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Reversal of Phenol and Naphthalene Effects on Ciliate Chemoattraction

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In an effort to address research needs in the area of rapid screening tests for aquatic toxicology, our lab engaged in a study of pollutant effects on protozoan chemoattraction. Among pollutants tested were metals and hydrocarbons. To ascertain whether inhibition observed after brief exposures to certain concentrations of the pollutants was irreversible, we examined the possibility of nullifying the inhibitory effect by removing protozoa from the toxicants after short exposures. Earlier work (Berk et al. 1985) showed that inhibitory effects of metals could be removed, and we report here the nullification and reversibility of effects of phenol and naphthalene on certain ciliates.

MATERIALS AND METHODS

The freshwater ciliates were species of Tetrahymena. One, Tetrahymena sp., was isolated from the Rivanna River in Charlottesville, Virginia, and the other, T. pyriformis was obtained from Carolina Biological Supply Company. Other ciliates used in the study were marine species. One of these, Paranophrys sp., was isolated from ocean water samples from Wachapreague, Virginia. The other, Miamiensis avidus was obtained from cultures of Dr. A.T. Soldo who isolated this species from shallow waters of the South Florida region.

Marine ciliates were maintained on bacterized cerophyl medium (Soldo and Merlin 1972) to which a bacterial strain isolated from Wachapreague, Virginia, was added. Prior to each experiment the bacteria were grown on an agar medium (Berk et al. 1977). The cerophyl medium was inoculated with a small loopful of bacteria 24 hr before inoculating the cerophyl medium with ciliates. Ciliate cultures prepared in this manner were used after 48 hr of growth at 25 C. Tetrahymena was grown axenically in a medium containing per liter: 2 g proteose peptone, 1 g yeast extract, 0.5 mL of 0.4 M $MgSO_4 \cdot 7\ H_2O$, 1.5 mL of 2 M glucose and 10 mL of 0.25 M Na_2HPO_4 added to 0.25 M KH_2PO_4 to a final pH of 6.9. The glucose and phosphate mixtures were autoclaved separately and added aseptically.

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The Tetrahymena were washed and collected by centrifugation in a table-top centrifuge at 85 x g with conical 10 mL tubes. Cells were rinsed twice with concentrated (ten-fold) Osterhout's solution containing per liter: 1.05 g NaCl, 0.023 g KCl, 0.010 g $CaCl_2$, 0.085 g $MgCl_2 \cdot 6 H_2O$ and 0.040 g $MgSO_4 \cdot 7 H_2O$. All testing with Tetrahymena was carried out using a ten-fold concentration of Osterhout's solution made from a hundred-fold stock solution. The ten-fold concentration of the salts solution more closely matched the osmolality of the medium. The bacterized marine cultures were cleaned and concentrated by a density-layering migration technique similar to that for cultures of Paramecium (Van Wagtendonk and Soldo 1970). The final washed stock of ciliates was suspended in filter-sterilized seawater from Wachapreague adjusted to 26 ppt salinity.

Phenol and naphthalene were dissolved (separately) in seawater or Osterhout's solution and mixed with the ciliate suspension to achieve the desired final toxicant concentration for testing inhibition of attraction. A saturated solution of naphthalene was prepared by adding naphthalene crystals to seawater or Osterhout's solution and stirring on a magnetic stirring plate for 24 hr in a sealed flask. This procedure was used by Pearson and Olla (1980), and Rogerson et al. (1983) used a similar procedure for preparation of saturated solutions of hydrocarbons for toxicity tests with freshwater ciliates. For the present experiments, aliquots were drawn from the saturated stock solution each time a test was run, because naphthalene may leave a solution over time due to its high volatility. For the present study, 50% saturated solution of naphthalene was used for exposing ciliates, i.e., equal volumes of the saturated solution and ciliate suspension were combined. The saturated solution was passed through a 0.22 μm filter before adding it to the ciliate suspension. For reversibility tests using naphthalene and Tetrahymena, a 25% saturated solution was used. All tests were conducted at 25 C.

