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Stream Pollution

MEASUREMENT OF TOXICITY OF ORGANIC WASTES TO MARINE ORGANISMS *

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Establishing criteria for quality of industrial effluents for disposal in the marine environment requires a somewhat different approach than in fresh water. Since most fresh water is potentially potable, taste, odor, and water quality in addition to effect of the waste to aquatic life become of prime importance; whereas, in brackish and sea water the main interest is in the effect of wastes on the marine ecological system. Historically, priority has been given to acute toxicity of waste to individual fish with little attention given to sub-lethal stresses on the entire community. Greater attention is now directed toward toleration of pollution (1).

Some organic wastes cause turbidity, color change, or slicks, and are aesthetically and usually ecologically undesirable. Effluents of these types can be detected easily and controlled visually or with relatively simple instrumentation; however, the dissolved toxic materials must be observed by either chemical or bio-assay techniques. Chemical evaluation of toxic components in effluent streams has received extended emphasis by some industries. These techniques are valuable when considering in-plant treatment but have not given satisfactory results in the field because chemical analyses usually give little or no information on the physiological state of the organisms, the history of the water, temperature, and other factors which become important in toxicity relations. Also, industrial effluents are chemically complex, and the necessary techniques for separating and determining all of these have not been developed, or chemical methods lack the sensitivity necessary to determine the small concentrations of materials which may be toxic. In complex wastes the presence of a number of different toxicants is expected. When mixed, these may create antagonisms or synergisms which will produce entirely different toxicity than that of pure components. As the mechanism of toxicity becomes better understood, chemical or biochemical techniques will be found increasingly useful. For the present, bio-assay procedures circumvent some of the problems of uncertainty by directly determining the effect of the waste on organisms, and can be done under conditions which simulate the natural environment to a very close degree.

Ideally, the effect of effluent streams should be studied in the waste-receiving water body. For this method to be effective, a large number of sampling stations must be taken over an extended period of time, and a rather complete knowledge of the hydrography, biota, chemistry, and mixing rates must be obtained. A number of

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broad-scale studies have been undertaken in an attempt to determine changes in the types, kinds, and numbers of organisms in fresh water streams and lakes and in coastal estuaries and bays resulting from release of sewage and industrial wastes.

Rather extensive studies of many fresh water basins have been undertaken in recent years (2) (3) (4) (5) (6) (7). Along with these studies a number of specific techniques for indicating pollution have evolved (8) (9) (10). The effects of pollution on coastal waters have also received extensive attention (11) (12) (13) (14) (15) (16) (17) (18) (19) (20). Extensive studies of a more specialized nature have been undertaken to determine the effect of pollution on oyster and shellfish growth and mortality (21) (22) (23). Techniques for evaluating the over-all effect of pollution by *in situ* measurements of primary productivity of estuarine areas have been developed by Odum and Hoskin (24) and Park *et al.* (25); however, these techniques have not yet had extensive use as over-all pollution indicators. Such information, while giving valuable data on the effect of wastes on productivity of an area, does not necessarily reflect shifts that may occur due to change in kind of flora or fauna. Studies of this type coupled with detailed studies of the plankton community should give evidence of the over-all effect of added contaminants.

Because of the difficulty of obtaining adequate environmental data concerning changes in normal processes caused by pollution, it is usually expedient to supplement survey work with both chemical and biological evaluation of effluent streams. Coupling such information with characteristics of the estuary or receiving water systems allows the investigator to make recommendations for effluent clean-up and to predict the effects of the effluent on the marine community.

Bio-assays

Studies on application of bio-assays to toxicity of sewage and industrial wastes effluents are recorded (26) (27) (28) (29) (30) (31) (32). Relatively little work has been reported on similar data for marine fish. Daugherty (33) has proposed essentially the same method as adopted for fresh water fish with the exception of substituting a salt water species for the test organism. The usual method of reporting toxicity is based on the standard TL_m levels which indicate concentration of median lethality of the waste material to the test organism. These tests are useful in many cases as a control, but sub-lethal stress data are more useful in estimating the over-all effect of effluents in the aquatic system. To obtain these values many factors must be considered. Among these are temperature, dissolved oxygen, carbon dioxide, pH, alkalinity, history of organisms and water system, light penetration, ionic strength, presence of other organic contaminants, synergisms, and antagonisms.

