Bulletin 69

WATER QUALITY MONITORING: BACTERIA AS INDICATORS

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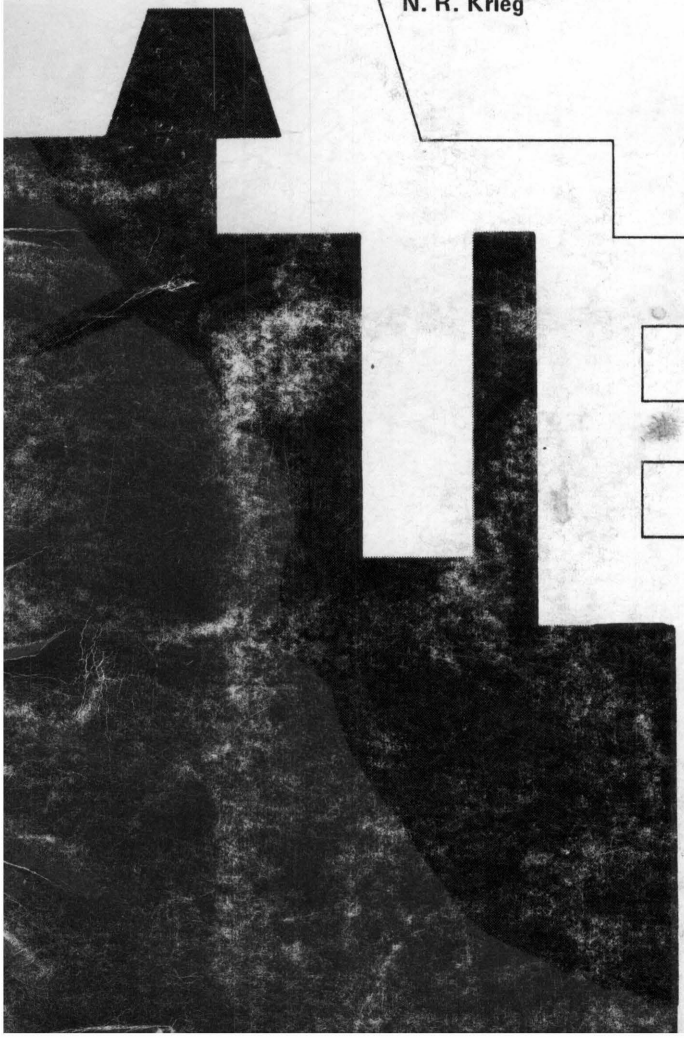

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February 1974

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The research on which this publication is based was supported by the Virginia Polytechnic Institute and State University, Research Division, and by the Office of Water Resources Research, United States Department of the Interior, as provided by the Water Resources Act of 1964.

VPI-VWRRC-BULL 69

A publication of
Virginia Water Resources Research Center
Virginia Polytechnic Institute and State University
Blacksburg, Virginia 24061

PREFACE

Multiple use of water is a technique worthy of consideration if we are to make the most of our available water resources. Simply stated, multiple use means that an industry would draw water from a stream, use it, and return all or most of it to the stream for the neighboring landowner to use for, say, irrigation or watering livestock. However, there is more to the technique than that. If the "ABC Corporation" discharges too much zinc or lead in its return of water to the stream, the effect on "Farmer Doe's" cattle or vegetable crops could be severe.

It is therefore incumbent on the ABC Corporation to clean up its waste before discharge. (Federal law demands zero discharge of pollutants by 1985). It would be uneconomical for ABC to process all its waste if, say, zinc is present only some of the time. Therefore, a monitoring system is needed so that ABC can check for certain pollutants at specified levels.

Recent research has led to the development of several monitoring systems using biological organisms most notably bluegill fish as sensors. The research described in this publication made use of a "swimming" bacteria, one visible even under low power magnification, to develop and standardize a monitoring system sensitive to some 10 to 15 substances in relatively low concentrations.