Stock solutions of phenol were made and diluted to be mixed with the ciliate suspensions. Phenol, unlike naphthalene, is very soluble in water, e.g., 1 g dissolves in 15 mL water (Windholz 1983). Phenol concentrations from 50-200 mg/L were used to test inhibition. All concentrations of phenol and naphthalene were determined to be sublethal after a 30-60 min exposure. The chemoattraction test required the ciliates to be exposed for only 15 min. Control suspensions consisted of ciliates suspended in either seawater (for the marine species) or Osterhout's solution (for the freshwater species) without added toxicant solution, but with equal volumes of seawater or Osterhout's solution added in place of the toxicant solution. Numbers of ciliates were adjusted to be equal in both the experimental and control tests, since this aspect proved to be important in previous studies of chemoattraction inhibition (Berk and Mills 1986).

To determine attraction to yeast extract, suspensions of washed cells were placed in troughs to which 5-μL capillary tubes containing the yeast extract, seawater, or Osterhout's solution were added separately. Earlier

determinations of attraction of Tetrahymena sp. to yeast extract were made in Dryl's solution (Van Houten et al. 1975). After 15 min capillaries were removed, and ciliates were enumerated as described below. For determination of inhibition of chemoattraction, the control treatments used yeast extract prepared in seawater or Osterhout's to a final concentration of 0.15%, whereas the yeast extract for the experimental tests was prepared by diluting a 0.3% yeast extract solution to 0.15% with a toxicant solution in order to provide the same toxicant concentration in the attractant as in the experimental ciliate suspension.

One mL of each test and control solution containing protozoa was placed in four replicate chambers made from 1-dram vials cut in half vertically to result in small glass troughs. Washed protozoa were used within approximately 2 hr, since long periods of starvation cause more cells to be attracted (Berk and Mills 1986). Levandowsky et al. (1984) used Tetrahymena starved for at least 6 hr to test for attraction to various compounds. Since it is unknown whether starvation affects the ciliates' sensitivity to phenol and naphthalene, the protozoa were used relatively soon after washing, and controls were always conducted simultaneously with experimental tests to avoid misinterpreting inhibition due to increased chemoattraction with time. Approximately 3 x 10^3 cells/mL were used in all tests. Before testing, the troughs were acid-washed, rinsed with deionized water, and coated with a silicone compound to prevent ciliates from accumulating on the bottom of the trough. Acid-washed 5- L capillary tubes were filled with the attractants and placed in the appropriate experimental or control troughs containing the ciliate suspensions. The ciliates were exposed for 15 min, after which the capillaries were removed, and the contents of each were expelled into separate small drops of water (seawater or Osterhout's) on a glass microscope slide. The water was used to increase the volume of the drop on the slide to prevent evaporation and drying of capillary contents before cells could be counted. A small drop of Lugol's solution was then added to each drop to fix and stain the ciliates for enumerating by direct microscopic examination at 100X magnification. Numbers of ciliates in the control and experimental capillaries were compared. Data are presented the ratio of numbers in control capillaries to numbers in experimental capillaries.

To distinguish between inhibition of motility and inhibition of chemosensory mechanisms, additional controls were incorporated into the protocol using T. pyriformis. The test for effects on random movements involved comparisons of numbers of ciliates in capillaries containing only a modified Osterhout's solution when the ciliates were suspended in 0, 25, 50, and 75% saturated solution of naphthalene and in 0 and 200 mg/L phenol. Table 1 summarizes the tests for each species.

To determine whether the inhibitory effects of phenol and naphthalene could be removed by washing, suspensions of ciliates were tested for inhibitory effects as described above, and in addition, other suspensions of

Table 1. Experimental conditions for various ciliate species

Species	Treatment	
	Phenol	Naphthalene
Tetrahymena sp.	inhibition test at 100, 200 mg/L, reversal test at 200 mg/L	inhibition test at 50% saturation, reversal test at 25% saturation
Tetrahymena pyriformis	random movement test at 200 mg/L	random movement test at 0, 25, 50, 75% saturation
Miamiensis avidus	inhibition test at 60, 75, 100 mg/L, reversal at 100 mg/L	inhibition test at 50% saturation
Paranophrys sp.	not determined	inhibition test at 50% saturation

ciliates were exposed to naphthalene or phenol for 15 min and subsequently washed of the toxicants by repeated centrifugation as described above. The exposed, washed cells were then tested for chemoattraction to yeast extract and compared with controls which had not been exposed to the toxicants. Analysis of variance tests (ANOVA) were used to determine significant differences between experimental and control treatments, and between the washed and unwashed cells in the reversibility tests.

