European J Appl Microbiol Biotechnol (1981) 12:123–127

European Journal of Applied Microbiology and Biotechnology

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In-Vitro Radiometry and Microscopy for Sensitive Measurement of Toxicity in Activated Sludge

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Summary. Inhibitory effects of an anti-microbial agent on a model activated sludge system were examined. Using microbial counts and $^{14}CO_2$ evolution from radiolabeled starch and cellulose, inhibitory effects of small concentration increments of $HgCl_2$ could be monitored as could the rapid recovery of microbial viability after 24 h. Mercury dissipation accounted for part of this recovery but bacterial resistance to mercury is also implicated.

Introduction

Due to a minimal understanding of the role of microorganisms in the wastewater treatment process, few biological parameters are routinely monitored at many treatment facilities (Bucksteeg 1966; Johnson 1978; Nelson and Lawrence 1980; Patterson et al. 1969). The five-day biochemical oxygen demand (BOD_5) measurement is the most commonly used parameter to evaluate the efficiency of wastewater treatment systems (Hammer 1975). However, it is time consuming and supplies no information on microbial interactions within the activated sludge. It is our intention to define sensitive parameters which can be used to monitor microbial viability in activated sludge.

Activated sludge floc utilizes carbon substrates for the synthesis of new cell material and obtains energy through the oxidation of these substrates to CO_2, water and other by-products. The production of excess biological material in wastewaters reflects, in part, the rate of substrate utilization by activated sludge (Patterson et al. 1969). Numerous methods have been developed to measure the evolution of $^{14}CO_2$ from bacterial degradation of radiolabeled substrates (DeLand and Magner 1970; Nelson and Lawrence 1980; Saba and DiLuzio 1966; Steinberg 1960). E. U. Buddemeyer (Buddemeyer 1974) developed an apparatus composed of a twocompartment liquid scintillation vial which was used for continuous measurement of $^{14}CO_2$ evolution from ^{14}C-glucose by pure cultures of *E. coli.* This technique was refined (Isbister et al. 1980) and utilized with high sensitivity for mixed microbial populations and for other ^{14}C-labeled substrates.

Sludge microbes utilize a wide range of carbon substrates as energy sources, making the degradation of these substrates critical to the wastewater treatment process (Blanchard et al. 1976; Degrémont 1979; Lawrence and McCarty 1970; Weddle and Jenkins 1971). The ubiquity and abundance of cellulose in wastewaters (Broadbent 1973) warrants careful examination of its degradation. Starch is a polysaccharide found in the waste effluent of many industrial processes and is also representative of the easily degradable carbohydrates found in activated sludge (Stavenger 1979). The U.S. Environmental Protection Agency requires the examination of both cellulose and starch degradation in certain environmental monitoring studies (Environmental Protection Agency 1978). We have used a modification of Buddemeyer's technique to monitor the microbial degradation of ^{14}C-starch and ^{14}C-cellulose in a laboratory sludge system. Sensitivity of the technique was examined by using mercuric chloride to inhibit starch and cellulose degradation. Mercuric chloride is a well known anti-microbial agent (Rheinheimer 1971; Hamdy and Wheeler 1978; Hedgecock 1967; Thomas et al. 1977) which has been demonstrated to inhibit the activated sludge treatment process (Houtmeyers et al. 1980; Patterson et al. 1969; Reinheimer 1971; Yamada et al. 1969). We have demonstrated that a significant ($P < 0.05$) decrease in substrate degradation (as measured by cumulative $^{14}CO_2$ production) after mercuric chloride

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treatment of activated sludge is an indicator of microbial toxicity by comparing radiometric data to bacteria and protozoa counts.