In addition to standard bio-assays with the use of fish, a few special studies have been reported on the effect of effluents on other marine organisms or to fish in the actual environment. Chin and Allen (34) studied the toxicity of insecticides to the shrimp *Penaeus aztecus* and *Penaeus setiferus*. Hood *et al.* (35) have used the phytoplankton *Chlamydomonas sp., Porphyridium cruentum, Nitzschia closterium* (Ehr) Wm. Smith, and an unidentified diatom in determining the effect of chlorinated hydrocarbon wastes to photosynthesis. Vivier (36) used fish in live boxes in the receiving stream as an index of toxicity, and Harrington and Bidlingmayer (37) investigated the effects of dieldren on the toxicity of fish and invertebrates of a salt water marsh.

Relatively few data have been obtained on the sub-lethal stresses of organic wastes to organisms. For prac-

tical purposes data must be obtained which indicate whether the organisms endemic to an area can prosper as a species in an environment containing the toxic effluent material. To answer this question, it is necessary to look, not merely at the ability of the individual fish or organism to survive, but also at the ability to compete with other organisms of the environment for survival. The ability to reproduce, which may include access to spawning grounds, and the ability of eggs and young stages to develop and grow must be considered. Of basic importance to any study is the effect of contaminants on the ability of food organisms to survive in the polluted area.

In the marine system the phytoplankton represent a large portion of the total basic food supply; thus, studies with these organisms would seem to give information far more easily interpreted in terms of the efficacy of effluent treatment or dilution in an estuarine condition. Tests with organisms of trophic levels other than fish would also be of interest.

In any case, it is important that information be obtained on the metabolic effects of wastes on organisms rather than only their tolerance to acute toxicity. Such data may be obtained by measuring the effect of effluents on respiration, photosynthesis, or on specific enzyme systems of the organism. Weiss and Botts (32) investigated the effect of wastes on oxygen consumption rates of sunfish and minnows. Weiss (38) has determined the effect of organic insecticides on the enzyme system acetylcholinesterase. Previous preliminary work has been done (35) (39) (40) to determine the effect of wastes on the photosynthesis by marine phytoplankton. The results of a continuing study on the effect of industrial effluents on metabolism of crustacea and phytoplankton as compared to acute toxicity to marine fish and grass shrimp are given in this paper.

Experimental

Acute Toxicity

Cyprinodon variegatus.—Acute fish toxicities were determined by the tentative A.S.T.M. method, D 1345-54T, in which the estuarine minnow, *Cyprinodon variegatus,* was used as the test organism. Parallel experiments using this fish and *Fundulus similis* gave similar results with phenol as the toxic material. In all tests the oxygen concentration of the test bottle was measured daily and maintained at near saturation. Temperatures were maintained at 25 ± 1°C and the fish were acclimated a minimum of 10 days and a maximum of 30 days from the time of gathering. Natural sea water obtained in Galveston Bay and adjusted to a chlorinity of 30 per cent was used as dilution water.

Palaemonetes.—The crustacean, *Palaemonetes* (grass shrimp), was used as a bio-assay organism employing the same techniques and conditions used for the fish.

Metabolic Effects

Artemia salina.—Commonly known as brine shrimp, these have been used by Hood (39) and by Okubo (41) as assay organisms for toxicity of effluents. This organism was selected because it is available as eggs which remain viable for several years and are easily accessible from pet shops. Hatching was accomplished by placing the eggs in sea water at a temperature of approximately 25°C for 24 hr. A detailed description of culturing methods is given by Bond (42). Organisms suitable for study of the effects of wastes on metabolic processes were prepared first by washing the shrimp eggs with several portions of sterile distilled water. Then the organisms were concentrated by removal of the water through a Buchner funnel. To a Fernbach flask equipped with a cotton plug and necessary glass tubing were added 1.5 l of 90-per cent sea water, and the

