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Epilithic Extracellular-Enzyme Activity in a Zinc-Contaminated Stream

K. R. Chappell, R. Goulder

Department of Applied Biology, University of Hull, Hull HU6 7RX, United Kingdom

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Most dissolved organic material in natural waters exists as high-molecular-weight, polymeric compounds. These represent a potential source of organic matter for catabolism and growth and of essential inorganic nutrients (e.g., PO_4 of phosphoric esters and NH_3 of proteins and polypeptides) but they are not immediately available to microorganisms because only low-molecular-weight compounds can be conveyed by transport systems through cytoplasmic membranes into microbial cells. The problem is resolved by extracellular enzymatic hydrolysis of the polymers to oligomeric and monomeric molecules. The extracellular enzymes involved are produced by heterotrophic microorganisms and microalgae. They function in periplasmic spaces, and bound to cell walls or non-living surfaces, and free in the surrounding water (Chróst 1991).

The development of highly-sensitive fluorimetric assays (Hoppe 1983) has encouraged the study of extracellular enzymes in aquatic environments. It has been shown that substantial extracellular-enzyme activity is associated with epilithic biofilms in streams. In calcareous headstreams in Northern England, the extracellular-enzyme activity bound to 1 cm^2 of stone surface equalled, on average, the free enzyme activity in 270 mL (ß-D-glucosidase), 330 mL (ß-D-galactosidase), 270 mL (ß-D-xylosidase), 120 mL (phosphatase), and 160 mL (sulphatase) of surrounding water (Chappell and Goulder 1992). Evidently, the breakdown of epilithic extracellular-enzyme function, in response to toxic pollution, would adversely affect the epilithic microbiota. In the present paper we report extracellular-enzyme activity on stones from a long-term zinc-contaminated stream in Northeast England, and contrast this with activity on stones from a neighboring uncontaminated stream.

The streams studied were the Rivers West and East Allen in Northumberland. They flow northwards, roughly parallel, about 5 km apart in adjacent valleys, for about 18 km before merging to form the River Allen, a tributary of the River South Tyne. The area was mined for mineral ores in the nineteenth century and abandoned zinc-rich spoil heaps are a feature of the West Allen catchment. Abel and Green (1981) and Abel (1989) described much reduced abundance and diversity of invertebrates in the West Allen, where zinc concentrations were up to ten times greater than in the East Allen. They found, however, that other chemical variables, including pH and conductivity, were similar in both streams; potentially-toxic heavy metals other than zinc (Cu, Cd, Pb, Ni, Co) were detectable in neither stream.

Correspondence to: R. Goulder

MATERIALS AND METHODS

Sampling was done on three days in October-November 1992 at two matched riffle sites on the West and East Allen, about 5.5 km and 6.5 km downstream of the respective sources. These sites were designated by Abel (1989) as site 7 (National Grid Reference NY 801 487) and site VI (NY 848 488). At each site, replicate water samples for zinc analysis were collected in acid-washed high-density polyethelene bottles, and three small stones were collected into an acid-washed polythene bag for assay of biofilm zinc. Field pH and temperature were measured using a pHox System type 42D pH meter and a mercury-in-glass thermometer. A further water sample was taken for laboratory determination of conductivity using a Jenway model 4070 conductivity meter. Twenty small stones for enzyme assay and chlorophyll determination were collected into individual sterile polythene bags. Three further stones were collected into a sterile polythene bag for determination of metabolic activity and abundance of epilithic bacteria. All samples were packed in ice for transit.

Zinc was determined by differential-pulse anodic-stripping voltammetry (ASV), using a Princetown Applied Research polarographic analyser (model 364) and static-mercury-drop electrode (model 303). Procedure was based on the manufacturer's application briefs and on Hart and Davies (1978) and essentially followed Milner and Goulder (1984). In stream water, ASV-labile zinc (Hart and Davies 1978) was measured in samples buffered 3:1 v/v with pH 4.8 acetate buffer, without prior acid digestion. This fraction was likely to be of greater biological relevance than bound, non-reducible, zinc. The biofilm, on each set of three stones collected for zinc assay, was scrubbed into 300 mL of ultra-pure water using an acid-washed nylon tooth brush. Replicate sub-samples (1 mL) of this suspension were digested by evaporation to dryness at 50 ^{o}C with 2 mL of Aristar concentrated nitric acid. Total biofilm zinc was then determined after redissolving in 10 mL 3:1 v/v ultra-pure water:pH 4.8 buffer.