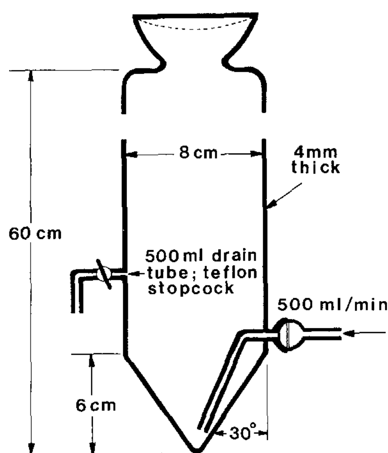

Fig. 1. Semi-Continuous Activated Sludge Chamber

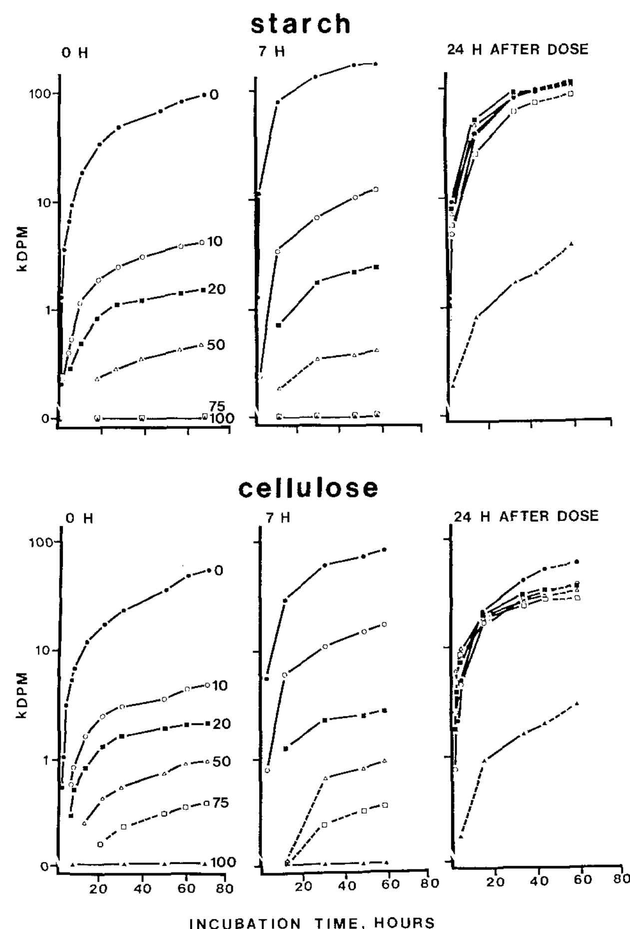

Fig. 2. $^{14}CO_2$ evolution from ^{14}C-starch and ^{14}C-cellulose degradation from 0, 10, 20, 50, 75, and 100 ppm $HgCl_2$ treated activated sludge. *Dashed line* indicates points below the limits of quantitation (Curie 1968) or extrapolation to data points beyond 56 h

Materials and Methods

Model Activated Sludge Chamber

An all-glass activated sludge reaction chamber (Fig. 1) is equipped with a teflon stop-cock for sample withdrawal and an aeration inlet supplying 500 ml/min positive pressure (providing a dissolved oxygen content of 6.0 mg/l) to the bottom of the reactor.

Operating Conditions

Activated sludge was obtained immediately prior to initiation of the experiment from Back River Sewage Treatment Plant, Dundalk, Maryland. The activated sludge solids were adjusted to 1,000 mg/l with aged tap water and amended with 20.5 ml/l of synthetic sewage nutrients (13.0 g/l each of glucose, nutrient broth, beef extract and K_2HPO_4 and 2.5 g/l of $(NH_4)_2SO_4$ which maintained a pH of 7.4 in all chambers throughout the experiment. One liter of the adjusted, activated sludge was acclimated in each of 6 reaction chambers by aerating for 24 h at 23 °C. Following the acclimation period, aeration was ceased for 0.5 h to allow the solids to settle. Two-thirds of the supernatant was then drawn off, 20.5 ml additional synthetic sewage nutrients added, the volume re-adjusted to 1 l, and aeration resumed.

Dosing and Sampling

Mercuric chloride ($HgCl_2$) from a freshly prepared stock solution was added to each reaction chamber for a final concentration of 0, 10, 20, 50, 75, and 100 ppm. Each sludge chamber was monitored for several biological and chemical parameters at 0, 1, 3, 7, and 24 h after $HgCl_2$ dosing.

Biological Parameters

Protozoa were counted by phase-contrast microscopy by placing one 25 μl drop from each activated sludge sample on a microscope slide, and covering with a 22 x 22 mm coverslip (# $1^1/_2$). Bacteria were enumerated using standard plate-count techniques (American Public Health Association 1975). Serial dilutions to 10^{-6} were prepared from 1.0 ml activated sludge, and spread-plated in duplicate on Nutrient Agar (Baltimore Biological Laboratories). Plates were incubated at 37 °C for 48 h and *colony-forming units* (CFU) were counted on plates within the statistically valid range (30–300 CFU/plate). Plate count and protozoa count reproducibility from activated sludge was ± 5%.

Microbial degradation of starch and cellulose was monitored by an in-vitro radiometric technique (Buddemeyer 1974; Buddemeyer et al. 1976; Isbister et al. 1980). At each sampling, duplicate activated sludge aliquots (0.1 ml) were evenly mixed with 0.2 μCi water soluble U-^{14}C starch (NEC-730, New England Nuclear) or 0.2 μCi U-^{14}C cellulose (NEC-732-H, New England Nuclear). The ^{14}C-cellulose was custom synthesized into a finely ground powder for use in radiometric assays and was aseptically mixed with sterile, deionized water into a uniform suspension. Samples were then placed in a sterile 0.5 dram vial which was placed inside a 20 ml glass liquid scintillation vial containing a filter strip saturated with scintillation cocktail and immediately sealed. Cumulative $^{14}CO_2$ evolution was continuously monitored during the experiment by comparing the results from sterile, acidified $NaH^{14}CO_3$ (0.1 μCi, NEC-086, New England Nuclear) to direct counting of $NaH^{14}CO_3$ in HP Ready-Solv Liquid Scintillation Cocktail (Beckman).