flask was autoclaved. After complete cooling, 3 g of the shrimp eggs were added to this culture medium and allowed to hatch at room temperature. Aeration was provided through a sterile cotton plug. To feed the shrimp, a few drops of a suspension of approximately 100 g of compressed baker's yeast mixed with 200 ml of sterile sea water were added every 24 hr. This method of culture, while not providing bacteria-free cultures, did provide an organism of low bacteria-count which was found suitable for respiration studies. To determine the effect of wastes on respiration, an aliquot of shrimp was removed from the culture flask and added to a 300-ml oxygen bottle containing gradient amounts of waste material. The bottles were completely filled with sterile sea water and allowed to stand for a period of 24 hr. The amount of oxygen consumed in each bottle was subtracted from the amount consumed in the control bottle, the difference representing the relative effect of waste concentration on the respiration of the shrimp. In cases where the waste interfered with the Winkler's reaction for DO, it was necessary to modify the experiment in order to circumvent this difficulty. In this case, the organisms were exposed to the waste material for a period of time, filtered, washed, and placed in sterile sea water for a growth period of approximately 24 hr. The oxygen utilization during this period was used as an index of the effect of exposure to the waste on the respiration of the organism.

Acute toxicity of wastes to brine shrimp was also determined. The number of living individuals in the culture medium before starting the experiment was determined by counting the total organisms in an aliquot of cultures, after killing with formalin, and subtracting from this the number of non-motile organisms observed prior to killing. A ruled 2- × 3- × ½-in. lucite chamber and a 4-power binocular microscope were found satisfactory for counting. An aliquot of the shrimp culture was exposed to the waste material, and after a period of 24 hr the number of dead organisms in the culture was again estimated and the percentage killed was determined.

Phytoplankton.—Unicultures of the Chlorophyceae, *Platymonas* sp. and *Chlamydomonas* sp.; Xanthophyceae, *Mischococcus* sp.; Bacillariophyceae, *Nitzschia closterium* (Ehr) Wm. Smith and an unidentified diatom; and Rhodophyceae, *Porphyridium cruentum,* were used to determine the effect of wastes on photosynthesis. The nutrient medium used to grow and maintain the cultures consisted of Ketchum and Redfield's (43) Solution A, 2.0 ml; Ketchum and Redfield's (43) Solution B, 0.5 ml; Arnon's (44) micro-nutrients, 0.5 ml; Bold's (45) soil extract, 2.0 ml; $Na_2SiO_3 \cdot 9H_2O$, 0.0466 g; $Na_2MoO_4 \cdot 2H_2O$, 4×10^{-5} g; and 1,000 ml filtered sea water. The cultures were sub-cultured about one week prior to use in the experiments. Twenty-four hours prior to the experiment, the cultures were centrifuged, washed three times with sterile sea water, and to the subcultures was added 1×10^{-4} mole of potassium nitrate, and 3.7×10^{-4} mole KH_2PO_4 to the medium to insure rapid growth throughout the experiment. Solutions were adjusted to pH 8.2 and sterile conditions were maintained throughout.

Measurement of Inhibition to Photosynthesis

The amount of photosynthesis occurring in algal cultures in a period of 24 hr in the presence of gradient amounts of waste was determined by the method of Steemann-Nielsen (46). Sodium bicarbonate containing 0.5 μc of carbon-14 was added to a total volume of 150 ml of medium. The cultures were grown in presence of 900 foot-candles of incident light energy from fluorescent tubes. Dark bottles which did not permit light to enter

were used to determine absorption of carbon-14. After 24 hr the organisms were removed by filtration through 0.45μ membrane filters, washed with 1 N hydrochloric acid, desiccated, and the carbon-14 activity determined on a gas flow proportional counter. Efficiency of this machine has been determined to be about 33 per cent for carbon-14. The inhibition to photosynthesis caused by the waste was indicated by the reduced rate of carbon-14 uptake from that of the control maintained under exactly the same conditions.

Oxygen Evolution Measurements

In some cases the evolution of oxygen by the photosynthesizing organisms was used as an indication of photosynthesis. In this case, 300-ml BOD bottles were filled to the 250-ml mark with nutrient medium and the proper amount of waste material and water was added to fill the bottles. Parallel runs were made with dark bottles from which light was excluded. Cultures were incubated for a 24-hr period under conditions similar to that for carbon-14 uptake experiments after which the amount of oxygen in each bottle was determined. The difference between the oxygen in the light bottle and that in the dark bottle indicated gross photosynthesis of the cultures. This was compared to the controls as an index of inhibition. In the case of some wastes, interference to the obtained Winkler method for oxygen was observed. In these cases, the organisms were exposed to gradient concentrations of waste for a period of time, removed from the cultures, washed free of waste with sterile sea water, and then placed in standard BOD bottles in the presence of uncontaminated nutrient solution. The organisms were allowed to grow for a period of 24 hr, after which the oxygen was determined as above. The effect of exposure time on the waste material for the green algae *Chlamydomonas* sp. was reported earlier by Hood *et al.* (35). It was observed that the maximum effect due to exposure occurred in the first 60 min, and this exposure time was usually used in these studies.