RESULTS AND DISCUSSION

All species of ciliates were attracted to yeast extract. Phenol was more inhibitory to a marine species, Miamiensis, than to the freshwater species, Tetrahymena sp. (Fig. 1). Phenol was not tested with Paranophrys, as cultures died before they could be tested. At 100, 75, and 60 mg/L phenol the ratio of control to experimental Miamiensis cells in capillaries was 5.5 (range= 3.1 -9.5 over 4 experiments), 3.6 (ave. of 4 replicate troughs), and 2.3 (ave. of 4 replicate troughs), respectively. Concentrations of 50 mg/L and lower were not significantly inhibitory to this species, i.e., chemoattraction responses were not significantly different from controls at low phenol concentrations. Phenol was significantly inhibitory ($p < .05$) at 100 mg/L for Tetrahymena sp., but the control to experimental ratio was only 1.5 (range= 1.4 - 1.6 over 4 experiments), and at 200 mg/L the ratio was 4.7 (range= 3.0 - 7.4 over 4 experiments). Inhibition of chemoattraction by phenol exposure was actually reversed after the cells were washed, i.e., after a 15-min exposure followed by removal of the phenol, the ciliates showed a stronger attraction toward the yeast extract than controls. This change was significantly different from controls for Tetrahymena sp. exposed to phenol. For Miamiensis exposed to phenol, the response was also

reversed after washing, however, due to one capillary containing unexplainably high numbers of cells, the reversal was not statistically different from controls. The response of Miamiensis after washing was still significantly different from that of unwashed cells, indicating that the inhibitory effect of phenol was nullified and the chemoattraction responses of the cells were restored at least to control levels. In Fig. 2, values greater than 1 indicate that chemoattraction was suppressed, whereas values less than 1 indicate that chemoattraction was enhanced.

One-half the saturation concentration of naphthalene was significantly inhibitory to all three species tested. The ratio of control to experimental cells of Tetrahymena sp. ranged from 4.0- 8.5 over 4 separate experiments, whereas the ratio for Paranophrys sp. was 3.2 (ave. of 4 troughs of one experiment). Miamiensis was the least sensitive (ratio= 1.7).

Since the solubility of naphthalene is lower in seawater, the marine and freshwater organisms would not be exposed to exactly the same concentration. We did not directly measure the dissolved naphthalene; however, Pearson and Olla (1980) have measured 19.1 mg/L as the dissolved naphthalene concentration in seawater at saturation at 21.5 C, and the solubility in distilled water at 25 C has been determined to be approximately 30 mg/L (Seidell 1940). Since the Osterhout's solution contained salts, stock naphthalene concentrations of the present study would have been slightly lower than 30 mg/L for the freshwater species.

Results of movement tests showed that random movement of T. pyriformis was not inhibited in the presence of phenol, indicating that inhibition is likely due to interference with chemosensory responses. Naphthalene, however, inhibited movement at concentrations greater than 25% saturation, but this inhibition was not enough to account for the overall decrease in numbers attracted to capillaries from naphthalene-exposed suspensions, also indicating that chemosensory responses were affected.

Reversal of the inhibitory effect of naphthalene was evident after the protozoa had been exposed for 15 min to 25% saturation concentration and subsequently washed (Fig. 2). The chemoattraction response after washing was very strong, and greatly different from the controls that had never been exposed to the naphthalene. This phenomenon was repeated 3 times, and consistent with results obtained a year earlier using Tetrahymena sp.

The 15-min chemoattraction assay measures a sublethal response to toxicants in a very short period compared with more standard toxicity tests, and it should provide useful screening information. The nature of the rapid assay makes it difficult to draw comparisons with toxic concentrations reported for other organisms, as most studies involve exposure for 24 hr or more and often test for lethality. However, results of the protozoan assay should be easily compared with results of the 15-min EC_{50} values of the Microtox assay, a screening test which uses luminescent bacteria.

