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Increase in Epiphytic Bacteria Downstream of a Sewage Works Outfall: Monitoring Using Artificial Hydrophytes

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Epiphytic bacteria on submerged freshwater macrophytes may be quantified by direct microscopic counting of *in situ,* phenolic-aniline-blue stained, bacteria (Hossell and Baker 1979). Thus, Kang and Goulder (1996) used *in situ* counting to demonstrate increased abundance of bacteria, per unit leaf area, on water starwort, *Callitriche* L. sp., and Canadian pondweed, *Elodea canadensis* Michx., downstream of sewage works outfalls. Such increases in epiphytic bacteria are potentially important in promoting biopurification of organically-enriched river waters. *In situ* counting is, however, very labor-intensive. An alternative, more economic, approach to the quantification of epiphytic bacteria is the preparation of a suspension of epiphyton by treatment of the host plant in a stomacher, followed by direct counting of bacteria in the suspension by epifluorescence microscopy (Rimes and Goulder 1986). The plant material is dried and weighed after stomaching, and results are expressed as bacteria per unit dry weight of host macrophyte. Interpretation of results is, however, complicated by the fact of substantial within-species and between-species variation in the surface area:dry weight ratio of submerged macrophytes (Fry and Humphrey 1978). This complication might potentially be overcome by the use of artificial plants which have uniform surface area, and hence readily allow determination of abundance on an areal basis. In the work described here, we investigated the effect of a sewage works outfall on density of epiphytic bacteria, assayed by direct epifluorescence counting of bacteria in epiphyte suspensions produced by stomaching of both real plants *(Callitriche* and *E. canadensis)* and artificial plants. Since pennate diatoms, principally *Cocconeis* Ehr. sp., were also a substantial component of the epiphyton, these too were counted on the artificial plants.

The watercourse studied was the Driffield Canal, a disused navigation channel in East Yorkshire, Northeast England. The canal is fed at its head by clean, calcareous, stream water, and flows slowly (modal flow 0.5 cu m/s) for 8 km before joining the Frodingham Beck, and then the River Hull after a further 1 km. Aquatic macrophytes are abundant but only *Callitriche* and *E.*

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canadensis occur throughout. The canal receives effluent from a sewage works, 0.6 km downstream of the canal head, at National Grid Reference TA 032 568. The works serves a population of 10,700 people, has conventional percolating biological filters, and a mean daily flow of 9,850 cu m. Kang and Goulder (1996) showed that the effluent caused appreciable enrichment of the canal water. Thus, in September-November 1993, comparison of samples from up to 10 km downstream of the outfall, with samples from 0.5 km and 0.25 km upstream, showed significant increase in conductivity, turbidity, BOD, phosphate concentration, algal-growth potential by *Selenastrum* bioassay, and abundance of bacterioplankton.

MATERIALS AND METHODS

Submerged plants of *Callitriche* and *E. canadensis* were collected using a pole and hook, and were manipulated in the field using forceps and scissors. Shoots were cut at 5 cm behind the apex and transferred, five shoots per bag, to sterile polythene stomacher bags. Artificial aquarium plants, a green plastic imitation of water-weed, *Egeria densa* Planchon (from Rolf C. Hagen Ltd, Castleford, UK), chosen as the closest available to a replica of *E. canadensis,* were weighted and submerged in depths of 1-1.5 m, at two matched sites both immediately upstream of disused lock gates. On retrieval, after 35 d or 49 d submergence, 10-12 cm lengths of plastic shoot were cut and transferred, one per bag, to sterile stomacher bags. All samples were packed in ice shortly after collection.

Epiphyte suspensions were prepared by addition of 50 mL of 0.2 µm-filtered pure water to the stomacher bags, followed by 5 min treatment in a Colworth stomacher-400 (A.J. Seward Ltd, London) (Fry *et al*. 1985). The suspensions prepared from real shoots were then strained through sterile 400 µm nylon mesh to remove plant fragments. Sub-samples of epiphyte suspensions (10 mL) were preserved for up to 2 wk with 2% formaldehyde.

To determine dry weight of *Callitriche* and *E. canadensis* shoots, the fragments retained by the mesh, and residue in the stomacher bags, were dried at 80 °C and weighed. Allowance for soluble plant fractions, and small fragments which passed the mesh, was made by evaporating 30 mL of the epiphyte suspension to dryness and weighing the residue. The surface area of artificial shoots was obtained from area determination of representative leaves and stem lengths, made using a leaf-area meter (model LI-3000, Lambda Instruments Corporation, Lincoln, Nebraska) and a digital electronic caliper (RS Components Ltd, Corby, UK), respectively.

The concentration of epiphytic bacteria in the suspensions was determined by the acridine-orange direct-count method (Daley 1979). Bacteria were stained with acridine orange (10 mg/L, 10 min), concentrated on black, 0.2 µm pore-size, polycarbonate membrane filters, and counted at x1250 magnification using a Nikon, Alphaphot epifluorescence microscope. At least 400 cells per preparation were counted; 95% confidence intervals were <±10%.

