisms, Dendryphiella salina and Trichocladium achrasporum fared best in the advanced screen.

Bioaccumulation

The four species described above were studied with radioactive atrazine to determine the sorption (adsorption and uptake) component of atrazine loss in artificial seawater plus 0.1% glucose and 0.02% NH_4NO_3. The percentage of added atrazine that could be found as sorbed material ranged from 1.9-9.9%. A portion (0.8-2.2%) was loosely bound and easily dissociable while another portion (0.2%-4.6%) was released only by sonification of the cells and extraction with methanol. Only P. prolifica had significant incorporation of radioactivity into cellular constituents, which might be indicative of degradation products.

The adsorption process was studied in greater detail with Dendryphiella salina using sodium azide as an uptake inhibitor. Atrazine was easily desorbed by successive artificial seawater washes indicating a relatively loose binding.

Periconia prolifica

Because data indicated that P. prolifica could mediate the degradation of the molecule, it was selected for a detailed study of all the interactive modes. Time course experiments demonstrated that under both low and high nutrient conditions, atrazine loss from the medium occurs primarily during the stationary growth phase of the fungus (from day 15 to 23 of a 23-day incubation). Loss of atrazine from the medium was maximized at increased glucose concentrations. Nitrogen concentration and salinity had less effect on atrazine loss.

PART II

Baseline Parameters of Cellulolytic Activity

Preliminary screening of eight species of fungi for cellulolytic activity using an assay measuring solubilization of dye from dyed cellulose and a radiometric assay using 14C-cellulose led to Dendryphiella arenaria as the subject of further investigation. Growth of D. arenaria in shake culture in an artificial seawater medium containing dyed cellulose revealed that although the fungus rapidly cleared the medium of particulate cellulose, dye was not released until after 8-10 days of incubation. This result suggested that the cellulolytic enzymes might be acting in close association with the cell wall.

During the initial screen with the dyed cellulose, we observed that not all organisms utilized the cellulose and released dye with the same pattern relative to growth. This suggested that perhaps the enzymes were localized differently in the various species.

Radiometric Assay for Cellulose Degradation

Since the dye-release assay could not serve as a general indicator of overall cellulolytic activity, it was necessary to develop an alternative assay for screening for

herbicide effects. We turned to a radiometric assay involving the measurement of $14CO_2$ which evolved following metabolism of 14C-cellulose. The assay was conducted in biometer flasks which are 250 ml Erlenmeyer flasks with a side arm connecting to a trap of NaOH. The base can be sampled periodically, neutralized and counted by liquid scintillation counting to determine the radioactivity evolved as $14CO_2$.

Effect of Herbicides on Fungal Growth, Respiration and Cellulolytic Activity

The respirometer flask was used to test the effects of three herbicides--atrazine, 2,4-diphenoxyacetic acid (2,4-D) and alachlor--on the mycelial weight, metabolism of 14C-glucose and metabolism of 14C-cellulose of several species of fungi, including Dendryphiella arenaria, D. salina, Zalerion maritimum and Z. varium. The only consistent effect observed was a stimulation of mycelial weight in the Dendryphiella species by 10 ppm 2,4-D. These studies are still underway.

The effect of atrazine on fungal growth has been studied also in connection with Part I: In the preliminary screen on solid medium, some species appeared to be stimulated by 30 ppm atrazine and others were inhibited. The effect on solid medium was quantitated by following radial growth from plugs. Z. varium cultures growing in the medium containing atrazine showed significantly less radial growth, relative to controls, by the eighth day of incubation ($p < 0.025$). Atrazine similiarly inhibited D. salina cultures by the second day of incubation ($p < 0.005$). When the radial growth of D. arenaria was compared in the presence and absence of atrazine, no statistical differences were observed. However, the medium containing atrazine severely inhibited the development of pigmentation in the older central portions of the fungal colony Subsequent microscopic examination of the growing margins of D. salina revealed that the atrazine-treated cultures had an overall decrease in hyphal branching with the initiation of branching occurring farther back from the hyphal tips Branching was also observed to be less acute in the atrazine-exposed mycelium. No morphological differences were noted for atrazine-exposed D. arenaria.