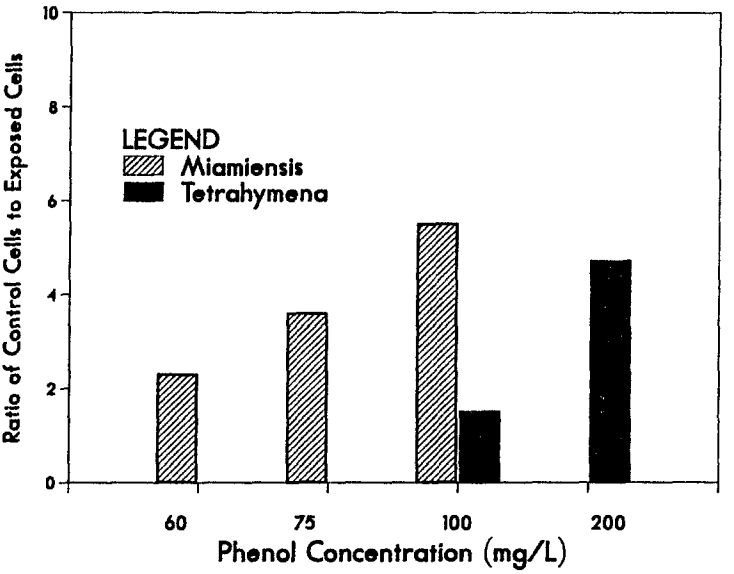

Figure 1. Inhibition of chemoattraction by Tetrahymena and Miamiensis exposed to various concentrations of phenol. Values of bars are ratios of numbers of ciliates in capillaries from control suspensions to numbers of ciliates in capillaries from phenol-exposed suspensions. Larger ratios indicate greater sensitivity to phenol.

The mechanisms of inhibition of attraction to yeast extract are not known at this time; however, for T. pyriformis it appears that movement and chemosensory responses are affected by naphthalene, whereas movement is not affected by phenol. In either case, the ability to reach the attractant is suppressed in toxicant-exposed ciliates compared with unexposed ciliates.

Kittredge et al. (1974) studied chemoreception in the intertidal crab Pachygrapsus crassipes exposed to naphthalene and water soluble extracts of crude oil. Their results showed that feeding responses of the crabs were inhibited at very low concentrations (1-100 μg/L). Their approach, however, involved a 24-hr exposure to naphthalene followed by washing and testing for inhibition.

Although naphthalene concentrations used in the present study were orders of magnitude higher than those used for crabs (Kittredge et al. 1974), they were determined to be sublethal to protozoa up to 60 min of exposure. In fact, Rogerson et al. (1983) found that the initial aqueous concentration of naphthalene necessary to cause lethal toxicity to Tetrahymena ellioti exceeded the solid solubility, i.e., saturated solutions of naphthalene were not toxic after 24 hr exposures.

The mechanisms for reversal of phenol to naphthalene effects are not known at this time. Perhaps a resistance mechanism compensating for the inhibitory effect was triggered such that washing ciliates after only a 15-

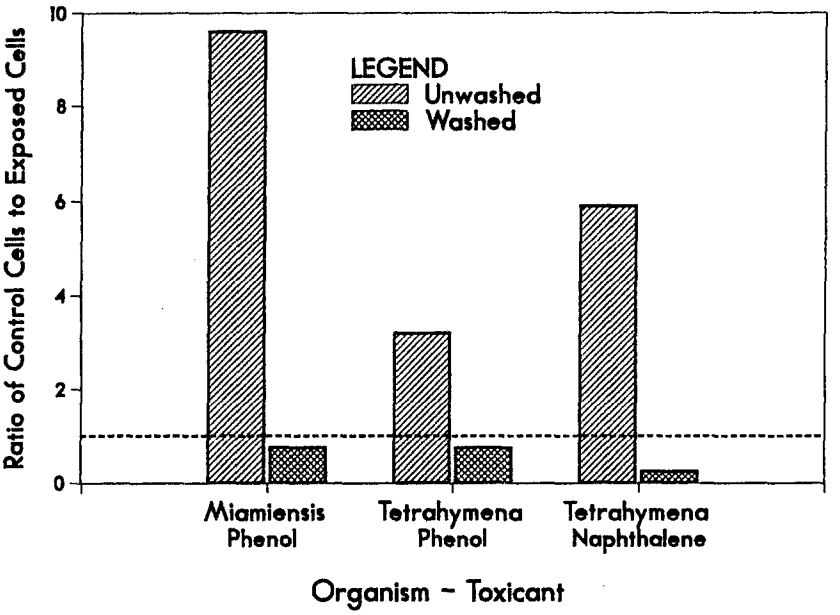

Figure 2. Inhibition of chemoattraction by Tetrahymena sp. and Miamiensis avidus exposed to 200 and 100 mg/L phenol, respectively; and inhibition of Tetrahymena sp. by 25% saturated naphthalene. Bar values above 1 indicate inhibition, and values below 1 indicate stimulation of chemoattraction.