Table 1. Density of epiphytic bacteria on *Callitriche* sp. and *Elodea canadensis* collected from the Driffield Canal at sites upstream and downstream of the sewage works outfall

	Density of epiphytic bacteria($x10^{10}$/g)	
	Callitriche	*E. canadensis*
Distance from outfall (km)	Mean(Range)CV	Mean(Range)CV
Upstream sites		
0.5	**1.5(1.1-1.8)23**	**1.9(0.5-4.2)103**
0.25	**2.3(1.7–3.0)27**	**0.8(0.2-1.3)75**
Downstream sites		
1.6	**2.3(1.5-2.9)31**	**1.2(0.2-1.7)71**
2.1	**1.6(0.8-2.2)46**	**0.7(0.3-0.9)52**
3.4	**2.7(2.5-3.0)12**	**1.2(1.0-1.5)24**
6.4	**2.4(1.5-3.4)40**	**1.0(0.6-1.5)50**
10.0	**1.8(1.0-2.2)32**	**1.4(0.9-1.7)25**

Values are per unit dry weight of host macrophyte; CV=Coeficient of variation(%), n=3 or 4 samples. Each sample comprised an epiphyte suspension prepared by treating five shoots in a stomacher. There was no significant difference between bacterial density on upstream and downstream shoots of either *Callitriche* or *E. canadensis;* two-tailed Mann-Whitney U-test, P>0.05, n_1 (upstream samples)=6, n_2 (downstream samples)=16. Shoots were collected 27 September to 10 November 1993.

Pennate diatoms, in the suspensions obtained by stomaching of artificial shoots, were counted in a Fuchs-Rosenthal haemacytometer, using bright-field microscopy at x100 magnification. A mean of about 75 diatoms per preparation was counted; 95% confidence intervals were mostly <±25%. Abundance of bacteria on real shoots was expressed per unit dry weight; values for artificial shoots were calculated per unit surface area.

RESULTS AND DISCUSSION

Densities of epiphytic bacteria on shoots of *Callitriche* and *E. canadensis,* collected September-November 1993, at sites from 0.5 km upstream to 10 km downstream of the sewage works outfall, are summarized in Table 1. These values were potentially underestimates because treatment in a stomacher does not release all epiphytic bacteria; e.g. Rimes and Goulder (1986) obtained a mean release efficiency of 62% from watercress, *Nasturtium officinale* R. Br., and fool's watercress, *Apium nodiflorum* (L.) Lag. There was apparently considerable within-site and between-site variation along the canal. Ranges were from 0.8-3.4 x 10^{10}/g dry wt on *Callitriche* and 0.2-4.2 x 10^{10}/g dry wt on *E. canadensis.* The density of epiphytic bacteria on both species showed no significant increase downstream of the outfall (P>0.05; Mann-Whitney U-test).

Table 2. Density of epiphytic bacteria and pennate diatoms on artificial plants which were submerged in the Driffield Canal upstream and downstream of the sewage works outfall

	Distance **from outfall**		
	0.1 km upstream	**2.1 km downstream**	
Duration of submergence	**Mean(Range)CV**	**Mean(Range)CV**	**P**
Baciteria(x10⁷/cm²)			
35 **days**	**1.7(1.5-1.9)10**	**10.1(8.1–12.3)13**	**<0.01**
49 days	**1.8(1.6-2.0)7**	**11.5(9.4-13.3)11**	**<0.01**
Pennate diatomsx10⁴/cm²)			
35 **days**	**2.4(1.2-3.5)39**	**2.1(1.2-4.0)41**	**NS**
49 days	**3.0(0.8-7.6)71**	**2.2(0.7-3.9)48**	**NS**

Values are per unit surface area of artificial plant; CV=Coeficient of variation(%), n=8 or 10 samples. Each sample comprised an epiphyte suspension prepared by treating a single plastic shoot in a stomacher. Comparisons between sites were made using the two-tailed Mann-Whitney U-test; NS=P>0.05. The plastic plants were put into the canal on 8 December 1993.

The apparent lack of downstream increase in epiphytic bacteria, per unit dry weight of host plant, suggested that the epiphytic bacterial population did not respond to enrichment. However, Kang and Goulder (1996) used *in situ* counting to show downstream increase in density of bacteria per unit leaf area of *Callitriche* and *E. canadensis.* Thus, the apparent lack of response to enrichment, and the irregular within-site variation of bacteria per unit dry weight, may have been caused by variation in surface area:dry weight ratio, related to variation in plant morphology. This suggestion was supported by the results from artificial plants (Table 2). The abundance of bacteria on plastic plants was much greater downstream of the outfall. The density of pennate diatoms, in contrast, was not significantly different downstream of the outfall; this observation agreed with the *in situ* recording of diatoms by Kang and Goulder (1996) which showed no consistent downstream increase in the percentage of leaf surface covered by diatoms.

The data in Table 2 also show that the population increase over the first 35 days was rapid but that there was very little further increase between days 35 to 49. Hence, steady-state bacterial densities, which represented between-site differences, had probably been achieved.

Artificial plants have frequently been used in studies of the nutrient requirements of epiphytic algae in fresh waters (e.g. Cattaneo and Kalff 1979; Fontaine and Nigh 1983; Morin 1986). The present study showed that they may potentially be used to

demonstrate and monitor the effects of sewage works effluent on abundance of epiphytic bacteria and diatoms.

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