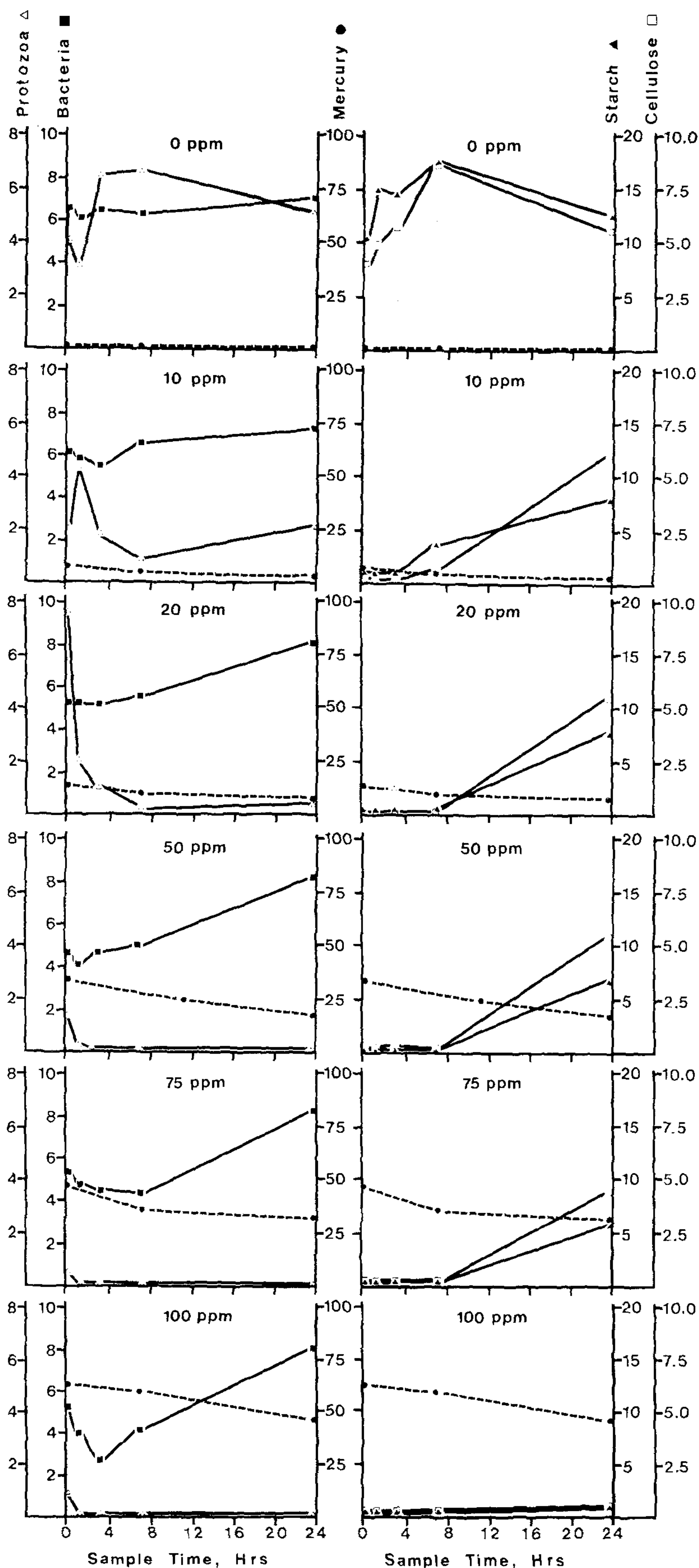

Fig. 3. Effects of 0, 10, 20, 50, 75, and 100 ppm $HgCl_2$ on protozoa (x 10^3/ml), bacteria (log total viable counts) and maximum ^{14}C-starch and ^{14}C-cellulose degradation (kDPM) in activated sludge. Maximum ^{14}C-starch and ^{14}C-cellulose degradation represents cumulative $^{14}CO_2$ evolved at 56 h

Table 1. Recovery of Hg from activated sludge at 0, 7, and 24 h after dosing with $HgCl_2$

