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Recalcitrant High-Molecular-Weight Material, an Inhibitor of Microbial Metabolism in River Biofilms

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Recalcitrant high (>1,000)-molecular-weight materials (>1K materials) have been proposed to inhibit the metabolic activity of river biofilms. Evidence is presented to support the hypothesis that the mode of action is through occlusion of the surface of the biofilm by recalcitrant >1K materials. The evidence includes the following. (i) The phenomenon was particularly prominent in brown-water rivers, which are rich in recalcitrant >1K material. (ii)Temporal changes in inhibition intensity were observed, which coincided with seasonal changes in the relative recalcitrance of river water >1K materials. (iii) Stores of intracellular carbon (poly-β-hydroxyalkanoates) were progressively compromised by increasing the supply of recalcitrant >1K materials. (iv) Nontoxic synthetic analogs of recalcitrant >1K materials were also demonstrated to be capable of producing an identical inhibitory effect. Finally, it was suggested that the application of recalcitrant >1K materials may represent a novel approach to the control of deleterious microbial communities.

Ford and Lock (9) recently described an experiment that was designed to determine the relative nutritional value of materials with a molecular weight of >1,000 (>1K materials) to river biofilms. The study produced some rather unexpected findings.

It was predicted that the removal of >1K material (>70% of the organic nutrient supply) would reduce microbial activity within the biofilms. However, contrary to these expectations, the treatment frequently resulted in dramatic increases in metabolic activity, rather than the decreases that were anticipated (9, 10).

A number of studies of aquatic bacterial communities have noted an apparently inhibitory activity of natural high-molecular-weight materials (2, 9, 10, 18, 23). It was therefore suggested that such responses could be due to either (i) the removal of a "toxic" inhibitor within the >1K fraction or (ii) an occlusion mechanism by which recalcitrant >1K material retards the entry of smaller, more labile moieties. Of these two possibilities, the latter was considered the most likely mode of action on the following grounds: (i) there is a finite surface area through which microbially labile constituents of river water can gain access to the biofilm, and (ii) an excess of microbially recalcitrant >1K material could occlude this surface, impeding the access of labile materials. Thus, removing the >1K material could increase the flux of labile <1K materials reaching the microorganisms within the biofilm, which in turn, could stimulate the observed higher levels of metabolic activity. However, no experiments were conducted to test this hypothesis.

This study was designed to test the hypothesis that recalcitrant (>1K) materials can reduce labile (<1K) nutrient availability and thus metabolic activity. For the hypothesis to be tenable, a number of criteria should be satisfied. (i) Brown-water rivers, in which the >1K material is considered to be more resistant to microbial catabolism (recalcitrant) (1), should show the inhibitory phenomenon with greater intensity and consistency than other rivers. (ii) Cyclical changes in inhibitor "potency" should be observed during natural changes in the relative recalcitrance of river water >1K material (11). (iii) The >1K material must be confirmed to be recalcitrant and not to promote microbial growth. (iv) Biofilm nutritional status should be inversely related to the concentration of recalcitrant >1K material. (v) Nontoxic synthetic analogs of recalcitrant >1K material should produce similar inhibitory effects.

MATERIALS AND METHODS

Phase 1: a three-river temporal survey of the effects of >1K-material removal. The basic approach to the first phase of the study involved collecting field-cultured biofilm over a 20-month period from three (previously described [10]) North Wales rivers. These included the Conwy, a brown-water river in which the >1K material is considered to be highly recalcitrant (1) and where a strong inhibition response would be anticipated, and two low-color rivers, the oligotrophic Aber and the more eutrophic Clywedog. On return to the laboratory, the biofilms were perfused for 4 days with either <1K material only or waters containing the full spectrum of dissolved materials. The effects upon microbial activity were then assessed by microcalorimetry, a technique that represents the most all-encompassing measure of metabolism available (22).