The system is constructed so that it can be used effectively by a relatively untrained lab technican. Standardization for other substances is a simple process.

Special acknowledgement is accorded the following who generously gave their time to a critical review of the manuscript: Dr. Thomas Bott, Limnologist, Stroud Water Resources Center, Avondale, Pennsylvania; Dr. R. N. Doetsch, Professor of Microbiology, University of Maryland; and Dr. John Cairns, Jr., Director, Center for Environmental Studies, Virginia Polytechnic Institute and State University.

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ABSTRACT

A rapid, simple, and inexpensive standardized method has been developed for biological monitoring of levels of toxicants in industrial effluent using motility of *Spirillum volutans* as an indicator. The system was sensitive to zinc, nickel, copper, mercury, and lead ions at concentrations of 2 or 3 ppm; cetyl pyridinium chloride at 1 ppm; aniline at 30 ppm; and other compounds in a similar concentration range. The sensitivity to zinc ions was comparable to that of monitoring systems using fish. Combinations of metals were effective when each was present at a level lower than its minimum effective concentration when used alone.

The response, which is visible by darkfield microscopy, is an immediate cessation of bacterial motility due to uncoordination of the flagella.

The method was developed for in-plant use in testing effluents for uncoordinating concentrations of toxic agents before discharge into a stream.

INTRODUCTION

Monitoring of water quality with respect to industrial pollutants, both in streams and in effluents about to be discharged into streams, is an essential component of the multiple-use management of water resources [1]. Biological monitoring has received considerable attention as a useful supplement to (but not a substitute for) monitoring by chemical or physical methods [2]. Biological monitoring will not identify the pollutants in a complex effluent, but it could indicate the occurrence of an adverse biological effect which might be difficult to predict by analyzing physical and chemical parameters alone. Because there is no one representative aquatic organism which can serve as an index of what is harmful to the entire aquatic community, it is desirable to have systems available which could utilize a variety of species.

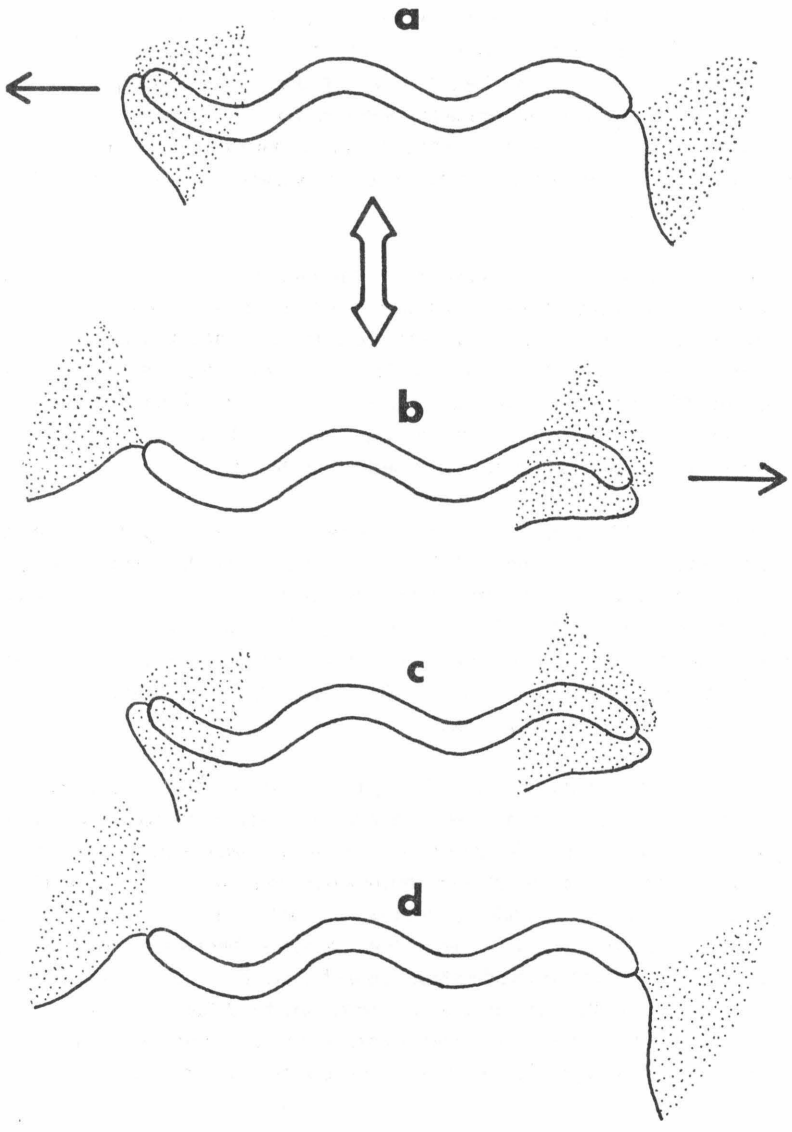
Jackson and Brungs [3] suggested the maintenance of fish (or other test organisms of local importance, such as oysters or shrimp) in aquaria receiving various dilutions of effluent in a continuous-flow situation. They stated that this system, using death or signs of distress in test organisms as a warning signal, would verify the biological acceptability of the effluent. It could also show possible synergistic effects of the mixture of effluent and receiving stream water, if upstream water was used as the diluent.

