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Comparison of Methods for Inhibiting Bacterial Activity in Sediment

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Dilute formaldehyde was the most suitable treatment to inhibit sediment bacteria, since bacterial activity remained low during long-term incubations and the chemical changes in the sediment were minimal. The inhibiting effects of $HgCl_2$, autoclaving, and gamma radiation were diminished during longer incubations; these treatments also caused increases in dissolved nutrients.

Bacteria and other microbes develop high densities in sediments (5), and by affecting nutrient release both directly (through mineralization) and indirectly (by altering pH and oxygen conditions), they may also make a major contribution to internal loading in lakes (1, 6, 17, 20). In order to determine the relative roles of chemical, physical, and microbiological processes in the sediment, the microbiological activity in the sediment should be somehow excluded. An appropriate method should inhibit bacterial activity effectively but not change the geochemistry of the sediment (e.g., pH and adsorption capacity) or supply sediment with nutrients. However, very seldom has attention been paid to these side effects. This study compared the effectiveness of formaldehyde, $HgCl_2$, autoclaving, and gamma radiation in inhibiting the bacterial activity in sediment and studied their impact on dissolved nutrients and pH.

The topmost sediment layer (0 to 1 or 0 to 2 cm) was sampled from a 10-m-deep plane in the Enonselkä Basin of Lake Vesijärvi, a eutrophic lake in southern Finland (61°05′N, 25°30′E) during overturns in October (1992 and 1993) and May (1993). The water content of the sediment varied between 89 and 91% (oven dried at 60°C), with a loss of organic matter of 12 to 13% on ignition at 550°C and a carbon content of 4.4 to 4.6% (Leco CHN-900 analyzer). The sedimentation rate in the Enonselkä basin is very high (up to 2 cm $year^{-1}$) (11). Therefore, the samples represented recently sedimented material. Several samples were bulked, homogenized carefully by mixing manually, packed in 0.5-liter polyethylene containers, and stored in cold (2°C) and darkness. The only change observed during storage was the appearance of a thin iron hydroxide layer on the walls of the containers.

Acid-free formaldehyde (buffered with $CaCO_3$; Merck) was added to the sediment samples to give final concentrations of 0.01 to 1%. $HgCl_2$ was used at a final concentration of 92 mg of Hg $liter^{-1}$, which was higher than the 50 mg $liter^{-1}$ recommended by Lee et al. (10) for sediment traps but lower than the 176 mg $liter^{-1}$ used in sediment studies by de Montigny and Prairie (2). Autoclaving was carried out at 120°C for 20 min. The doses used in the gamma radiation were 25 and 50 kGy.

Both treated and live control samples were incubated in 120-ml centrifuge tubes in darkness at 17°C (an average summer temperature of the lake at a 10-m depth). The sediment was preacclimated at 17°C for 16 h before the incubation. Each tube received 30 ml of sediment and 30 ml of treatment solution (either formaldehyde solution, $HgCl_2$ solution, or distilled water; distilled water was added to the live, autoclaved, and radiated samples). The tubes were closed with silicon stoppers and mixed for 30 min prior to incubation. One set of samples (three replicates) was analyzed immediately after mixing. Other sets (three to six replicates) were analyzed after 1, 7, and 21 days from the start of the incubation.

After incubation, the pH of the sediment suspension was measured, and the suspension was centrifuged. The supernatant, filtered through a 0.2-μm-pore-size Nuclepore polycarbonate filter, was analyzed for soluble reactive P (SRP) and $NO_3^- + NO_2^-$ according to Murphy and Riley (14) and Wood et al. (25), respectively, and for NH_4^+ by the phenol hypochlorite method (21). Dissolved organic carbon was analyzed by a high-temperature combustion technique (18).

Bacterial activity in the sediment was determined by the incorporation of [^{3}H]thymidine into DNA (measured by the dialysis method [12, 13]) and by the total incorporation of [^{3}H]thymidine and [^{14}C]leucine (by the combustion technique described by Tuominen and Kairesalo [22], except that 80% ethanol, including 100 mg of thymidine and leucine $liter^{-1}$, was used to rinse unincorporated thymidine and leucine). The dialysis method was performed principally according to the method of Moriarty and Pollard (13), with the following additions: the incubation was terminated by filtering the samples through Nuclepore polycarbonate filters (pore size, 0.2 μm); after dialysis, a further step to separate the DNA was performed by heating the samples in 5% trichloroacetic acid at 90°C for 40 min.