min exposure left the cells with a better ability to follow a chemical gradient. Kittredge et al. (1974) observed recovery of inhibition in crabs exposed to naphthalene after washing, and Sabourin (1982) found that naphthalene toxicity to blue crabs increased with time up to 24 hr after which respiratory components returned to control levels. Berk et al. (1985) found that chemotaxis inhibition caused by 15-min exposures to Cd and Cu could be nullified for certain marine and freshwater ciliates after washing the protozoa.

For the present study, yeast extract was used as the attractant because it was strongly attractive for all the species used. Hellung-Larsen et al. (1986) found that of several attractants tested with Tetrahymena, only yeast extract, proteose peptone, and a platelet derived growth factor could attract unstarved cells. Levandowsky et al. (1984) found attraction to L-histamine, agmatine, cimetidine, and several of the amino acids by Tetrahymena thermophila; and Antipa and Norton (1984) reported that Paramecium showed positive chemotaxis toward a heat-stable 500 dalton factor produced by bacteria in a minimal medium.

Reversal of inhibitory effects may be an indication that the protozoa would have adapted to the inhibitory concentrations of certain chemicals. Studies using longer-term exposures are currently under investigation.

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REFERENCES

Antipa G, Norton J (1984) Chemoreception by Paramecium of bacterial signals produced by Escherichia coli in a strictly defined minimal medium. J Protozool 31:13A

Berk SG, Gunderson JH, Derk LA (1985) Effects of cadmium and copper on chemotaxis of marine and freshwater ciliates. Bull Environ Contam Toxicol 34:897-903

Berk SG, Brownlee DC, Heinle DR, Kling HJ, Colwell RR (1977) Ciliates as a food source for marine planktonic copepods. Microbial Ecol 4: 27-40

Berk SG, Mills BA (1986) Factors affecting chemotaxis inhibition tests with protozoa. J Testing Eval 14:140-144

Hellung-Larsen P, Leick V, Tommerup N (1986) Chemoattraction in Tetrahymena: on the role of chemokinesis. Biol Bull 170:357-367

Kittredge JS, Takahashi FT, Sarinana FO (1974) Bioassays indicative of some sublethal effects of oil pollution. Proc Tenth Ann Conf Mar Tech Soc, Washington, D.C.

Levandowsky M, Cheng T, Kehr A, Kim J, Gardner L, Silvern L, Tsang L, Lai G, Chung C, Prakash E (1984) Chemosensory responses to amino acids and certain amines by the ciliate Tetrahymena: a flat capillary assay. Biol Bull 167:322-330

Pearson WH, Olla BL (1980) Threshold for detection of naphthalene and other behavioral responses by the blue crab, Callinectes sapidus. Estuaries 3:224-229

Rogerson A, Shiu WY, Huang GL, Mackay D, Berger J (1983) Determination and interpretation of hydrocarbon toxicity to ciliate protozoa. Aquatic Toxicol 3:215-228

Sabourin TD (1982) Respiratory and circulatory responses of the blue crab to naphthalene and the effect of acclimation salinity. Aquatic Toxicol 2:301-318

Seidell A (1940) Solubilities of organic compounds, vol 2, D. Van Nostrand Co., Inc., New York, p 647

Soldo AT, Merlin EJ (1972) The cultivation of symbiote-free marine ciliates in axenic medium. J Protozool 19:519-524

Van Houten J, Hansma H, Kung C (1975) Two quantitative assays for chemotaxis in Paramecium. J Comp Physiol 104:211-223

Van Wagtendonk WJ, Soldo AT (1970) Methods used in the axenic cultivation of Paramecium aurelia. In: Prescott DM (ed) Methods in cell physiology, Academic Press, New York, pp. 117-130

Windholz M (1983) The merck index, Merck & Co., Inc., Raway, New Jersey, p 7117

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