Field culture of biofilms. Six 2-m lengths of 1.5-mm diameter glass beads on nylon monofilament were attached to a Plexiglas plate and placed out in each of the three rivers such that they colonized naturally with biofilm microorganisms. A minimum of 8 weeks of colonization was allowed prior to each experiment. Darkened conditions were maintained by placing the above-described apparatus within an incubation system consisting of a 10-cm-diameter pipe fitted with a light trap consisting of two consecutive 90° bends at either end (10). The heterotrophic processes (which are of primary interest in a study of organic carbon metabolism) could thus be studied in relative isolation from autotrophic processes.

Water fractionation. All water samples were filtered through Whatman GF/F glass fiber filters to remove particulate material (17). From half of each sample, >1K material was then removed by ultrafiltration with a Millipore Tangen-

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tial Flow Ultrafiltration Cell fitted with a nominal 1K-cutoff filter stack (9).

Perfusions with control and <1K fractions. On return from the field, biofilm-coated beads were evenly distributed into a series of microcalorimeter flow cells (15). Half the flow cells were perfused with the <1K fraction, and the other half were perfused with the unmodified water. After 4 days of perfusion (9) in the dark at river temperature, activity measurements were performed. Four-day perfusions were found necessary because of the slow responses of river biofilms to radically altered nutrient regimens (16).

Microcalorimetry. The semiadiabatic flow microcalorimeter described by Lock and Ford (15) was used to determine metabolic activity according to a previously described protocol (10).

Phase 2: effects of addition of two sources of >1K material. The basic approach to the second phase of the study involved first collecting samples of river clywedog-cultured biofilms during the spring and summer of 1988. Upon return to the laboratory, the biofilms were perfused for 4 days with >1K-material-free waters to flush out any remnant >1K materials and then challenged for a further 4 days with the perfusion media described below. The impacts upon microbial growth rate and endogenous carbon reserves were then determined.

Perfusion media. (i) Control (>1K-material-free) waters. The control >1K-material-free perfusion waters were prepared as described under "Water fractionation" above, by using low-color river Clywedog water.

(ii) Natural recalcitrant >1K-material-amended waters. Water from the humic-rich river Conwy (10) was fractionated as described above, except that the >1K material was retained and the <1K material was discarded. Harvested >1K material was then added to >1K-material-free waters from the Clywedog prepared as described in (i) above, in increasing quantities. A range of 0, 25, 50, 100, and 200% of the original river Conwy >1K concentration was prepared.

(iii) "Model" recalcitrant >1K-material-amended waters. To >1K-material-free Clywedog waters as described in (i) above, 10 mg of one of a variety of molecular weight preparations of polyvinylpyrrolidone (PVP) was added, such that a range of perfusion waters was prepared. These included 10,000-, 40,000-, and 360,000- nominal-molecular-weight preparations.

Endogenous carbon reserves measurement. The procaryotic storage product poly-β-hydroxyalkanoate (PHA) was monitored as an indicator of the endogenous carbon reserve status (4, 14) by using the ion exclusion high-pressure liquid chromatography (HPLC) technique described by Karr et al. (14) but modified for biofilm samples (10). The technique involves digestion of the biofilm in hot H_2SO_4 to convert the PHA to crotonic acid prior to HPLC analysis by using an Aminex HPX-87H ion exclusion column (Bio-Rad), with 0.014 N H_2SO_4 eluent at 0.7 ml min^{-1}. A 100-μl injection volume was maintained throughout. PHA concentrations were expressed in terms of crotonic acid as described by Karr et al. (14).