All the isotopes were added at saturation level concentrations (tested to be 750 nM for thymidine and 15 μM for leucine). The samples were incubated at 17 to 18°C, and the optimum incubation time was 20 min. [*methyl*-^{3}H]thymidine (1.74 to 1.81 TBq $mmol^{-1}$; Amersham) and L-[U-^{14}C]leucine (11.5 to 11.7 GBq $mmol^{-1}$; Amersham) were used in the form of a dilution of 1/20 (vol/vol) with an unlabelled thymidine or leucine solution (same concentration). In some of the experiments, [*methyl*-^{3}H]thymidine at 185 GBq $mmol^{-1}$ was used undiluted.

The results of the autoclaved samples after 1 day of incubation were taken as blanks indicating the degree of thymidine and leucine adsorption into the sediment during incubation.

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TABLE 1. Average relative bacterial activities (percentage of the live sample activity) in sediment suspension after 1-day, 1-week, and 3-week incubations

Incubation time	Treatment	Avg bacterial activity (%) by[a]:		
		Dialysis	Combustion	
			Thymidine	Leucine
1 day	None (live sample)	100	100	100
	Formaldehyde (1%)	3	13	11
	Autoclaving[b]	0	0	0
	Radiation			
	25 kGy	0	3	0
	50 kGy	0	1	0
	$HgCl_2$	0	3	4
1 week	None (live sample)	100[c]	100[d]	100[d]
	Formaldehyde (1%)	10	8	8
	Autoclaving	43	42	156
	Radiation			
	25 kGy	20	27	77
	50 kGy	10	12	35
	$HgCl_2$	7	12	33
3 weeks	None (live sample)	100[c]		
	Formaldehyde (1%)	0	3[e]	17[e]
	Autoclaving	97	81[e]	118[e]
	Radiation			
	25 kGy	53	43[e]	188[e]
	50 kGy	63	94[e]	276[e]
	$HgCl_2$	17	30[e]	57[e]

[a] Dialysis, incorporation of [^{3}H]thymidine into DNA; combustion, total incorporation of [^{3}H]thymidine or [^{14}C]leucine.
[b] The results for the autoclaved samples on day 1 were taken as blanks and were subtracted from all the results.
[c] 97% of the activity in the live sample after 1 day.
[d] 99% of the activity in the live sample after 1 day.
[e] Calculated as a percentage of activity in the live sample after 1 week (the results for the live samples after 3 weeks of incubation had to be rejected).

The results of these blanks were 11% (thymidine, dialysis method), 5% (thymidine, combustion technique), and 11% (leucine, combustion technique) of the activity in the live samples and were subtracted from all the final results.

In terms of bacterial activity, the three methods showed similar trends (Table 1). However, the leucine incorporation method gave higher relative activities than did the thymidine incorporation method. This might have been caused by the uptake of leucine by organisms other than bacteria, since high concentrations of leucine were needed to reach the saturation level in sediment samples (contrary to observations of water samples by Kirchman et al. [8]). Thymidine incorporation is, on the contrary, more specific to heterotrophic bacteria (12).

After 1 day of incubation, all treatments had lowered the bacterial activity to 0 to 13% of the activity in the corresponding live samples (Fig. 1; Table 1). Thereafter, bacterial activity in the autoclaved and radiated samples began to rise, and after 3 weeks of incubation, it was at the same level as that in the live samples. The increase in the bacterial activity between the 1-day and 3-week incubations was statistically significant (analysis of variance, $\alpha = 0.05$). Our results contrast with those of some earlier studies in which radiation (25 to 60 kGy) was reported to inhibit colony formation on plates (19, 24). However, Wolf et al. (24) noted that extractable Mn concentrations increased in soil samples, and therefore they did not recommend the use of gamma radiation for soil sterilization. In addition, only a small fraction of aquatic bacterial populations grows on plates (23). Therefore, bacterial activities may be high regardless of the fact that a low level of colony growth on plates is observed.