Cairns et al. [2,4] have developed an in-plant monitor using the movement patterns and breathing rate of fish as indicators. One disadvantage of such biological methods is the length of time required for the indicator organisms to die or exhibit sub-lethal effects. Cairns et al. [4] have emphasized the need for bioindicators capable of exhibiting more rapid responses to industrial pollutants. The present investigation deals with the development of such a biological monitor for in-plant use.

Spirillum volutans is an unusually large, helical, aquatic bacterium. Hylemon et al. [5] recently characterized the available strains in detail. Of special interest, however, is the occurrence of a rotating fascicle of flagella at each cell pole, visible even under low microscope magnifications by darkfield illumination. During normal swimming, the polar fascicles form oriented cones of revolution (Figure 1a). The bacteria frequently reverse their direction, and during this process both polar fascicles re-orient simultaneously (Figure 1b), with the tail fascicle becoming the head fascicle and vice versa [6,7]. Krieg et al. [7] described flagellar uncoordination of two types (depending on the agent used) in response to low concentrations of various

Figure 1

Flagellar Orientation of Coordinated and Uncoordinated
Cells of *Spirillum volutans*



solutes: (1) dual-head coordination, with both fascicles assuming the head orientation (Figure 1c), or (2) dual-tail uncoordination, with both fascicles assuming the tail configuration (Figure 1d). Although the flagella still rotate at high speed, the uncoordinated cells are unable to swim because of the opposing propulsion at the cell poles. Caraway and Krieg [8] investigated the flagellar uncoordination further, and described a third type of uncoordination (dual straight tail) in which the fascicles extended in straighter fashion away from the cell poles. They also expanded the list of chemical agents known to cause the various types of uncoordination. Because many of these chemical agents can be found in industrial wastes, there appeared to be the possibility of applying flagellar uncoordination (and the consequent loss of bacterial motility) to the problem of biological monitoring of pollutants in industrial effluents.