Microbial growth rate. The rate of growth (as DNA synthesis) was estimated by using the thymidine incorporation technique of Moriarty and Pollard (19) but with the improved DNA extraction procedure of Findlay et al. (5, 6). Twenty beads were transferred to a scintillation vial containing 1 ml of water (as used in the perfusion) with 20 μCi of thymidine and 10 nmol of nonlabelled thymidine. Samples were incubated for 1 h in darkness at river temperature. A preliminary experiment was conducted at each river to ensure that (i) the rate of thymidine incorporation was linear over at least double the 1-h incubation period and that (ii) 10 nmol was sufficient to prevent de novo synthesis and thus prevent intracellular isotope dilution, by using the technique of Moriarty and Pollard (19, 20). Incubations were terminated by the addition of 125 μl of formaldehyde, and the samples were washed four times with 5% formaldehyde. DNA was extracted with 3 ml of an extraction cocktail (0.3 N NaOH, 25 mM EDTA, 0.1% sodium dodecyl sulfate) over 12 h. After precipitation in an ice bath with 480 μl of 3 N HCl and 600 μl of 50% trichloroacetic acid, samples were centrifuged at 4°C, the supernatant was discarded, and the precipitated nucleic acids were hydrolyzed for 1 h at 100°C with 1 ml of 5% trichloroacetic acid. After recentrifugation, a 0.5-ml aliquot of supernatant was counted in 4.5 ml of hydrofluor (National Diagnostics) scintillation cocktail on a Beckman LS7000 scintillation counter.

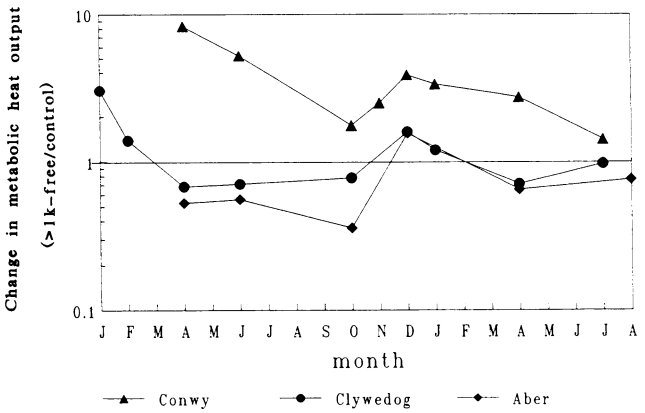

FIG. 1. Temporal variation in the response of biofilm metabolic heat output upon >1K material removal. Expressed as (>1K-material-free heat output) / (control heat output); thus, >1 indicates an increase, <1 indicates a decrease. (Sampled 1987 to 1988.)

RESULTS

The results of phase 1, the field survey, showed that Conwy (brown-water) biofilms responded in a manner significantly different from those of the two low-color streams (analysis of variance, $P < 0.05$) upon removal of >1K material. This consistently resulted in increases (range, 41 to 833%) in heat output throughout the study. In contrast, the two low-color streams generally responded with decreases (range, 3 to 64%) in metabolic heat output over most of the year (Fig. 1). However, in winter, they too exhibited heat output increases (range, 19 to 201%) upon removal of the higher-molecular-weight fraction.

In the second phase of the study, the addition of a wide range of concentrations of natural recalcitrant >1K material from the river Conwy (Fig. 2) failed to produce statistically significant increases in DNA synthesis (*t* test). However, in contrast, a marked inverse relationship between the concentration of recalcitrant material and biofilm PHA content was observed ($r = -0.89$, $P < 0.05$, multivariate correlation analysis). The maximum response was a reduction to 70% of the original PHA concentration ($P < 0.05$, *t* test). Similar responses were observed upon addition of PVP (Fig. 3) in that no significant effect on DNA synthesis was observed (*t* test) and yet significant reductions in biofilm PHA content occurred ($P < 0.05$). However, the extent of the reduction