Three epilithic extracellular-enzyme systems were assayed (one per sampling day), i.e., phosphatase, ß-D-glucosidase, leucine aminopeptidase. Procedure was based on Chappell and Goulder (1992). Each stone was incubated in 100 mL of autoclaved stream water from the collection site plus 100 μmol/L of a model substrate which on enzymic hydrolysis yielded a fluorescent product. Substrates were 4-methylumbelliferyl (MUF) phosphate, MUF ß-D-glucopyranoside, and L-leucine 7-amido-4-methylcoumarin. Incubations were in an orbital incubator at 80 oscillations/min for 0.75-1.5 h in darkness at river temperature (7-8 ^{o}C). Post-incubation stream water was mixed 1:0.08 v/v with pH 10 BDH buffer, and fluorescence intensity was read at 455 nm, under excitation at 366 nm, using an EEL 244 fluorimeter. Allowance for non-enzymatic hydrolysis of substrates and/or fluorescent impurities was made by subtracting the mean fluorescence intensity of two control incubations from which stones were omitted. The fluorescent product (4-methylumbelliferone or 7-amino-4-methylcoumarin) was quantified using straight-line calibration graphs prepared from buffered standard solutions made up in autoclaved stream water.

To investigate epilithic bacteria, the biofilm was scrubbed from three stones, using a sterile tooth brush, and combined in 300 mL of sterile 0.2 μm-filtered stream water. The suspensions were treated in a stomacher (Colworth 400) for 5 min to ensure homogeneity. Metabolic activity, as V_{max} for glucose mineralization, was obtained by incubation of suspensions, diluted 10x with sterile stream water, at the presumed-saturation ^{14}C-glucose concentration of

Table 1. Comparison of environmental variables, epilithic extracellular-enzyme activity, and epilithic microbial variables in the Rivers West Allen and East Allen.

	West Allen	East Allen		
	Mean (range) CV	Mean (range) CV	n	P
Stream-environment variables				
ASV-labile zinc (mg/L)	1.6(0.80-2.6)45	0.11(0.09-0.12)13	6	**
Biofilm zinc ($\mu g/cm^2$)	41.9(28.0-51.2)21	5.75§(2.8-15.0)80	6	**
pH	7.7(7.68-7.72)0.3	7.78(7.76-7.8)0.3	3	-
Conductivity ($\mu S/cm$)	225(220-230)2	114(109-121)6	3	-
Temperature (^oC)	7.8(7.0-8.0)10	7.8(7.0-8.0)10	3	-
Stone size (cm^2)	45.4(28.0-65.1)21	45.1(23.2-77.2)23	60	NS
Epilithic extracellular-enzyme activity				
Phosphatase ($nmol/cm^2/h$)	4.88(0.89-10.8)59	2.99(1.03-7.06)65	20	*
ß-D-glucosidase ($nmol/cm^2/h$)	0.95(0.43-2.08)43	1.08(0.55-2.07)38	20	NS
Aminopeptidase ($nmol/cm^2/h$)	13.2(2.23-21.9)46	15.8(4.62-26.5)35	20	NS
Epilithic microbial variables				
V_{max} (ng glucose/cm^2/h)	7.8(6.1-9.2)20	1.2(0.5-2.2)67	3	-
Total bacteria ($x10^8/cm^2$)	2.0(1.2-3.0)38	1.2(0.8-1.8)36	6	*
Chlorophyll *a* ($\mu g/cm^2$)	1.65(0-5.20)73	1.42(0.22-4.16)62	60	NS

Between-stream statistical comparisons were made using the two-tailed Mann-Whitney U-test; **=P<0.01, *=P<0.05, NS=P>0.05; - indicates insufficient data for statistical analysis. CV=coefficient of variation (%), n=number of determinations: § indicates a mean for biofilm zinc calculated with an anomalous value of 15.0 included, with this value omitted results were 3.9 (2.8-5.3) 26.

210 $\mu g/L$. Five 10 mL replicates and two acidified blanks were incubated in darkness for 2 hr at river temperature. Incubations were stopped by acidification and $^{14}CO_2$ produced was captured, and assayed by liquid-scintillation counting. Procedure and calculation followed Goulder (1987). Total bacteria in replicate sub-samples of the suspensions were counted using epifluorescence microscopy following staining with acridine orange and concentration on 0.2 μm polycarbonate membranes (Goulder 1988).

The abundance of epilithic microalgae, as chlorophyll *a*, was measured on all the stones used for enzyme assay. After incubations, each stone was transferred to 100 mL of 95% ethanol. Chlorophyll was extracted by boiling for 2 min and was quantified spectrophotometrically (Jespersen and Christoffersen 1987). The surface area of stones was obtained by wrapping in aluminium foil of known weight:area ratio, trimming excess and weighing the foil.