Chlorophyll Determination

Chlorophyll concentration in the algae was determined by the method of Richards (47).

Wastes Investigated

Waste No. 1 was a lighter chlorinated hydrocarbon mixture containing some ethers and alcohol. The hydrocarbons were largely of 3 carbon units with limited substitution of chlorine. This mixture had a specific gravity of 0.95.

Waste No. 2 was black liquor obtained from Champion Paper and Fibre Company. It had a gravity of 1.265 g/cu cm at 60°C and contained 45.6-per cent solids; it had a pH of 13 and an alkalinity of 1.7 me/g of material.

Waste No. 3 was a heavy chlorinated hydrocarbon mixture. The hydrocarbons were largely of 3 carbon units with heavy substitution of chlorine. The mixture had a specific gravity of 1.34.

Waste No. 4 was an aqueous alkaline solution containing mercaptans and sulfides and was saturated with Waste No. 1 and No. 3. It had a specific gravity of 1.14.

Waste No. 5 was a mixture of heavy chlorinated hydrocarbons with an alkaline aqueous phase containing mercaptans and sulfides. This waste was produced at a later time than Wastes 3 and 4.

Waste No. 6 was reagent grade phenol.

Wastes immiscible with water were shaken vigorously with water, allowed to stand for 24 hr at 25°C and were centrifuged to separate phases. The water portion was considered saturated and dilutions of this material were used as test material. If the wastes consisted of water soluble and also im-

miscible wastes, a homogenized mixture of the two was added to water as above. The mixture of the two was considered saturated if immiscible material remained in contact with the aqueous phase.

Results and Discussion

Work on materials contained in Waste 3 gave the data presented in Figure 1. These data indicate that the test organisms which represent a rather broad spectrum of classes of marine plants do not respond to the same concentration levels with respect to this waste. *Porphyridium cruentum* and *Chlamydomonas* sp. appear to be most resistant of the organisms tested and even in this case the concentration of wastes of 10^{-4}-per cent saturated (5×10^{-5} g/l) affects photosynthesis adversely. In the case of *Mischococcus* sp. and the diatom, much lower concentrations are inhibitory. Experiments with other wastes have shown that with some wastes inhibition to all phytoplankton tested occurs at nearly the same concentration level, and at times the comparative sensitivity of the various organisms may shift depending on the nature of the waste material.

The effect of Waste No. 2, commonly known as black liquor, on photosynthesis of *Nitzschia closterium, Platymonas* sp., and the unidentified diatom is shown in Figure 2. Data on the per cent lethality of this waste to zooplankton obtained from a natural environment are also given. Concentrations of black liquor which completely inhibited photosynthesis (1 g/l) killed only about 25 per cent of the zooplank-