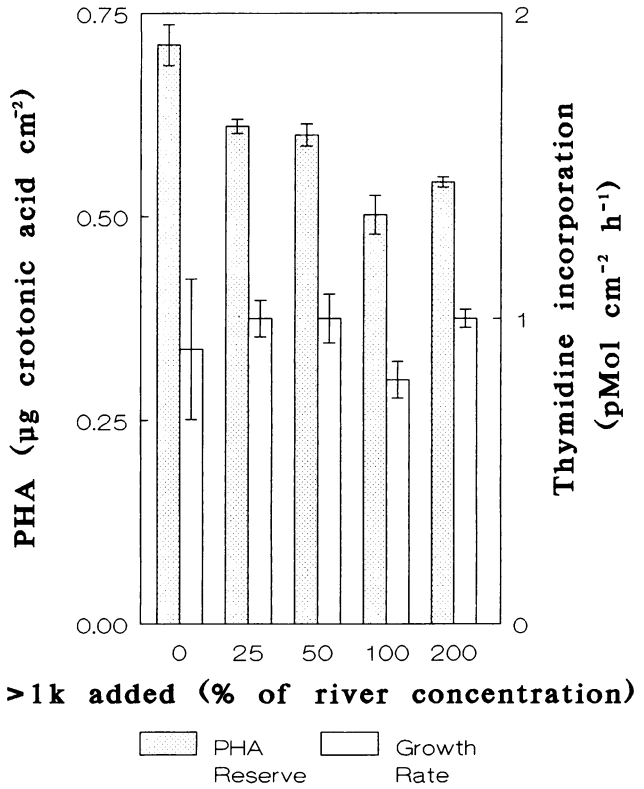

FIG. 2. Effect on Clywedog biofilm DNA synthesis and PHA reserves of the addition of recalcitrant (brown-water) >1K material from the river Conwy. Error bars indicate standard error; $n = 6$.

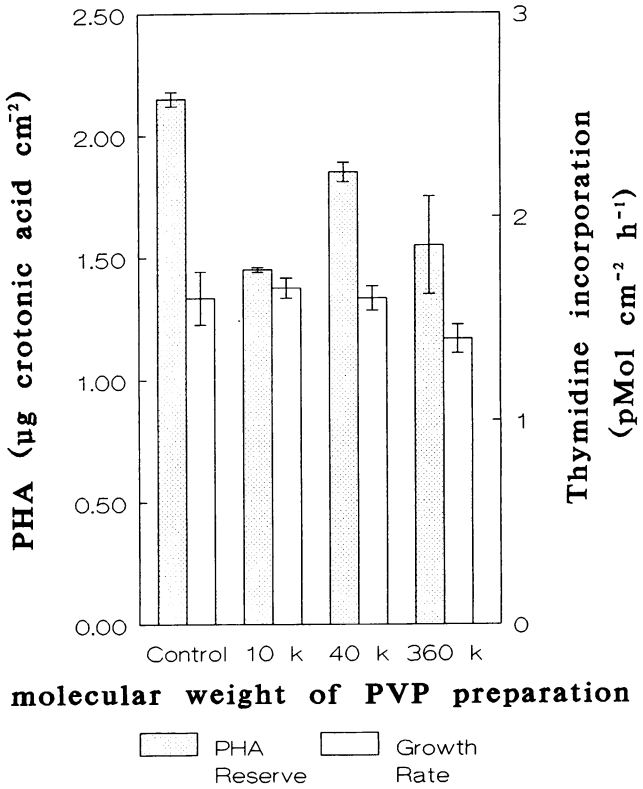

FIG. 3. Effect on Clywedog biofilm DNA synthesis and PHA reserves of the addition of various molecular weight preparations of polyvinylpyrrolidone. Error bars indicate standard error; $n = 6$.

appeared to be dependent upon molecular weight, with a minimal reduction of 14% produced by the 40,000-molecular-weight form and the maximum of 31% produced by the 10,000-molecular-weight fraction.

DISCUSSION

The results of these studies appear to support the hypothesis of Ford and Lock (9) that recalcitrant >1K materials can inhibit microbial metabolism, through an occlusion mechanism which apparently reduces the availability of labile <1K nutrients. Five criteria concerning biofilm properties and functioning were proposed to test the hypothesis.

The first criterion required that biofilms from brown-water streams (whose brown color is due to large quantities of recalcitrant humic substances [1]) should exhibit the inhibitory phenomenon with greater intensity and consistency than low-color streams, which have a considerably lower humic content. This was very clearly demonstrated to be the case, and additional support can also be drawn from observations of this phenomenon in brown-water Canadian streams (9) and also in a German brown-water stream (10).