In liquid shake culture, the mycelial weight of Z. varium was inhibited by concentrations as low as 55 ppb. However, when the release of $14CO_2$ from labeled glucose was monitored, no effect of atrazine on Z. varium respiration was observed. This inconsistency has not yet been explained. Similarly, the Dendryphiella species are not inhibited by up to 30 ppm atrazine by either criteria (mycelial weight of $14CO_2$ evolution) in liquid culture. Clearly, more work would be required to clarify the complicated nature of atrazine's effects on fungal growth.

DISCUSSION

Our studies demonstrate that a number of environmentally important species of estuarine fungi interact with atrazine in various modes, including adsorption, uptake, cometabolic metabolism and, in a few cases, degradation to the extent that the degradative products are utilizable for growth. It is also important to note, however, that many species showed no evidence of interaction with atrazine in the preliminary screen. Clearly, the estuarine fungi cannot be considered to be a uniform group of organisms in this regard.

Adsorption was shown in the advanced screen to be a relatively minor component of the overall loss from the medium for most organisms. However, a detailed study of the adsorptive capability of Dendryphiella salina showed that the ability of the fungi to adsorb atrazine is comparable to that of other aquatic bacteria though less than that of

algae (Geller 1979; Valentine and Bingham 1976). Therefore, the fungi must be considered as one component of a microbial population which may be responsible for adsorbing atrazine in an aquatic environment. The adsorption study also demonstrates that the adsorbed atrazine is easily desorbed. Microorganisms which have adsorbed atrazine in one location of the estuary may subsequently release it as they move to other parts of the estuary which lack atrazine or which have pH or temperature favoring desorption. Thus, the estuarine biota which otherwise would not be exposed to atrazine may have to interact with it due to its transport on the cell surfaces of marine fungi and bacteria.

Based on the study with four species, we conclude that some fungi are able to internalize atrazine; a significant amount of atrazine was found as the intact molecule inside the cell at the end of the incubation period. This atrazine may not be as readily available for release to other parts of the estuary as the adsorbed portion. Nevertheless, the fungi are in a continuous state of growth and lysis; as the mycelia lyse, internalized atrazine may well be reintroduced into the environment. If the fungi have been redistributed as components of detritus, then they may be considered a vehicle for redistributing atrazine within the estuarine environment. Furthermore, as detrital particles are consumed by filter-feeders this internalized atrazine may be transferred to these organisms, another step up the food chain.

Definitive evidence of atrazine degradation was found only for two species, Leptosphaeria oraemaris and Periconia prolifica, which were able to utilize atrazine for growth. The fact that both L. oraemaris and P. prolifica were able to derive sufficient nitrogen for growth suggests strongly that the molecule was indeed metabolized beyond the dealkylation stage, at least to deamination, and perhaps involving ring cleavage. It is likely that other species may be degrading the molecule to some extent since others showed evidence of utilization in the preliminary screen or substantial loss in the co-metabolic screen. This study was directed at exploring the variety of interactive modes and detailing degradation for one species. However, on the basis of our data, it would be possible to select a number of species to examine for degradation, either by generation of $14CO_2$ from the side-chain or ring-labeled atrazines in the absence of other carbon or nitrogen sources, or by examining mycelial extracts for specific accumulating metabolites.

Periconia prolifica was the species selected for detailed study on the basis of its consistently good performance by all the screening criteria. The observation that atrazine uptake and metabolism by this organism occurs entirely during the stationary phase can be accounted for in various ways. Possibly the mycelium is not permeable to atrazine during the growth phase or the active transport enzymes responsible for its uptake are specific to the stationary phase. It is also possible that the enzymes responsible for its degradation are expressed as a component of secondary metabolic pathways and therefore are only present during this phase. A final alternative we have considered is that the degradation may be due to a combination of physicochemical and biological processes. The pH of the high glucose-high nitrogen culture becomes acidic during the stationary phase and acid facilitates the hydrolysis of atrazine to hydroxyatrazine. Other organisms are able to utilize hydroxyatrazine more readily than atrazine (Goswami and Green 1971), but that has not yet been established for P. prolifica.