Sample Time (h)	Nominal Addition (mg/l)		Hg Recovery (%)		
	$HgCl_2$	Hg	Filtrate	Solids	Total
0	10	7.4	15.4	70.9	86.3
0	20	14.8	10.4	77.7	88.1
0	50	36.9	11.8	79.7	91.5
0	75	55.4	9.2	76.7	85.9
0	100	73.9	11.6	75.1	86.7
7	10	7.4	12.4	66.6	79.0
7	20	14.8	7.3	59.1	66.4
7	50	36.9	7.8	65.0	72.8
7	75	55.4	7.0	56.9	63.9
7	100	73.9	7.8	73.7	81.5
24	10	7.4	4.6	58.1	62.7
24	20	14.8	4.9	43.6	48.5
24	50	36.9	5.2	43.8	49.0
24	75	55.4	3.3	53.7	57.0
24	100	73.9	8.1	56.3	64.4

Chemical Parameters

Fifty ml samples were withdrawn from each chamber at 0, 7, and 24 h after $HgCl_2$ dosing for duplicate pH readings. Samples were then immediately frozen for subsequent mercury analysis. Following the experiment, thawed samples were filtered and mercury content was determined in both the solid and aqueous phases of the sludge by the Dithizone Method (American Public Health Association 1975).

Results and Discussion

High sensitivity measuring the toxic effects of $HgCl_2$ on ^{14}C-starch and ^{14}C-cellulose degradation in activated sludge (Fig. 2) was obtained using the in-vitro technique. Altered kinetics were rapid and well demonstrated among slight concentration increments of $HgCl_2$. The differences in kinetics were statistically significant (t-test, $P < 0.05$), and the inhibition due to $HgCl_2$ persisted from 0 to 24 h after dosing. At the 24 h sampling, however, ^{14}C-starch and ^{14}C-cellulose degradation recovered in all chambers, degree of recovery decreasing with increasing $HgCl_2$ concentration. The average reproducibility of evolved $^{14}CO_2$ from replicate ^{14}C-starch and ^{14}C-cellulose samples was 13.3% and 10.5%, respectively.

Initial bacteria and protozoa counts in all $HgCl_2$ treated chambers decreased with increasing $HgCl_2$ concentration (Fig. 3). In contrast, bacteria counts after 24 h were significantly ($P < 0.05$) greater than those of the control, whereas protozoa remained sensitive to all concentrations of $HgCl_2$ throughout the study. Neither bacterial nor protozoal species composition, bulking characteristics nor gross sludge morphology in the control chamber changed significantly over the course of this experiment. Approximately 88% mercury was recovered in the solid (75%) and aqueous (13%) phases of the treated sludge immediately after dosing, whereas only 56% total mercury was recovered from the treated sludge after 24 h (Table 1).

The dramatic increase in bacteria counts, and recovery of ^{14}C-starch and ^{14}C-cellulose degradation after 24 h in the treated chambers can be explained in part by the loss of mercury from the system accompanied by the development of bacterial resistance to residual levels of mercury. Mercury-resistant microorganisms adsorb and metabolize mercury (Landa 1978; Sayler et al. 1975) leading to the subsequent volatilization of organic and inorganic mercury from the system (Leong 1973; Vaituzis et al. 1975). The development of plasmid-mediated mercury resistance and of microbial dissipation of applied $HgCl_2$ from an activated sludge system has been documented (Houba and Remacle 1980; Nakamura et al. 1975). In fact, we have isolated bacteria from the $HgCl_2$ treated flasks which were resistant to up to 100 ppm $HgCl_2$ incorporated into both solid and liquid media. Furthermore, the absence of protozoa, [microbial predators] at the higher treatment levels resulted in an increase of bacteria ten-fold higher than the control.

We have demonstrated that microbial metabolism in an activated sludge system can be monitored through simple and inexpensive techniques. The radiometric method described herein has advantages over standard plate counts and BOD_5 determinations in its rapidity, ease of continuous measurement and sensitivity to small increments of toxins. Used in conjunction with standard wastewater plant parameters such as BOD_5, the technique may provide important information on microbial interactions within activated sludge. Integration of the radiometric method into a waste treatment plant would require the acquisition of in-plant, baseline data. Significant deviations from the norm would indicate viability problems, signaling plant operators to respond appropriately.

The assay may also serve as a laboratory aid in assessing the toxic effects of pesticides in the waste treatment process. Since the U.S. EPA requires chemical manufacturers to determine the effects of certain chemicals on a model activated sludge system, the radiometric technique we have described would help supplement current testing parameters.

Acknowledgements. This work was supported by Borriston Research Laboratories, Inc.

We wish to thank D. Orlando, C. Kroboth, T. Christison, S. Rose, L. Vilcheck, K. Schroader, and P. Montgomery for their excellent technical assistance on this project.

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Received September 10, 1980