The second criterion required cyclical changes in inhibitor potency which corresponded to seasonal changes in the relative recalcitrance of >1K material in river waters to be observed. Work by Geller (11) has shown high-molecular-weight materials to become more labile to microbial catabolism during the summer months. As such, the elevation in metabolism associated with the removal of >1K material was predicted to be less in summer than winter. This proved to be the case in all three rivers. This shift in apparent recalcitrance may be the consequence of low winter temperatures on the extracellular enzymes which degrade the >1K material. Since low winter temperatures have been shown to dramatically reduce biofilm extracellular enzyme activities (12), then the low rates of catabolism could give the >1K organic matter the appearance of greater recalcitrance.

The third criterion required that addition of recalcitrant >1K material should not promote increased microbial growth. The demonstration that neither natural (Conwy) nor synthetic (PVP) sources of >1K material stimulated DNA synthesis (reproductive growth) or PHA accumulation (unbalanced growth [21]) confirms the poor nutritional value, and hence recalcitrance, of the added >1K material.

The fourth criterion required that application of increasing loadings of >1K material should progressively deprive the biofilm of access to labile exogenous <1K material, leading (through starvation) to an enforced mobilization of the endogenous carbon reserves (3, 4). This was clearly demonstrated by a substantial 30% depletion in just 4 days of perfusion.

Finally, the fifth criterion required that a nontoxic recalcitrant synthetic analog of >1K organic matter (PVP) would cause nutrient deprivation (and thus PHA depletion) similar to that which would occur with natural >1K material. This proved to be the case, with each of the molecular weight preparations of PVP inducing a PHA depletion response. PVP is an inert compound frequently used as a plasma extender because it is pharmacologically benign. It also represents a high-molecular-weight material of a nature very different from that naturally occurring in the rivers of North Wales, and thus the microbial communities are unlikely to be

acclimated to its utilization (13). As a consequence, the compound should have appeared highly recalcitrant to the biofilm. The observed PHA depletion response therefore lends support to the hypothesis that the inhibitory effect was physicochemical by nature rather than toxicological.

The degree of endogenous reserve depletion appeared to be influenced by molecular weight. The molecular weight of natural river water material has also been shown to have an influence on microbial growth in other studies. Ford (7, 8) observed an inverse relationship between microbial metabolic activity and the percentage of organic material in the molecular weight range 1,000 to 10,000. Meyer et al. (18) found materials of this same molecular weight range to promote less microbial growth than material of higher or lower molecular weight. In our study, it was interesting that 10,000-molecular-weight PVP was the form resulting in the greatest depletion of the stores of biofilm PHA, which would appear broadly consistent with these other studies (7, 18). Such findings may be related to differences in the ability of the various size fractions to diffuse into the biofilm matrix.

In conclusion, we suggest that evidence from any one of the criteria chosen (in isolation) to test the hypothesis (that >1K organic matter reduces metabolism of <1K matter) would be relatively weak. However, when all five are considered, this study lends substantial support to the hypothesis of Ford and Lock (9). Future studies will, however, need to be directed towards a further clarification of the mechanism of the inhibition to establish whether indeed it is a physicochemical phenomenon (as these studies suggest) rather than a toxicological one. Finally, if this phenomenon proves to be general to all aquatic systems, then this suggests the possibility of practical application in biofilm control, in situations in which conventional biocides are inappropriate or environmentally unacceptable. It may prove possible to control fouling biofilms by the addition of natural or synthetic microbially recalcitrant compounds.