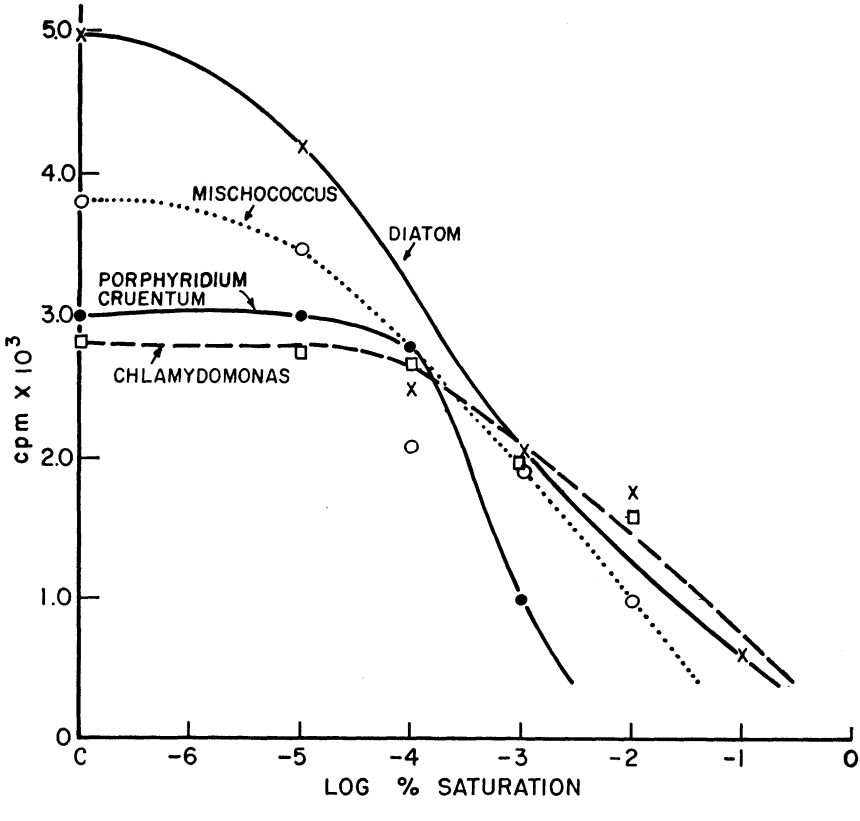

FIGURE 1.—Effect of Waste No. 1 on marine phytoplankton photosynthesis. C-14 uptake used as indicator of photosynthesis; 0.5 c $HC^{14}O_3$ added to each 150 ml culture; growing period, 22 hr.

ton organisms. Inhibition to photosynthesis occurred in *Platymonas* sp. organisms at a concentration of about 10^{-3} g/l; for *Nitzschia,* 10^{-2} g/l; and for the diatom, at about 0.16 g/l.

Ideally, zooplankton organisms endemic to the marine environment would be used for determining the effect of wastes on metabolism of this trophic level. There are few natural zooplankton that may be cultured in the laboratory in quantities sufficient to make these tests possible, and investigations were therefore undertaken to obtain an organism which would give representative data, and yet provide an organism readily available to the investigator. Investigations were thus undertaken with *Artemia salina,* the brine shrimp, as a test organism. In Figure 3, comparative data are presented on the effect of various wastes, including phenol, on the oxygen consumed by these organisms in the presence of gradient amounts of wastes. In the case of phenol, inhibition to respiration is initiated at 10^{-4} g/l and complete inhibition occurs at about 10^{-1} g/l. For comparison, data for the inhibition to photosynthesis of the phytoplankton *Platymonas* sp. are given for Waste No. 1. Brine shrimp appear to be much more resistant to inhibition to respiration than are the *Platymonas* sp. to photosynthesis. Only a 10^{-4}-per cent saturated solution initiates inhibition to photosynthesis by *Platymonas* sp.; whereas, little inhibition to respiration occurs even at the 10^{-2}-per cent saturation level for brine shrimp.

In Figure 4 comparative data are presented for lethality of Waste No. 5 to brine shrimp and fish, and also for the effect of the waste on respiration of the brine shrimp. These data indicate that brine shrimp are much less resistant to acute toxicity than fish but

