

Temporal and Geographical Distributions of Epilithic Sodium Dodecyl Sulfate-Degrading Bacteria in a Polluted South Wales River

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Received 17 July 1987/Accepted 11 November 1987

Epilithic bacteria were isolated nonselectively from riverbed stones and examined by gel zymography for their ability to produce alkylsulfatase (AS) enzymes and thus to metabolize alkyl sulfate surfactants such as sodium dodecyl sulfate. The percentages of AS⁺ isolates from stone epilithon at five sites from the source to the river mouth were measured on five sampling days spread over 1 year. The results showed that (i) the prevalence of epilithic AS⁺ strains (as a percentage of all isolates) was much higher at polluted sites than at the source; (ii) when averaged over the whole river, percentages of AS⁺ strains were significantly higher at the end of summer compared with either the preceding or the following winter; (iii) analysis of site-sampling time interactions indicated that water quality factors (e.g., biochemical oxygen demand and dissolved oxygen concentration) rather than climatic factors determined the distributions of epilithic AS⁺ isolates; (iv) constitutive strains were the most prevalent (7.2% of all isolates), with smaller numbers of isolates with inducible (4.5%) and repressible (1.7%) enzymes.

Use of detergents containing synthetic surfactants that commonly possess strongly anionic groups such as sulfonate (C-SO₃⁻) or ester sulfate (C-O-SO₃⁻) has increased dramatically, in terms of volume and range of applications (12, 15), since their introduction on a commercial scale over 40 years ago. Early problems with foaming in sewage works and receiving waters led to a parallel growth in studies of microbial degradation of surfactants. Such studies fall into two broad types. First, biodegradability of pure or mixed (commercial) surfactants by mixed microbial flora from river water or sewage has been assessed by a variety of methods such as Organisation for Economic Cooperation and Development screen, river water die-away, and Sturm mineralization tests (8, 9, 21). A second, more fundamental, approach has been the identification of metabolic intermediates and pathways in single species growing in pure culture, with the purification and characterization of the relevant enzymes including the physiological control of their synthesis (3, 5, 6, 21).

Although these studies make a valuable contribution in assessing environmental acceptability of various detergent formulations, they leave unanswered a third aspect pertinent to such assessments, namely, the abundance and distribution of surfactant-degrading bacteria in receiving-water ecosystems and the factors that affect the distribution. Several isolates able to achieve primary biodegradation of linear alkyl sulfates (e.g., sodium dodecyl sulfate [SDS]) have been described (2), and the ability to degrade primary alkyl sulfates is common among pseudomonads (5, 6, 13, 20). Alkyl sulfate degradation is initiated by alkylsulfatase enzymes which remove inorganic sulfate from the substrate to give an alcohol that is readily assimilated by central metabolic pathways present in bacteria. Therefore, the presence of SDS-degrading alkylsulfatase enzymes is synonymous with the ability to accomplish complete mineralization of SDS.

A preliminary study from our laboratory (22) showed that alkylsulfatases were widespread among mixed epilithic-

planktonic bacterial samples from a polluted river and that the number of strains producing the enzymes (AS⁺) was significantly higher at a polluted site than at the source, both in absolute terms and as a proportion of all isolates.

The present work extends that pilot study to an analysis of the distribution of SDS-degrading bacteria in epilithic samples at five sites along the river course and five sampling times spread over a 12-month period.

MATERIALS AND METHODS

Materials. Bacto-Peptone, Casamino Acids, and Bacto-Agar were obtained from Difco Laboratories (Detroit, Mich.). Lysozyme was purchased from Sigma (London) Chemical Co. (Poole, United Kingdom). All other reagents were supplied by BDH (Poole, United Kingdom). Growth medium and diluents for serial dilutions were sterilized by autoclaving (121°C and 101 kPa for 15 min).