MATERIALS AND METHODS

Spirillum volutans (American Type Culture Collection No. 19554) was transferred daily in a casein hydrolysate-succinate-salts (CHSS) medium having the following composition in distilled water (g/l): "Vitamin-free," salt-free casein hydrolysate (obtainable from Nutritional Biochemical Co., Cleveland, Ohio), 2.5; succinic acid, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; $(NH_4)_2SO_4$, 1.0; $FeCl_3 \cdot 6H_2O$, 0.002; and $MnSO_4 \cdot 20$, 0.002. The pH was adjusted to 6.8 with KOH and the medium was dispensed in 80-ml quantities into 250-ml Erlenmeyer flasks and sterilized at 121°C (15 psi) for 15 min. Optimal growth was obtained in 24 hr at 30°C when 1.5 ml of a previous 24-hr culture was used as the inoculum. Cultures were incubated without shaking. Although *S. volutans* is an obligate microaerophile [9], it was not necessary to adjust the concentration of oxygen in culture flasks when such a large inoculum was used. Sterile medium was stored in the dark to prevent the formation of peroxides which inhibit growth.

Motility was observed in a defined test medium (DTM) having the following composition (g/l): $MgSO_4 \cdot 7H_2O$, 0.05; $(NH_4)_2SO_4$, 1.0; ethylenediamine tetra-acetic acid (EDTA), 0.0073; N,N-Bis-2-hydroxyethyl-2-aminoethane sulfonic acid (BES buffer), 0.2133. Distilled water passed through a Bantam standard multi-bed resin cartridge was used. The pH was adjusted to 6.8 with KOH. The medium was prepared at 10X concentration and was sterilized at 121°C (15 psi) for 15 min. This motility medium enabled *S. volutans* to respond to considerably lower concentrations of flagellar uncoordinating agents than did the use of the defined motility medium (DMM) previously described by Caraway and Krieg [8].

Most of the potential pollutants tested were taken from a list of hazardous polluting substances prepared by the Environmental Protection Agency [10]. Borosilicate glassware used with test compounds was cleaned with hydrochloric acid, washed with a non-ionic detergent in a laboratory glassware washer, and rinsed with distilled water followed by deionized water. Slides were boiled in 1% HCl, thoroughly rinsed, and boiled twice in deionized water and dried. In the initial phases of the work, the concentrations of zinc ions used were verified by atomic absorption spectrophotometry.

The standardized procedure developed for testing the effect of a chemical agent (or an industrial effluent) on motility was as follows. Cells from a 24-hr CHSS culture were centrifuged at 750 X g in a clinical centrifuge, washed once in 80 ml of 1X DTM, and suspended in 80 ml of 1X DTM. The suspension

was allowed to stand for 1 hr in DTM before use. The same preparation of cells could then serve as a stock cell suspension for use throughout an 8-hr working day; in fact, reproducible results have been obtained with cells that have remained in DTM for 10 hours. The initial 1-hr incubation was found to yield cells exhibiting more reproducible results.

Test compounds were prepared to 1.11X the desired test concentration in deionized distilled water, and 9 parts of a test solution were then added to 1 part of 10X DTM. (In practice, a suitable dilution of industrial effluent could be used). A 5-ml aliquot of stock cell suspension was centrifuged for 1 min, resuspended in the test solution, and immediately examined by darkfield microscopy at 125X. A Leitz Ortholux microscope equipped with N.A. 1.20 darkfield condenser and 150-watt XBO xenon burner was employed for microscopic observations; however, any darkfield microscope would suffice. The minimum effective concentration of a pollutant was defined as the minimum concentration necessary to eliminate reversing motility in more than 90% of the cells.

Normally motile cells and uncoordinated cells were photographed using the motility-track method of Vaituzis and Doetsch [11]. Time exposures of 5 sec, Kodak Plus-X Pan film, and a 35-mm Leica camera with microscope mount were used. As a check on the reliability of the method, the usual practice was to select test solutions "blind" and at random. The solutions included uncoordinating agents at their minimum effective concentrations, uncoordinating agents at levels too low to be effective, and also DTM blanks (no agents added). The number of solutions of each type was also unknown to the examiner. A second test was conducted in a similar manner, except that it was designed to show whether a person unaccustomed to working with these tests or with the test organism could correctly detect the presence of effective levels of pollutants. The person was instructed in the use of the microscope and was shown examples of normal and of uncoordinated preparations of *S. volutans*. He was then presented with eight test solutions to be examined using the standard method. These solutions included uncoordinating agents at their minimum effective concentrations, solutions containing uncoordinating agents at sub-minimal effective concentrations, DTM blanks, and ineffective agents.