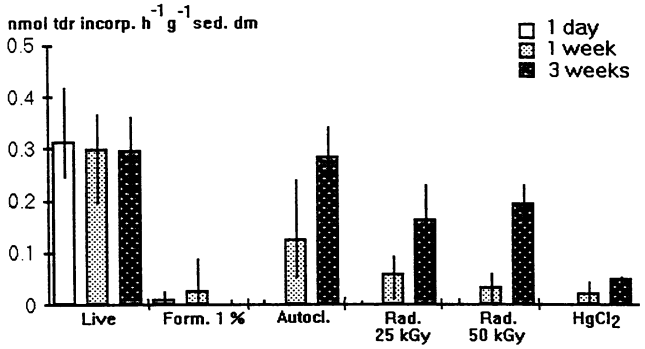

FIG. 1. Bacterial activity in differently treated sediment samples after 1-day, 1-week, and 3-week incubations. The activity was determined by the thymidine incorporation into DNA (nanomoles of thymidine [tdr] incorporated per hour per gram of sediment [dry mass] by the dialysis method; means and range of variation are given). Form., formaldehyde; autocl., autoclaving; rad., radiation.

The live control samples remained chemically unchanged during the experiment except for the peak concentration of NO_3^- on the first day of incubation (Table 2). This concentration was strikingly high but within the limits of the potential nitrification rate in freshwater sediment (4). Autoclaving and gamma radiation yielded high concentrations of dissolved organic carbon (Table 2), which provided a good environment for bacterial spores that had survived the treatment. In the autoclaved samples, the SRP concentration increased tremendously (Table 2). This was attributable to the release of P from bacterial cells and to anaerobiosis, judging from the appearance and smell of the sediment. Later, the SRP concentrations decreased, presumably because of chemical binding and microbiological uptake. Because autoclaving kills bacteria but not spores, one autoclavation is not sufficient to sterilize the sample. In some soil samples, for instance, respiration was found to increase within 30 h up to the initial level (15). Autoclaving may increase Mn^{2+} concentrations in soil samples 10-fold by causing anaerobic conditions (24). The same phenomenon resulted most obviously in high SRP concentrations in this study.

Gamma radiation creates free hydrogen and hydroxyl radicals. These radicals are very reactive and can act as reducing or oxidizing agents and cleave carbon-to-carbon bonds. Radiation causes carbohydrate polymers (e.g., cellulose) to depolymerize (3), which could be the reason for the observed high dissolved organic carbon concentration in the samples.

$HgCl_2$ inhibited bacterial activity quite effectively, but the activity increased statistically significantly during the 3-week incubation however (Fig. 1). $HgCl_2$ did not affect the sample pH or dissolved nutrient levels, except the NH_4^+ concentration (Table 2). The dissolved NH_4^+ concentration likely increased because of the following reasons: (i) when dying, the cells released nutrients, of which SRP was rapidly adsorbed chemically; (ii) $HgCl_2$ released NH_4^+ ions from the sediment material (also Ag and Cd have been observed to be released by $HgCl_2$ from degrading algal material [9]); or (iii) the presence of the Hg ion interfered with the analysis of NH_4^+. Wolf et al. (24) recommended the use of $HgCl_2$ to inhibit bacterial activity in soil because it caused minimal changes in the chemical and physical properties. Its mode of action is to combine with cellular proteins containing the sulfhydryl group (16).

TABLE 2. Concentrations of DOC, SRP, NH_4-N, and NO_3-N plus NO_2-N in supernatants of differently treated sediment samples