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REFERENCES

1. **Beck, K. C., J. H. Reuter, and E. M. Perdue.** 1974. Organic and inorganic geochemistry of some coastal plain rivers of the South-eastern United States. Geochim. Cosmochim. Acta **38:** 341.
2. **Chudoba, J.** 1985. Inhibitory effects of refractory organic carbon compounds produced by activated sludge micro-organisms on microbial activity and flocculation. Water Res. **19:**197–200.
3. **Dawes, E. A.** 1976. Endogenous metabolism and the survival of starved prokaryotes, p. 19–53. *In* T. G. R. Gray and J. R. Postgate (ed.), survival of vegetative microbes. Cambridge University Press, Cambridge.
4. **Dawes, E. A., and P. J. Senior.** 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. **19:**135–278.
5. **Findlay, S. G., J. L. Meyer, and R. T. Edwards.** 1984. Measuring bacterial production via rate of incorporation of ^{3}H thymidine into DNA. J. Microbiol. Methods **2:**57–72.
6. **Findlay, S. G., J. L. Meyer, and R. Risley.** 1986. Benthic bacterial biomass and production in two blackwater rivers. Can. J.Fish. Aquat. Sci. **43:**1271–1276.
7. **Ford, T. E.** 1984. A study of colloidal and dissolved organic carbon in rivers and their contribution to benthic microbial metabolism. Ph.D. thesis, University of Wales, Bangor.
8. **Ford, T. E., and M. A. Lock.** 1985. A temporal study of colloidal and dissolved organic carbon in rivers: apparent molecular weight spectra and their relationship to bacterial activity. Oikos **45:**71–78.
9. **Ford, T. E., and M. A. Lock.** 1987. Epilithic metabolism of dissolved organic carbon in boreal forest rivers. FEMS Microbiol. Ecol. **45:**89–97.
10. **Freeman, C., M. A. Lock, J. Marxsen, and S. E. Jones.** 1990. Inhibitory effects of high molecular weight dissolved organic matter on metabolic processes in contrasted rivers and streams. Freshwater Biol. **24:**159–166.
11. **Geller, A.** 1985. Degradation and formation of refractory DOM by bacteria during simultaneous growth on labile substrates and persistent lake water constituents. Schweiz. Z. Hydrol. **47:**27–44.
12. **Jones, S. E.** 1990. Extracellular enzyme activity in river biofilms. Ph.D thesis, University of Wales, Bangor.
13. **Kaplan, L. A., and T. A. Bott.** 1985. Acclimation of a streambed microflora; responses to dissolved organic matter. Freshwater Biol. **15:**479–492.
14. **Karr, D. B., J. K. Waters, and D. W. Emerich.** 1983. Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. Appl. Environ. Microbiol. **46:**1339–1344.
15. **Lock, M. A., and T. E. Ford.** 1983. An inexpensive flow microcalorimeter for measuring the heat production of attached and sedimentary aquatic microorganisms. Appl. Environ. Microbiol. **46:**463–467.
16. **Lock, M. A., and T. E. Ford.** 1985. Microcalorimeteric approach to determine relationships between energy supply and metabolism in river epilithon. Appl. Environ. Microbiol. **49:**408–412.
17. **Lock, M. A., P. M. Wallis, and H. B. N. Hynes.** 1977. Colloidal organic matter in running waters. Oikos **29:**1–4.
18. **Meyer, J. L., R. T. Edwards, and R. Risley.** 1987. Bacterial growth on DOC from a blackwater river. Microb. Ecol. **13:**13–29.
19. **Moriarty, D. J. W., and P. C. Pollard.** 1981. DNA synthesis as a measure of bacterial productivity in seagrass sediment. Mar. Ecol. Prog. Ser. **5:**151–156.
20. **Moriarty, D. J. W., and P. C. Pollard.** 1982. Diel variation in sea grass (*Zostera capricorni*) beds measured by rate of thymidine incorporation into DNA. Mar. Biol. **72:**165–173.
21. **Nickels, J. S., J. D. King, and D. C. White.** 1979. Poly-β-hydroxybutyrate accumulation as a measure of unbalanced growth of the estuarine detrital microbiota. Appl. Environ. Microbiol. **37:**459–465.
22. **Pamatmat, M. M.** 1982. Heat production by sediment; ecological significance. Science **215:**395–397.
23. **Saz, A. K., S. Watson, S. R. Brown, and D. L. Lowery.** 1963. Antimicrobial activity of marine waters. 1. Macromolecular nature of antistaphylococcal factor. Limnol. Oceanogr. **8:**63–67.