Hydrogen Sulfide Production from Ethion by Bacteria in Lagoonal Sediments

by J. C. SHERMAN, T. A. NEVIN, and J. A. LASATER

Oceanography Department
Florida Institute of Technology
Melbourne, Fla. 32901

INTRODUCTION

There is little quantitative information concerning the relationships of pesticides to the microorganisms in oceanic and estuarine environments, and only scattered data are available concerning pesticide persistence in aquatic environments. Currently used pesticides are generally organic in nature and are eventually decomposed in the environment by bio-chemical and physio-chemical processes. Parathion, for example, is altered to the non-toxic aminoparathion by certain yeasts found in soils (GOULD, 1966). Therefore, it is a reasonable assumption that pesticides or their degradation products will affect or be affected by microbial processes. Since there is evidence of microbial action or organophosphorous compounds, some of which contain sulfur, the interactions of sulfur containing pesticides and sulfur metabolizing microorganisms may be of significance to the equilibrium of the sulfur cycle. Further, studies thereof may contribute to the understanding persistence of pesticides in estuarine environments. In Brevard County, Florida, Ethion a sulfur containing organophosphate is widely used as a pesticide in the orange groves (INFORMAL COMMUNICATION FROM THE BREVARD COUNTY AGRICULTURAL STATION, 1971) and the Indian River-Banana River lagoonal system receives run off from these groves. Ethion is slightly soluble in water (MERCK INDEX, 1968). Oxidation increases its solubility in water and its anticholinesterase activity (GOULD, 1966). In soil, an increase in pH or moisture content enhances its degradation (GOULD, 1966). The probable degradation products include acetaldehyde, mercaptans, and short carbon chain acidic fragments. The persistence of Ethion in river water was studied in a laboratory experiment on water taken from the Little Miami River in Ohio (EICHELBERGER & LICHTENBERG, 1971). After a three-week period, only fifty per cent of the original Ethion was still present in the sample, whereas there was no loss of the compound from a distilled water preparation during this same period.

The present report describes the production of Hydrogen Sulfide in Ethion enriched lagoonal sediments and by bacterial subcultures derived therefrom.

METHODS AND MATERIALS

Fourteen sediment samples were taken at various sites in the Indian River, a saline lagoon, between Cape Kennedy to Vero Beach. The types of sediments varied from large grained sands to black peat-like material. Samples were taken from the upper regions of the sediment layer and were refrigerated until the enrichment processes were initiated.

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Prior to chemical analyses, the samples were centrifuged for twenty minutes at 5000 rpm, the supernatant decanted, and the sediment weighed. Two mls of 6M sodium hydroxide were added to minimize hydrogen sulfide (H_2S) loss. The samples were dried for 24 hours at 110°C and reweighed to determine the water contents, after correction for the sodium hydroxide additions. Residual Ethion was removed from the samples to which it had been added by extracting twice with pesticide-grade hexane.

Sulfate ($SO_4^=$) was determined gravimetrically by precipitation as barium sulfate, filtering out the precipitate, and weighing it. The method is described in SOIL MECHANICS FOR ROAD ENGINEERS (1952).

The method used to determine sulfide ($S^=$) in the sediment samples was essentially that described in STANDARD METHODS (1965).

At the same time samples were prepared for chemical analyses, the enrichment experiments were started.

The non-alkalinized portions of the centrifuged sediments were divided into four equal parts and placed into sterile petri dishes. Two ml of Ethion (equivalent to 0.816 gms of S) were added to each of two of the petri dishes. Each sample was then covered with the homogeneous supernatant H_2O collected during centrifuging. When there was not sufficient supernatant, sterile distilled water was used. One dish with, and one without added Ethion were placed in a candle jar. The jar was shut tightly after the candle was lighted to increase the available CO_2 in the jar. All of the plates were incubated for 20 days at room temperature in the dark. Sterile distilled water was added as necessary to the air incubated enrichments to restore water lost by evaporation.