RESULTS AND DISCUSSION

The minimum effective concentrations of compounds which were found to uncoordinate *S. volutans* are shown in Table 1. The uncoordinating effects of the metal pollutants were detected at 2 or 3 ppm. Certain other agents (cetyl pyridinium chloride, 1-naphthol) were also effective in this range, while others produced uncoordination at higher concentrations (ranging up to several percent for the alcohols tested). All results were reproduced on numerous occasions.

Table 2 indicates levels of some potential pollutants which failed to uncoordinate the spirilla. The most notable of these were the long-range, bioaccumulated poisons Aroclor 1242* (a polychlorinated biphenyl product) and the organochlorine insecticides Dieldrin and DDT. These compounds were insoluble in water and were dispersed in the aqueous medium after dissolution in 95% ethanol; the final test solutions consequently contained 0.475% ethanol in addition to the toxicant (10 ppm). These compounds appeared to form microscopic globules when dispersed in the medium and may thus have been prevented from contacting the cells effectively. Aroclor 1242 and Dieldrin have been shown to have long-term growth effects on algal populations grown in culture [12,13]. Other compounds such as phenol which failed to uncoordinate spirilla even at concentrations as high as 90 ppm (see Table 2) may possibly affect motility at higher concentrations [8].

The reliability of the testing method was confirmed by the procedures described in Materials and Methods. In every case, correct characterization of the motility response in the coded samples was achieved.

It is unlikely that an industrial effluent would contain only one potential pollutant; therefore, the effects of combinations of agents on motility were studied. An additive effect was observed when any two metals were used together, each at 1/2 its minimum effective concentration (i.e., such mixtures produced uncoordination). Cetyl pyridinium chloride at 1 ppm, which normally caused only a delayed uncoordination within 5 min (see Table 1), uncoordinated the cells immediately in the presence of 1 ppm Zn^{2+} (normally a sub-minimal effective concentration). A mixture of ethanol and n-proponal, each at 1/2 its minimum effective concentration, uncoordinated about half the cells; the rest were still able to swim but did not reverse direction.

* Registered trademark, Monsanto Co., St. Louis, Missouri

Table 1

Minimum Effective Concentrations (MEC) of Uncoordinating Agents

Agent	MEC*	Comments
Hg^{2+}	3 ppm	added as $HgCl_2$
Ni^{2+}	3 ppm	added as $NiCl_2 \cdot 6H_2O$
Zn^{2+}	3 ppm	added as $ZnSO_4 \cdot 7H_2O$
Cu^{2+}	2 ppm	added as $CuSO_4 \cdot 5H_2O$
Pb^{+}	2 ppm	added as lead acetate
U^{6+}	1 ppm	added as $UO_2(NO_3)_2$
Cetyl pyridinium chloride	1 ppm	Delayed uncoordination (occurs within 5 min); flagellar activity ceases within 15 min.
Sodium dodecyl sulfate (sodium lauryl sulfate)	200 ppm	Flagella completely immobilized and cells motionless; cells can swim at 100 ppm.
"7X" detergent**	1%	Delayed uncoordination (occurs within 1 min).
1-Naphthol	3 ppm	
1-Naphthylamine	90 ppm	
Hydroxylamine	40 ppm	
p-Nitrophenol	25 ppm	
Hydrazine	10 ppm	added as hydrazine sulfate

Table 1
(Continued)

Agent	MEC*	Comments
Aniline	30 ppm	added as the sulfate or oxalate
n-Propanol	2%	Effects of alcohols progressed during a period
Ethanol	4%	of a few min from slow unidirectional swimming to
Ethylene glycol	10%	dual-head uncoordination

*ppm by weight; % by volume. Concentrations expressed as the ion or compound listed under "Agent."
**Linbro Chemical Co., New Haven, Connecticut.