Catalysis by an acidic environment would be ecologically relevant. Many of the fungal species are isolated from decaying estuarine and marine plants in a damp environment, such as that found in accumulations of decaying plant material at high tide level in the salt marshes and estuaries. The pH is lowered as organic matter decomposes (Lynch and Poole 1979) and may lead to synergism between this environment and the fungi in mediating the degradation of pollutants such as atrazine.

The microenvironment of the fungi in the estuary is also relevant to their cellulolytic activity. The optimal pH of the extracellular endoglucanase activity in D. arenaria is 5.8 while the pH of the estuarine waters can range from 7.5-8.4 (Johnson and Sparrow 1961). In decaying plant material, this enzyme may well be functioning optimally. However, in a submerged environment the excreted enzymes presumably diffuse away from the fungus and are affected by the higher pH. The opposite situation may be true of the extracellular exoglucanase of T. achrasporum which had an optimal pH at 7.0.

The temperature optima of the cellulase components are characteristic of other cellulase systems. The high temperature optima may reflect temperature conditions of the fungal habitat since the accumulations of decaying plant material may be functioning as compost heaps with characteristically elevated internal temperatures. The activity of the cellulase enzymes over a broad temperature and pH range affords these species the capability of adaptation in their marine environment. Extrapolation of laboratory results to estimates of rates of degradation in the natural environment must consider localized conditions of temperature and pH. In addition, the presence of different cellulase components in cell-associated and filtrate fractions emphasizes the need to look beyond the culture filtrate for evidence of cellulolytic activity in a given species.

CONCLUSION

A theme running through the results of all parts of this research is the variability among species within the marine fungi. The differences in localization of enzyme components between D. arenaria and T. achrasporum are one important example of this. Variable metabolic capabilities were also observed with respect to the interaction with atrazine. The full range was observed, from several species demonstrating no interaction by any of our criteria, to those which interacted to a great extent and by a variety of modes. Finally, the effect of atrazine on fungal growth ranged from toxicity to neutrality to stimulation, and a variety of physiological effects besides direct toxicity were also observed for a variety of species. This variability has an important implication for ecological research in that the impact of a given environmental factor (e.g., pollutants) cannot be assessed in terms of simple enumeration of fungi. Rather, one must consider the specific impact upon individual fungal species which possess the specific physiological functions of interest.

Our studies have demonstrated that the estuarine fungi are capable of interacting with atrazine as it washes into the estuaries through the processes of adsorption, uptake and degradation. In this regard, the fungi can be considered as part of a "safety net" that prevents high levels of herbicide from reaching the waters of the Bay. This is a two-edged sword, however, as the fungi also serve as a vehicle for transporting atrazine to parts of the environment that otherwise might not be exposed to atrazine. Our studies have demonstrated that a portion of the atrazine removed from the surrounding medium can be released by desorption or lysis at a later time.

Furthermore, our studies have demonstrated the cellulolytic activity of these organisms that is responsible for degradation of the grass and wood substrates upon which they grow. We demonstrated that the various components of the cellulase complex are compartmentalized within the fungal mycelia and that only some components are excreted. Therefore, studies of cellulase activity must examine more than filtrate activities.

The physiological effects of atrazine on the fungi and their cellulolytic activity are varied and complex. No consistent inhibition of mycelial dry weight, respiration,

or cellulase activity was observed for most species, through other effects on morphology and growth on solid substrate were observed.

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LITERATURE CITED

Armstrong, D.E., and G. Chesters. 1968. Adsorption catalyzed chemical hydrolysis of atrazine. Environ. Sci. Technol. 2: 683-689.