Incubation time	Treatment	DOC[a] (mg liter^{-1})	SRP (μg liter^{-1})	NH_4-N (μg liter^{-1})	NO_3-N + NO_2-N (μg liter^{-1})
0 (start)	None (live sample)	9.4	11	960	25
	Formaldehyde (1%)	NA[b]	6.7	1.3	37
	Autoclaving	110	670	530	46
	Radiation				
	25 kGy	82	6.3	1,000	26
	50 kGy	99	5.7	520	31
	$HgCl_2$	8	6.0	1,100	27
1 day	None (live sample)	5.2	33	480	790
	Formaldehyde (1%)	NA	20	9.3	30
	Autoclaving	110	120	510	79
	Radiation				
	25 kGy	69	18	990	170
	50 kGy	93	42	520	85
	$HgCl_2$	15	57	1,200	85
1 week	None (live sample)	8.6	6.8	1,500	700
	Formaldehyde (1%)	NA	8.7	12	25
	Autoclaving	130	16	1,900	36
	Radiation				
	25 kGy	82	2.0	3,300	38
	50 kGy	92	1.0	3,300	180
	$HgCl_2$	19	4.8	3,400	58
3 weeks	None (live sample)	6.7	9.0	580	380
	Formaldehyde (1%)	NA	16	12	55
	Autoclaving	87	65	3,000	54
	Radiation				
	25 kGy	73	12	3,600	41
	50 kGy	79	25	3,600	92
	$HgCl_2$	14	10	2,000	200

[a] DOC, dissolved organic carbon.
[b] NA, not analyzed.

Regardless of buffering, a 1% concentration of formaldehyde lowered the pH by 2 units in the sediment samples (Table 3). A lower concentration, 0.04% in October 1992 (Fig. 2) or 0.02% in May 1993 and October 1993, effectively inhibited bacterial activity but did not cause any significant change in the pH. Other treatments did not affect the sample pH (except a small increase in the autoclaved samples).

Of the methods compared, the dilute formaldehyde solution (0.02 to 0.04%) proved to be the most promising for inhibiting bacterial activity in sediment. In particular, formaldehyde did not cause any changes in dissolved nutrient concentrations. The chronic influence of formaldehyde is based on its ability to kill emerging spores also (16) through reactions with vital organic nitrogen compounds such as proteins and nucleic acids. However, the influence on pH depends greatly on the formaldehyde concentration and the buffer capacity of the sediment, and therefore the formaldehyde concentration must be determined for each type of sediment separately. For instance, Lee et al. (10) successfully used dilute formaldehyde (0.11%) to terminate the bacterial activity in sediment trap samples, but a concentration of as high as 4% did not decrease the sample pH in a calcareous sediment (7).

TABLE 3. Sample pH after 1 day and 3 weeks

Treatment	pH	
	1 day	3 weeks
None (live sample)	6.4	6.2
Formaldehyde (1%)	5.0	4.0
Autoclaving	7.0	6.9
Radiation		
25 kGy	6.6	6.6
50 kGy	6.6	6.7
$HgCl_2$	6.0	6.7

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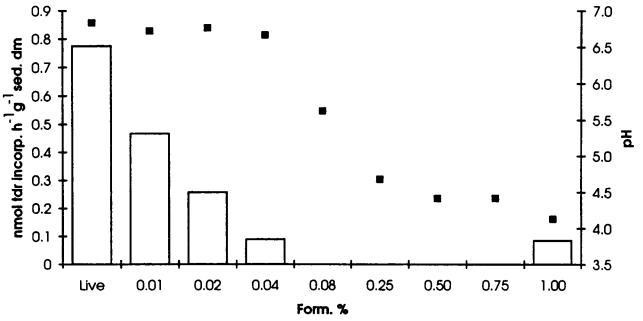

FIG. 2. Influence of formaldehyde concentration on sediment pH (squares) and bacterial activity (columns). Bacterial activity was determined by the combustion technique and expressed as nanomoles of thymidine (tdr) incorporated per hour per gram of sediment (dry mass). The absence of columns indicates that activity was not measured. Form., formaldehyde.