Table 2

Agents Which Did Not Produce Uncoordination at Levels Tested

Agent	Concentration	Comments
Aroclor 1242	10 ppm (in 0.475% ethanol)	no effect after standing 2 hr
Dieldrin	10 ppm (in 0.475% ethanol)	
DDT	10 ppm (in 0.475% ethanol)	
CCl_4	90 ppm	
isoamyl acetate	90 ppm	only effect was a slightly wobbly motility
nitrilotriacetic acid	90 ppm	neutralized with KOH; cells still normal after 75 min
H_3BO_3	90 ppm	
Sr^{2+}	90 ppm	added as $SrCl_2 \cdot 6H_2O$
o-nitrophenol	90 ppm	yellow solution; visually detectable
methylamine	90 ppm	
phenol	90 ppm	

In addition to additive effects, the possibility exists that two or more agents in an effluent may act synergistically, producing a response at levels well below the minimum effective concentration of the individual agents. A well-known example of such synergism was described by Doudoroff [15], who showed that minnows (*Pimephales*) survived 8 hr of exposure to either 8 ppm of Zn^{2+} or 0.2 ppm of Cu^{2+}. The fish, however, died within 8 hr in the presence of a mixture containing 1 ppm Zn^{2+} and 0.025 ppm Cu^{2+}. In the present study, a synergistic effect was caused by a combination of 0.5 ppm Zn^{2+} and 0.5 ppm Ni^{2+}. Neither of these metals caused uncoordination alone at 1 ppm. Such effects emphasize the usefulness of a biological monitor for pollutants, since the effects cannot be predicted from a knowledge of only physical and chemical parameters.

Preparation of a photographic demonstration of the lack or presence of motility in testing for pollutants would be advantageous in that a permanent record would be available for future reference. The feasibility of preparing such a photographic record is indicated in Figure 2, where the time-exposure method of Vaituzis and Doetsch [11] was employed for normal cells and for cells uncoordinated by 3 ppm Zn^{2+}. It can be seen that normal cells leave an exposure-track on the film because of their motion, while uncoordinated cells do not swim and consequently appear merely as overexposed "blobs" on the film.