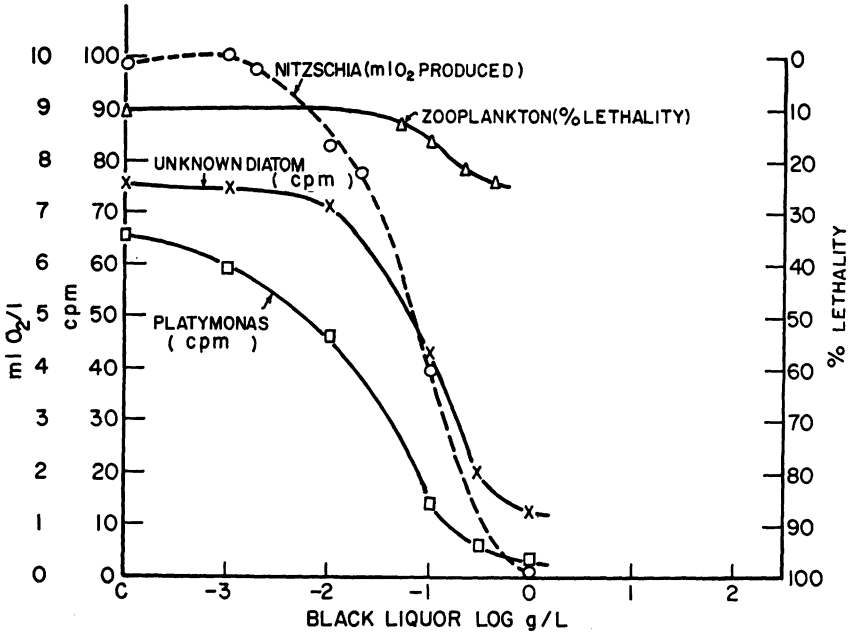

FIGURE 2.—Effect of Waste No. 2 (black liquor) on photosynthesis of marine phytoplankton. C-14 uptake used as indicator of photosynthesis for unknown diatom and *Platymonas* sp.; 0.5 c added to each 150 ml culture; growing period, 22 hr at 24°C. Oxygen evolution used as indicator of photosynthesis with *Nitzschia closterium*; growing period, 24 hr. Zooplankton obtained in ship channel at Institute of Marine Sciences Laboratory, Port Aransas, Tex. Exposure time, 120 min.

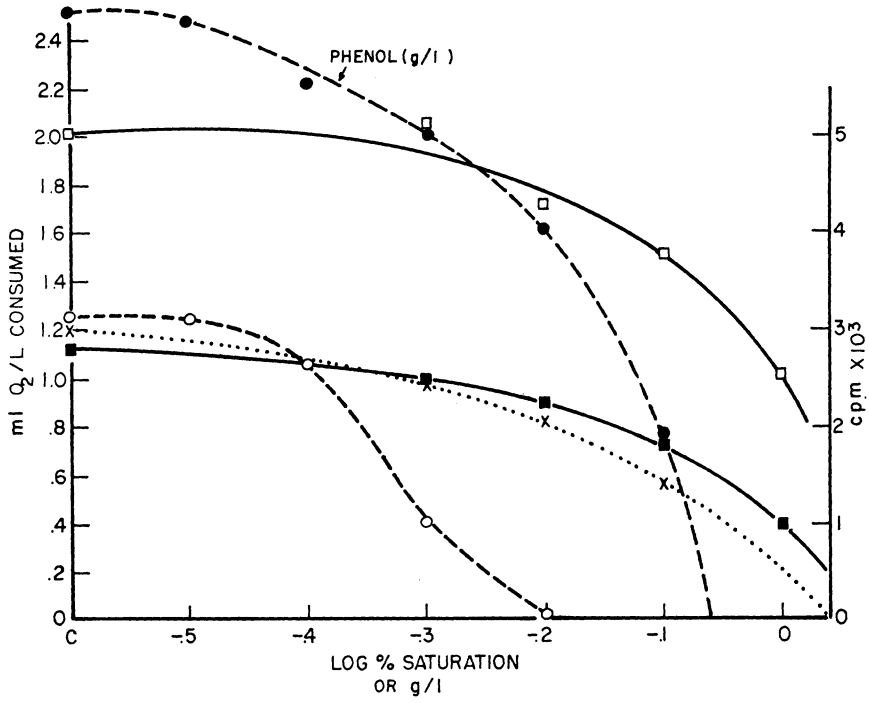

FIGURE 3.—Effect of different wastes on the respiration of *Artemia salina* as compared to effect on photosynthesis by *Platymonas* sp.

- ● - - - ● Phenol in g/l on inhibition of respiration of *Artemia salina.*
- □——□ Waste No. 3 in per cent saturation on inhibition of respiration of *Artemia salina.*
- ■——■ Waste No. 1 in per cent saturation on inhibition of respiration of *Artemia salina.*
- X · · · X Waste No. 4 in per cent saturation on inhibition of respiration of *Artemia salina.*
- ○ - - - ○ Waste No. 1 in per cent saturation on inhibition of photosynthesis of *Platymonas* sp.

Exposure time of *Artemia salina* to wastes was 2 hr; growth period was 24 hr.

inhibition to respiration in these crustaceans occurs at about the same concentration as does lethality to fish. These data suggest that the brine shrimp may yield results similar to fish toxicity data providing inhibition to respiration criteria are used in place of acute toxicity. The chief advantage of using these organisms lies in the ease of handling, relatively small samples of effluent required for tests, and the relative ease with which the data may be obtained. It is believed that tests based on these organisms could be applied to in-plant surveys to determine waste streams which are causing toxicity of plant effluents.