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REFERENCES

1. **Boström, B., I. Ahlgren, and R. Bell.** 1985. Internal nutrient loading in a eutrophic lake, reflected in seasonal variations of some sediment parameters. Verh. Int. Ver. Limnol. **22:**3335–3339.
2. **de Montigny, C., and Y. T. Prairie.** 1993. The relative importance of biological and chemical processes in the release of phosphorus from highly organic sediment. Hydrobiologia **253:**141–150.
3. **Desrosier, N. W.** 1963. The technology of food preservation, 2nd ed. Avi Publishing Company, Inc., Westport, Conn.
4. **Dodds, W. K., and R. D. Jones.** 1987. Potential rates of nitrification and denitrification in an oligotrophic freshwater sediment system. Microb. Ecol. **14:**91–100.
5. **Doremus, J. C., and L. S. Clesceri.** 1982. Microbial metabolism in surface sediments and its role in the immobilization of phosphorus in oligotrophic lake sediments. Hydrobiologia **91:**261–268.
6. **Gächter, R., and J. S. Meyer.** 1993. The role of microorganisms in mobilization and fixation of phosphorus in sediments. Hydrobiologia **253:**103–121.
7. **Gächter, R., J. S. Meyer, and A. Mares.** 1988. Contribution of bacteria to release and fixation of phosphorus in lake sediments. Limnol. Oceanogr. **33:**1542–1558.
8. **Kirchman, D., E. K'nees, and R. Hodson.** 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl. Environ. Microbiol. **49:**599–607.
9. **Lee, B.-G., and N. S. Fisher.** 1992. Degradation and elemental release rates from phytoplankton debris and their geochemical implications. Limnol. Oceanogr. **37:**1345–1360.
10. **Lee, C., J. I. Hedges, S. G. Wakeham, and N. Zhu.** 1992. Effectiveness of various treatments in retarding microbial activity in sediment trap material and their effects on the collection of swimmers. Limnol. Oceanogr. **37:**117–130.
11. **Liukkonen, M., T. Kairesalo, and J. Keto.** 1993. Eutrophication and recovery of Lake Vesijärvi (South Finland): diatom frustules in varved sediments over a 30-year period. Hydrobiologia **269/270:** 415–426.
12. **Moriarty, D. J. W.** 1990. Techniques for estimating bacterial growth rates and production of biomass in aquatic environments. Methods Microbiol. **22:**211–234.
13. **Moriarty, D. J. W., and P. C. Pollard.** 1990. Effects of radioactive labelling of macromolecules, disturbance of bacteria and adsorption of thymidine to sediment on the determination of bacterial growth rates in sediment with tritiated thymidine. J. Microbiol. Methods **11:**127–139.
14. **Murphy, J., and J. P. Riley.** 1962. A modified single-solution method for the determination of phosphate in natural waters. Anal. Chim. Acta **27:**31–36.
15. **Nowak, A., and H. Wronkowska.** 1987. On the efficiency of soil sterilization in autoclave. Zentralbl. Mikrobiol. **142:**521–525.
16. **Pelczar, M. J., Jr., E. C. S. Chan, and N. R. Krieg.** 1986. Microbiology, 5th ed. McGraw-Hill Book Co., New York.
17. **Ryding, S.-O.** 1985. Chemical and microbiological processes as regulators of the exchange of substances between sediments and water in shallow eutrophic lakes. Int. Rev. Gesamten Hydrobiol. **70:**657–702.
18. **Salonen, K.** 1979. A versatile method for the rapid and accurate determination of carbon by high temperature combustion. Limnol. Oceanogr. **24:**177–183.
19. **Sinke, A. J. C., and T. E. Cappenberg.** 1988. Influence of bacterial processes on the phosphorus release from sediments in the eutrophic Loosdrecht Lakes, The Netherlands. Ergeb. Limnol. **30:**5–13.
20. **Sinke, A. J. C., F. H. M. Cottaar, and P. Keizer.** 1993. A method to determine the contribution of bacteria to phosphate uptake by aerobic freshwater sediment. Limnol. Oceanogr. **38:**1081–1087.
21. **Solorzano, L.** 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. **14:**799–801.
22. **Tuominen, L., and T. Kairesalo.** 1992. A method for measuring the uptake of thymidine and leucine in sediment. Aqua Fenn. **22:**43–48.
23. **van Es, F. B., and L.-A. Meyer-Reil.** 1982. Biomass and metabolic activity of heterotrophic marine bacteria. Adv. Microb. Ecol. **6:**111–170.
24. **Wolf, D. C., T. H. Dao, H. D. Scott, and T. L. Lavy.** 1989. Influence of sterilization methods on selected soil microbiological, physical, and chemical properties. J. Environ. Qual. **18:**39–44.
25. **Wood, E. D., F. A. J. Amstrong, and F. A. Richards.** 1967. Determination of nitrate in sea water by cadmium-copper reduction to nitrite. J. Mar. Biol. Assoc. U. K. **47:**23–31.