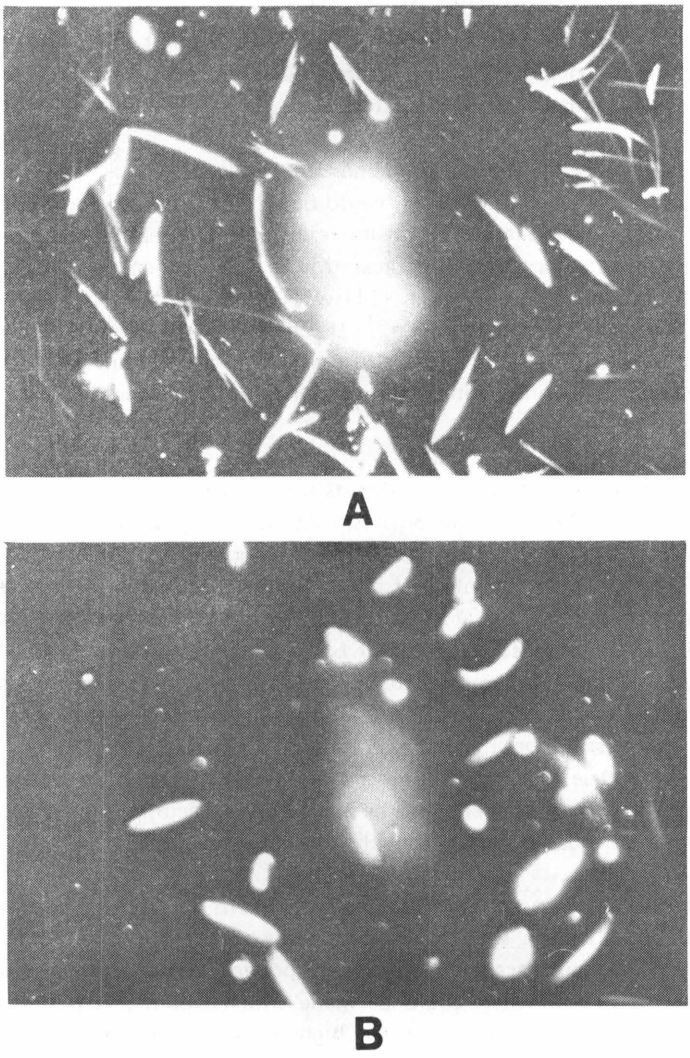
Because the effects of various toxicants vary widely among different species of aquatic life [16], no one organism can be used to predict the effect of pollutants on all other species, or on the aquatic ecosystem as a whole. Therefore, the use of a biological monitoring system would involve detecting the presence of toxicants at levels previously defined as harmful if discharged into the stream. These levels could be determined by actual study of the particular receiving stream, including such factors as its assimilative capacity, the presence of toxicants from other industrial sources, and the effects of the agents in question on its biotic community. Biological harm to a stream would, of course, have to be assessed not only in terms of dramatic effects such as fish kills, but in terms of the ability of the members of the ecosystem to function adequately throughout their complete life cycles.

Use of the *Spirillum volutans* system for effluent monitoring would involve determination of the sensitivity of the cells to the potential pollutants in question in the presence of the other components of the effluent. A suitable dilution of effluent then could be made which would just permit normal motility if the pollutants were at the highest level considered permissible for discharge. Upstream water could be used as the diluent to simulate the interaction of the effluent with the receiving stream water.

Figure 2

Motility-track Photomicrographs of *Spirillum volutans*

Magnification = 108 X. The large bright diffuse area in the center of each photomicrograph is the image of the xenon arc used for illumination.



Tests on incoming stream water would distinguish pollution originating from spills within the plant from toxicants already present upstream.

Using these techniques, uncoordination of the spirilla by effluent would indicate a rise in one or more toxic substances to an unacceptable level. The waste could then be subjected to physical or chemical tests to determine the identity and concentration of the particular toxicants involved. Meanwhile the waste could be shunted to a holding pond for further treatment if necessary.

In studies reported by Cairns et al. [4], in which bluegill movement patterns and breathing rates were tested for use in an industrial effluent monitoring system, zinc was employed as the toxicant and was detected at concentrations of a few parts per million. A latency period of several hours elapsed between the introduction of the zinc and the detection of the response, and the length of the latency period was inversely proportional to the concentration of toxicant employed. The lowest concentration of zinc used, 2.55 ppm, was detected by the breathing response after 52 hr, and the limit of sensitivity of the activity-pattern detection method was given as between 3.64 and 2.94 ppm for a 96-hr exposure. Thus it appears that the maximum sensitivities of the two biological monitors, using bluegills and using *S. volutans*, are comparable for zinc. With regard to other pollutants, possible similarities in sensitivity between the two monitoring systems remain to be determined as further data for the bluegill system are gathered. For toxicants which could be detected by either fish responses or flagellar uncoordination, the bacterial system would offer the advantages of immediate response, simplicity, and lower cost. In addition, the bacterial tests would always be performed on readily available, easily cultured organisms of controlled age and physiological state. This would eliminate some problems faced in monitoring with higher organisms, where aging, confinement, long-term low-level exposure to toxicants, or other physiological factors may affect the responses of the sensor organism [2].