Correll, D.L., J.W. Pierce, T.C. Wu. 1978. Studies of the transport of atrazine and alachlor from minimal till corn fields into Chesapeake Bay tidal waters. Proc. Northeastern U.S. Week Sci. Soc. Conf. (Suppl.) 32: 21-32.

Davis, D.E., J.D. Weete, C.G.P. Pillai, F.G. Plumley, J.T. McEnerney, J.W. Everest, B. Truelove, and A.M. Diner. 1979. Atrazine fate and effects in a salt marsh EPA-600/3-79-111, United States Environmental Protection Agency. Gulf Breeze, Fl. 84 p.

Geller, A. 1979. Sorption and desorption of atrazine by three bacterial species isolated from aquatic systems. Arch. Environ. Contam. Toxicol. 8: 713-720.

Gessner, R.V. 1980. Degradative enzyme production by salt marsh fungi. Bot. Mar. 23: 133-139.

Giardina, M.D., M.T. Giardi, and G. Filacchioni. 1980. 4-Amino-2-chloro-1,3,5-triazine: a new metabolite of atrazine by a soil bacterium. Agric. Biol. Chem. 44: 2067-2072.

Goswami, K.P., and R.E. Green. 1971. Microbial degradation of the herbicide atrazine and its 2-hydroxy analog in submerged soils. Environ. Sci. and Technol. 5: 426-429.

Johnson, T.W., Jr., and F.K. Sparrow, Jr. 1961. Fungi in Oceans and Estuaries. Weinheim. J. Cramer. New York, NY.

Kaiser, P., J.J. Pochon and R. Cassini. 1970. Influence of triazine herbicides on soil microorganisms. Pp. 211-233. In: Residue reviews. Ed., R.A. Gunther. Springer-Verlag, New York.

Kaufman, D.D., and J. Blake. 1970. Degradation of atrazine by soil fungi. Soil Biol. Biochem. 2: 73-80.

Kirk, T.K., E. Schultz, W.J. Connors, L.F. Lorenz, and J.G. Zeikus. 1978. Influence of Culture Parameters in Lignin Metabolism by Phanerochaete chrysosporium. Arch. Microbiol. 117: 277-285.

Kohlmeyer, J. and E. Kohlmeyer. 1979. Marine Mycology: The higher fungi. Academic Press, New York.

Lynch, J.M. and N.J. Poole, (eds.). 1979. Microbial ecology: A conceptual approach. John Wiley and Sons, New York, NY.

Odum, W.E. and J.E. Drifmeyer. 1978. Sorption of pollutants by plant detritus: a review. Environ. Health Perspectives. 27: 133-137.

Squros, P.L. and R.A. Quevedo. 1978. Role of marine fungi in the biochemistry of the oceans. VI. Interactions of Zalerion maritimum with the pesticides aldrin and dieldrin. Mycologia 70:431-448.

Valentine, J.P. and S.W. Bingham. 1976. Influence of algae on amitrole and atrazine residues in water. Can. J. Bot. 54: 2100-2107.

Wu, T.L. 1980. Dissipation of the herbicides atrazine and alachlor in a Maryland corn field. J. Environ. Qual. 9: 459-465.

Related Publications Available from Maryland Sea Grant College Program:

MacDonald, MJ and M.K. Speedie. 1982. Cell associated and extracellular cellulolytic enzyme activity in the marine fungus Dendryphiella arenaria. Can J. Bot. 60: 838-844. UM-SG-RS-82-09.

Shocken, M.J. and M.K. Speedie. "Interaction of higher marine fungi with the herbicide atrazine. II. Sorption of atrazine to four species of marine fungi. Bull. Environm. Contam. Toxicol. 29: 101-106. UM-RS-SG-82-05.

Schocken, M.J., M.K. Speedie, P.W. Kirk, Jr., Interaction of higher marine fungi with the herbicide atrazine. I. Survey of interactive modes. Mycologia (In press).