Further experiments were conducted to obtain a comparison of response to wastes between an additional member of the crustaceans, *Palaemonetes* sp., fish, and phytoplankton. In Figure 5 data are presented using phenol (Waste No. 6) as the toxic material. For this waste the TL_m for *Palaemonetes* occurred at a phenol concentration of about 90 per cent that for the fish. The phytoplankton showed some inhibition to photosynthesis at about 1 mg/l and 85-per cent inhibition occurred at 10 mg/l. The most marked difference between the inhibition to photosynthesis of phytoplankton and lethality to the other two organisms is the response of

the plants to very low concentrations of the phenol. Excessively high concentrations of phenol (200 mg/l) were required to completely inhibit photosynthesis. Similar data are given in Figure 6 for the response of the same organisms to Waste No. 5. In this case, the TL_m for *Palaemonetes* sp. occurred at a concentration of waste of 1.6-per cent saturated; whereas, for fish it occurred at 2.5-per cent saturated. *Platymonas* sp. showed greater sensitivity to the waste up to a concentration giving about 50-per cent inhibition to photosynthesis and then reached a plateau in which increasing concentrations caused relatively little increase in inhibition.

Summary and Conclusions

More sensitive tests of the sub-lethal stress placed on organisms by organic contaminants in sea water may be determined by measuring the inhibition to photosynthesis of several families of marine phytoplankton. These unicellular plants are found very sensitive to toxic materials although complete inhibition of photosynthesis may occur at greater concentrations of waste than is found lethal to fish.

Inhibition to respiration of *Artemia salina*, the brine shrimp, appears to occur at concentrations of a mixed waste of chlorinated hydrocarbon and alkaline sulfide which are lethal to fish, although lethality in these organisms occurs at a much higher concentration. In the phytoplankton *Platymonas* sp. photosynthesis was inhibited at concentrations of 10^{-4}-per cent saturation of a light fraction of chlorinated hydrocarbon waste (Waste No. 1); whereas, inhibition to respiration in *Artemia salina* occurred at about 10^{-2}-per cent saturation.

The crustacean *Palaemonetes*, commonly known as grass shrimp, are

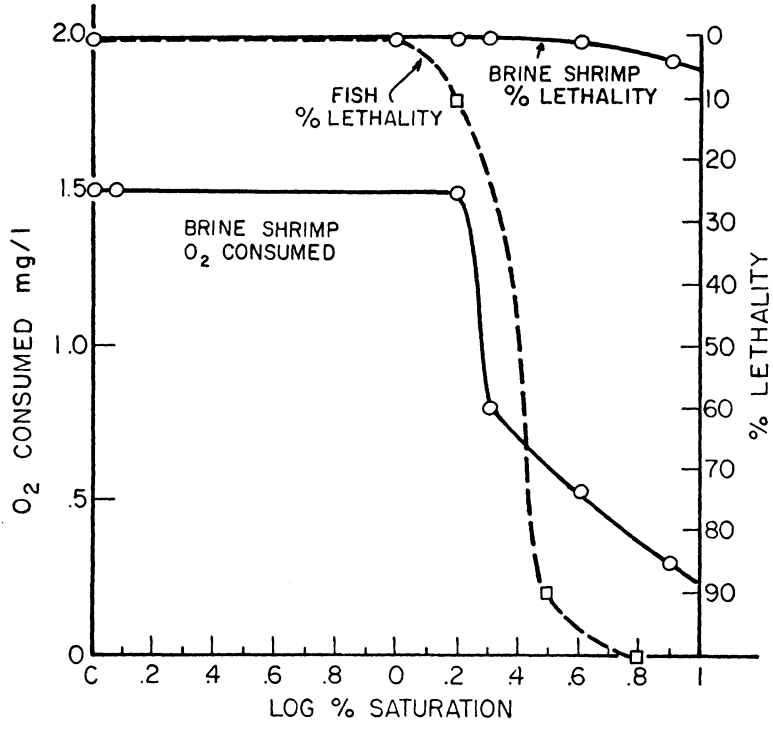

FIGURE 4.—Comparison of acute toxicity of *Artemia salina* and fish to inhibition of respiration of *Artemia salina* with Waste No. 5. Exposure time of *Artemia salina* to waste was 24 hr; growth period was 24 hr.