RESULTS AND DISCUSSION

The results of analysis (Table 1) confirmed that zinc was substantially more abundant in the West Allen. ASV-labile zinc in the water (mean 1.6 mg/L) was more than ten times greater than in the East Allen, and was reasonably similar to the mean concentration of 1.3 mg/L obtained by AAS in filtered samples from

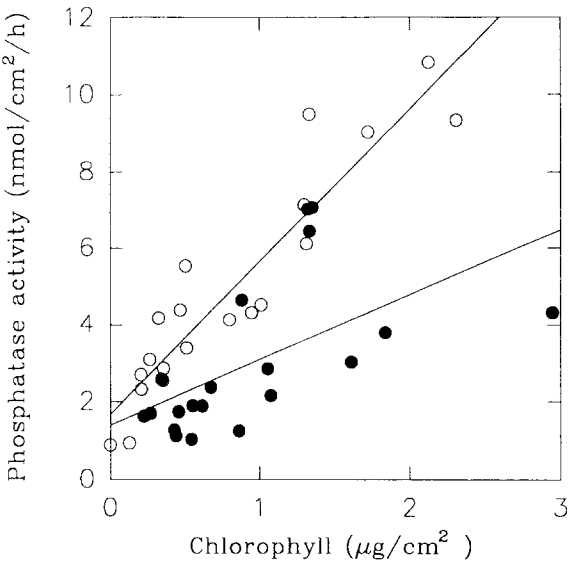

Figure 1. Relationship between epilithic phosphatase activity and algal chlorophyll *a* in the Rivers West Allen (o) and East Allen (•); each point represents one stone, n=20 stones for both streams but some points are obscured. Correlation coefficients were; West Allen 0.93 ($P<0.001$), East Allen 0.58 ($P<0.01$): regression coefficients (slope) were; West Allen 4.0, East Allen 1.7 nmol MUF-PO_4 hydrolysed/h/μg chlorophyll *a*.

the same site in 1979-80 (Abel 1989). Biofilm zinc on stone surfaces was also substantially higher in the West Allen. Values of pH were similar in both streams, also in agreement with (Abel 1989), but conductivities were somewhat higher in the West Allen. Water temperature, and mean size of stones used for enzyme assay, were virtually the same for both streams.

Despite high zinc levels, the epilithic extracellular-enzyme activity was not depressed in the West Allen (Table 1). Indeed, phosphatase activity was higher in the West Allen, while no significant between-stream difference was shown by ß-D-glucosidase or leucine aminopeptidase activity. Therefore, although extracellular-enzyme activity can be inhibited experimentally by addition of heavy metals to seawater (Vives-Rego et al. 1986) or to the products of bacterial culture (Bloquel 1989; Capalash et al. 1991), and immobilized enzymes have been suggested for use as indicators of pollution by metals in natural waters (Harrison and Flint 1987), there is no evidence that the zinc contamination of the West Allen was sufficient to suppress extracellular-enzyme activity.

The investigation of epilithic bacteria (Table 1) suggested that metabolic rate, as V_{max} for glucose mineralization, and abundance were both greater in the zinc-contaminated West Allen. Evidently, the epilithic bacterial community was well acclimatized to elevated zinc levels; the higher metabolism and abundance may have been related to lower invertebrate grazing pressure. Abel (1989) suggested that high zinc levels in the West Allen might inhibit detritus processing by decomposer microorganisms and so result in lack of food for, and depletion of, the invertebrate fauna; our results do not support that hypothesis.

Epilithic algae, as chlorophyll *a* density, were equally abundant in both streams (Table 1). Production of extracellular phosphatase in natural waters is frequently related to microalgae, unlike ß-D-glucosidase and leucine aminopeptidase which are usually associated with bacteria (Chróst 1991). A significant relationship between epilithic phosphatase activity and chlorophyll was found in both streams (Fig.1). This suggests that epilithic algae were a major source of phosphatase. The markedly greater regression coefficient for the West Allen shows that phosphatase activity per unit algal biomass was enhanced in the zinc-contaminated stream. Both plots in Fig.1 have similar intercept values (West Allen 1.7, East Allen 1.4 nmol/cm^2/h of phosphatase activity). This suggests that non-algal phosphatase activity was similar in both streams.

The work reported here was carried out in October-November 1992, following heavy autumn rainfall and high flows which will have dislodged algal material from stones. A very different situation was observed during a preliminary site visit in July 1992. There was then nothing distinctive in the appearance of stones in the East Allen, whereas stones in the West Allen had a conspicuous bright-green, loosely-attached algal layer, mostly 2-3 mm thick but up to 1 cm thick in places. This luxuriant algal biomass may have been related to lack of invertebrate grazing. Turning of a few stones revealed no invertebrates in the West Allen, in contrast to the East Allen where mayfly (Ephemeroptera) and caddis (Trichoptera) larvae were abundant. Microscopic examination of the algal layer showed it to be mostly filamentous chlorophycean algae, including much *Stigeoclonium*. Clearly, the excess of epilithic phosphatase activity in the zinc-contaminated West Allen (Table 1) might well have been even more pronounced had the work been done earlier in the year.

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