CONCLUSIONS

It appears from laboratory studies that the uncoordination of the flagella of *Spirillum volutans* by certain potential pollutants might be used as a rapid, simple, and relatively inexpensive bioindicator for the presence of such compounds in industrial effluent. The sensitivity of the system for Zn^{2+} appears to be comparable to that of monitors using fish as indicator organisms, and the bacterial response is detectable with a much shorter time lag.

The motility response to uncoordinating compounds is often affected by the presence of other components of the mixture; therefore, the concentrations necessary to produce a response must be determined individually for each effluent. As with any biological monitor, a response indicates the presence of conditions which may have adverse biological effects; the exact nature of these conditions and the corrective action to be taken would in general have to be determined by further testing.

LITERATURE CITED

1. Cairns, John, Jr., Kenneth L. Dickson, Richard E. Sparks, and William T. Waller, "A Preliminary Report on Rapid Biological Information Systems for Water Pollution Control." Jour. Water Poll. Control Fed., 42 (5, part 1): 685-703 (1970).

2. Cairns, John, Jr., John W. Hall, Eric L. Morgan, Richard E. Sparks, William T. Waller, and Garson F. Westlake, "The Development of an Automated Biological Monitoring System for Water Quality." Virginia Water Resources Research Center, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061. Bulletin 59 (February 1, 1973).

3. Jackson, H. W., and W. A. Brungs, Jr., "Biomonitoring of Industrial Effluents." Proceedings, Industrial Waste Conference, 2lst, Purdue University, L. (1): 117-124 (May 3, 4, 5, 1966).

4. Cairns, John, Jr., Guy R. Lanza, Richard E. Sparks, and William T. Waller, "Developing Biological Information Systems for Water Quality Management." Water Resources Bulletin 9: 81-99 (1973).

5. Hylemon, Phillip B., J. Scott Wells, Jr., Jean H. Bowdre, Thomas O. MacAdoo, and Noel R. Krieg, "Designation of *Spirllum volutans* Ehrenberg 1832 as Type Species of the Genus *Spirillum* Ehrenberg 1832 and Designation of the Neotype Strain of *S. volutans*." International Jour. Systematic Bacteriol. 23: 20-27 (1973).

6. Metzner, P., "Die Bewegung und Reizbeantwortung der bipolar gegeisselten Spirillen." Jahrb. Wiss. Bot. 59: 325-412 (1919).

7. Krieg, N. R., J. P. Tomelty, and J. S. Wells, Jr., "Inhibition of Flagellar Coorination in *Spirillum volutans*." Jour. Bacteriol., 94: 1431-1436 (1967).

8. Caraway, B. H., and N. R. Krieg, "Uncoordination and Recoordination in *Spirillum volutans*." Can. Jour. Microbiol., 18: 1749-1759 (1972).

9. Wells, J. S., Jr., and N. R. Krieg, "Cultivation of *Spirillum volutans* in a Bacteria-free Environment." Jour. Bacteriol., 90: 817-818 (1965).

10. Thompson, C. Hugh, "Selection and Designation of Hazardous Polluting Substances." Division of Oil and Hazardous Materials, Office of Water Programs, Environmental Protection Agency, Washington, D. C. (October 14, 1971).

11. Vaituzis, Z., and R. N. Doetsch, "Motility Tracks: Technique for Quantitative Study of Bacterial Movement." Applied Microbiol., 17: 584-588 (1969).

12. Morgan, Janet R., "Effects of Aroclor 1242 (a Polychlorinated Biphenyl) and DDT on Cultures of an Alga, Protozoan, Daphnid, Ostracod, and Guppy." Bull. Environ. Contam. Toxicol., 8: 129-137 (1972).

13. Cairns, John, Jr., "The Effects of Dieldrin on Diatoms." Mosquito News, 28: 177-179 (1968).

14. Mosser, J. L., N. S. Fisher, T. C. Teng, and C. F. Wurster. Polychlorinated biphenyls: toxicity to certain phytoplankters. Science, 175: 191-192 (1972).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