After the 20 day incubation, duplicate 5 ml amounts of thioglycollate medium (NIH FORMULA, DIFCO), dispensed into 13 x 100 mm tubes, were inoculated with 1 ml of the fluid from each of the enrichment cultures. The inoculated tubes were incubated for one week at 37°C. Nutrient agar (DIFCO) slants containing 0.01m Na_2SO_4 were prepared and a one and one-half inch piece of iron wire was added after sterilization, to detect H_2S production. The iron wire had been cleaned with dilute HCl and sterilized by flaming. A stab inoculum into a slant was made from each thioglycollate tube and was then incubated at 37°C for 48 hours. Parallel slants in which the Na_2SO_4 was replaced with Ethion were also inoculated and incubated. Nutrient agar (DIFCO) plates were then streaked with the culture from each slant in which FeS was detected. The plates were incubated in increased CO_2 at 35°C for 48 hours. Gram stained smears from typical colonies on the agar plates were observed microscopically.

The cultures grown in thioglycollate medium were subcultured into fresh thioglycollate medium. Cultures derived from the Ethion enrichments were inoculated into tubes to which Ethion had been added. Cultures derived from the controls received no added Ethion. After forty-eight hours of incubation at 37°C, they were again subcultured into nutrient agar stabs with iron wire and Ethion and into controls with no Ethion. The stabs were incubated at 37°C for twenty-four hours and observed for FeS production. Prior to addition to the culture media, the Ethion was effectively sterilized by passage through a presterilized 0.45 micron membrane filter.

RESULTS AND DISCUSSION

The literature indicates that the amount of work on the toxicity of pesticides on the macroscopic level far exceeds that on the effects of these chemicals on microbial populations. Several recent papers however (HORVATH 1972; EICHELBERGER & LICHTENBERG 1971) have reported the natural microbial degradation of pesticides and other organic compounds. Ethion is a sulfur containing compound and conditions in the Indian River indicate the presence of sulfur utilizing bacteria. Therefore, the possible interaction of a general group of microorganisms on the sulfur component of Ethion, was the overall intent of the study.

Ethion enriched sediment samples from the Indian River were used as the source of microorganisms for the experiments.

Upon enrichment, 3 of 14 sediments demonstrated significantly increase levels of sulfides after incubation. Ethion was the only extraneous source of sulfur. These data are summarized in Table I.

TABLE I

SULFIDE CONTENT OF SEDIMENT SAMPLES AFTER INCUBATION WITH AN ETHION ENRICHMENT

>mg $S^{=}$ per 100 g sample<

Sample	Initial	20 Day Incubation	
		Control	Ethion added
2	1.71	1.47	9.58
9	4.82	4.66	7.02
14	2.50	11.40	24.60

It is evident from the table that the rate of degradation in the sediments is not rapid. This may reflect either low numbers of active Ethion degrading organisms or a low metabolic rate since the calculated yield of sulfur per ml

of Ethion is 0.406g of $S^{=}$ per ml.

The sediment samples were not fixed immediately with sodium hydroxide and, therefore, the amounts detected initially cannot be taken as indicative of the $S^{=}$ concentration at the sample site. The large amount of sulfide determined in the control of sediment 14 reflects sources of sulfur other than Ethion in the sediment.

The sediments with added Ethion were subcultured in thioglycollate tubes with added Ethion and the controls were carried simultaneously. They were incubated at 37°C for 48 hours and all tubes showed gross and microscopic evidence of bacterial growth.

The subcultures were then inoculated into nutrient agar tubes to which iron wire had been added. The Ethion/no Ethion identity was maintained throughout the experiment. If bacterial degradation of the Ethion occurred, the wire would serve as a source of iron for the production of iron sulfide. Samples were incubated at 37°C and examined periodically. After twelve hours, production of iron sulfide in five different samples was observed. Three of the five were the same as those listed in Table I.

A parallel experiment was performed in which thioglycollate tubes with no Ethion were inoculated from the Ethion enriched sediments. Subsequently, agar stabs with added Na_2SO_4 and Fe wire were inoculated from the cultures in thioglycollate. The five samples which degraded the Ethion also produced FeS from the Na_2SO_4 and iron wire. The data for both experiments are presented in Table II.