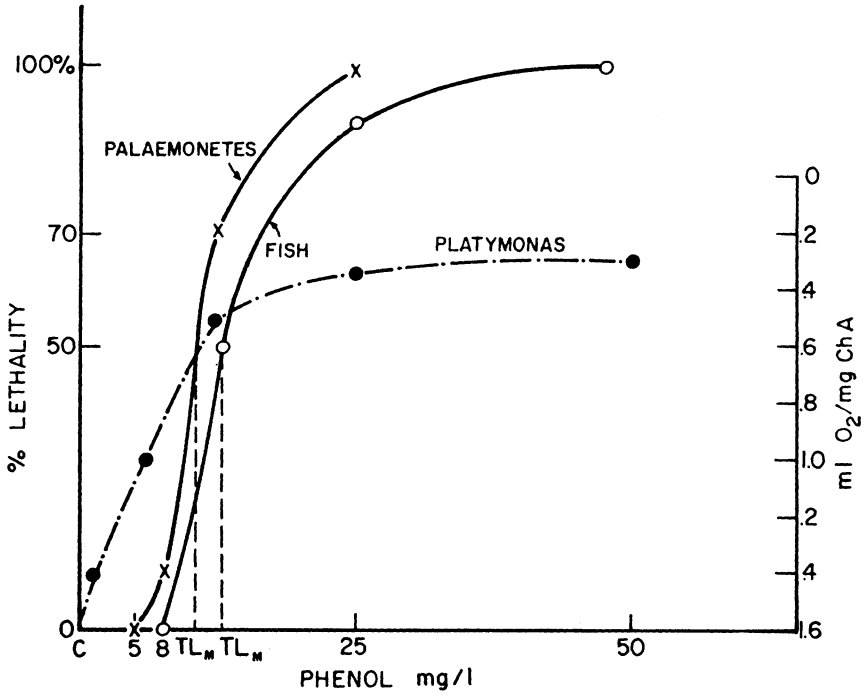

FIGURE 5.—Comparison of acute toxicity of phenol to *Palaemonetes* and fish, and to inhibition of photosynthesis in *Platymonas* sp. Photosynthesis indicated by evolution of oxygen because of Cholorophyll A in culture.

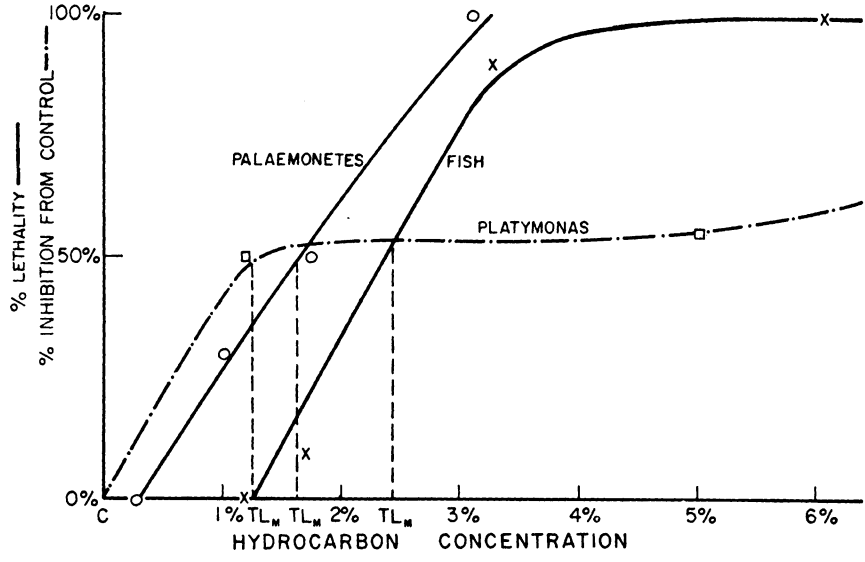

FIGURE 6.—Comparison of acute toxicity of heavy chlorinated hydrocarbon to *Palaemonetes* and fish, and to inhibition of photosynthesis in *Platymonas* sp. Photosynthesis indicated by per cent difference between C-14 uptake by control and sample. 0.5 μc $HC^{14}O_3^-$ added to each 150-ml culture.

somewhat more sensitive to wastes than are *Cyprinodon variegatus*, the fish used in these studies.

It is concluded that experiments on the metabolic effects of wastes give more useful information on the tolerance of important ecological groups of the environment than can be obtained from acute toxicity of wastes to fish or to other individual organisms. It is suggested that further work on metabolic effects of wastes, perhaps to include enzyme inhibition, will provide a better understanding of the effect of industrial effluents on the environment.

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