TABLE II

ENRICHMENT CULTURES EXHIBITING FeS* PRODUCTION WHEN INCUBATED WITH ETHION OR SULFATE

Sample Number	FeS from Sulfate	FeS from Ethion
2	+	+
8	+	+
9	+	+
13	+	+
14	+	+

*Sterile iron wire inserted into culture tubes

Nutrient agar plates were streaked with each of the 5 cultures and

incubated under increased CO_2 for 48 hours. Typical discreet colonies from the plates were subcultured in nutrient agar tubes with iron wire and Ethion to confirm FeS production from the pesticide. Microscopic examination of stained smears of the tube cultures indicated a spore-forming gram-positive rod. Since Ethion is a reducing agent, it is probable that the bacteria are members of the genus Clostridium.

Examinations of the culture tubes indicated four different conditions. Inoculated media which had an iron wire and Ethion gave evidence of darkening of the wire but also, and most significantly, gave evidence of FeS production at the stab-line of inoculation, after twelve hours of incubation. Upon further incubation of the culture, FeS discoloration spread from the inoculation stab throughout the tube. There was no FeS production at the surface of the slant, indicating further the probability that the reaction was anaerobic. Inoculated media which had iron wire but no Ethion added supported growth but not FeS production.

Uninoculated media to which both an iron wire and Ethion were added gave evidence of FeS production by a darkening of the surface of the wire. This indicated a chemical reaction of the Ethion with the wire. However, there was no evidence of FeS production throughout the medium. Uninoculated media which had an iron wire but no Ethion served as the experimental control. There was no growth, indicating no contamination and no outside uncontrolled source of sulfur other than Ethion. Prior to chemical analysis, the remaining Ethion was extracted out of the enrichment culture with hexane.

No effort was made during this study to pursue further the chemical mechanism of the degradation or to evaluate the breakdown products of Ethion.

In all except 3 samples (8, 12, 13), the sulfide concentration increased to some extent after exposure of the sediments to the Ethion. There were no corresponding decreases in sulfate concentrations, in fact 8 of the 14 samples showed a small to moderate increase therein, indicating some other source of sulfide sulfur, i.e., Ethion. The data are presented in Table IV.

TABLE IV

Mg PER 100 GRAMS OF SULFIDE AND SULFATE RECOVERED FROM ETHION ENRICHED CULTURES AND CONTROL CULTURES AFTER 20 DAYS INCUBATION AT ROOM TEMPERATURE

Sample #	Sulfide			Sulfate		
	Control	Ethion	Difference	Control	Ethion	Difference
1	1.41	3.77	1.36	300	540	140
2	1.67	8.77	7.10	415	265	-150
3	1.05	4.46	3.41	1360	1580	220

4	0.76	4.21	3.45	125	245	120
5	1.06	3.20	2.14	100	275	175
6	1.28	1.35	0.07	325	120	-205
7	0.54	1.34	0.80	215	170	- 45
8	1.50	1.43	0.07	320	370	50
9	5.59	7.23	1.64	1545	2235	690
10	0.50	12.60	12.10	925	170	-755
11	5.03	8.04	3.01	975	740	-235
12	2.45	1.38	-1.07	283	223	- 60
13	3.42	3.32	-0.10	340	460	120
14	12.55	20.38	7.73	1210	3820	2610

Based on the sample amount of sediment used in each environment culture observed there was no correlation between the theoretical and actual $S^{=}$ concentrations, but the stoichiometry of the biological reactions was not measured. The data do suggest that the production of sulfides and the production of sulfates proceed independently of each other.

The method of enrichment culture provides those selective features which lead to the predominence of a particular species. Therefore, this mechanism can be used to study biodegradability of pesticides in the environment. One type of biodegradability mineralization, is represented by complete degradation of the organic molecule to its basic elements. A second type of biodegradability co-metabolism (HORVATH, 1972), which might be just as significant is the conversion of the organic pesticide to lesser, but still complex decomposition products. Organisms capable of co-metabolic activity can also be obtained by enrichment techniques. Co-metabolism, although it does not bring about complete mineralization to the elements, may be an important mechanism in the initial or perhaps intermediate steps of natural microbial populations.

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