Isolation of bacteria: Samples were taken from the River Ely, which flows for 40 km through South Glamorgan into the Bristol Channel at Cardiff, Wales. The sample sites A through E were 0.7, 8.6, 15.9, 26.7, and 36.8 km from the source, respectively. The water quality ranged from clean water at the source (site A, which is chemical class 1, unpolluted [4]), through heavily polluted downstream sites (B, C, and D, chemical class 4, polluted) receiving various industrial and treated sewage effluents, to the estuary (site E). A flat stone (approximately 10-cm diameter) was taken from the riverbed at each of the five sites and returned to the laboratory. Epilithon, the slimy matrix containing a mixed population of bacteria and algae growing on submerged surfaces, was removed from each stone by scrubbing with a sterile toothbrush in sterile distilled water. Surface areas of stones were estimated by making tracings of each flat surface on good quality paper and comparing their weights with the weight of a known area. The volume of scrubblings was brought to 100 ml with sterile distilled water, and particulate material was dispersed by agitation in a stomacher for 5 min. Samples of stone scrubblings were diluted serially (10⁻¹ to 10⁻⁶) in diluent medium containing yeast extract, Bacto-Peptone and Casamino Acids, each 0.05% (wt/vol);

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TABLE 1. Three-way ANOVA 1 of AS⁺ prevalence for the factors sample site, sample number (time), and composition of growth medium (\pm SDS)

Factor and interaction	df	Mean square	F	Significance level (P)
Main effects				
Site	4	0.152	50.7	<0.001
Sample number (time)	4	0.003	11.0	<0.001
Medium (\pm SDS)	1	0.010	3.3	0.09
Interactions				
Two-way				
Site-time	16	0.060	20.0	<0.001
Site-medium	4	0.001	0.3	>0.75
Medium-time	4	0.001	0.3	>0.75
Three-way (site-medium-time)^a				
	16	0.003		

^a In the absence of replicate samples for each site on each occasion, the three-way interaction was used as the residual variance.

K₂HPO₄, 0.02% (wt/vol); and MgSO₄, 0.005% (wt/vol). Samples (0.1 ml) of each dilution were plated in triplicate on medium M, a nonselective medium based on casein-peptone-starch (10) and containing 2% (wt/vol) glycerol (1). Plates were incubated aerobically at 15°C, and colony counts were made after 7 days. For each sample, approximately 100 colonies were selected randomly and transferred to axenic culture on fresh plates of medium M. Lawns of each isolate were made on further plates of medium M and medium M supplemented with 0.03% (wt/vol) SDS (filter sterilized and added aseptically). Plates were incubated as above for 7 to 14 days, after which time the confluent bacterial growth provided sufficient material for the subsequent zymographic analysis. Approximately 500 isolates were tested (five sites, each yielding 100 epilithic isolates), with each isolate grown in the absence and presence of SDS. The sampling was repeated on five occasions spread over a 1-year period (sample 1, 22 January 1985; sample 2, 11 April 1985; sample 3, 3 July 1985; sample 4, 21 November 1985; sample 5, 3 February 1986).

Preparation of cell extracts and gel zymography. Bacteria were harvested with sterile cotton buds and suspended by vortexing the cotton bud in 0.4 ml of a solution containing 0.1 M phosphate (pH 7.5), 0.58 M (20%, wt/vol) sucrose, and 0.01 M EDTA. The bud was removed and stored frozen in 20% (vol/vol) glycerol as a stock culture. The suspended cells were lysed by adding 4 mg of lysozyme in 0.1 ml of the phosphate-EDTA-sucrose buffer and incubating for 60 min at 37°C. Triton X-100 (100 μ l of a 10% [vol/vol] solution) was added to complete lysis of inner membranes. Bromophenol blue tracker dye (20 μ l of 0.04% [wt/vol] in 50% [vol/vol] glycerol) was added to the lysate, and a sample (100 μ l) was subjected to vertical slab polyacrylamide gel electrophoresis based on methods described previously (19, 22). Gel zymography was used to detect alkylsulfatase enzymes because large numbers of samples can be examined conveniently and constitutive-inducible enzymes and multiple enzymes can be distinguished easily. Gels were stained by incubating them in 10 mM SDS in 50 mM Tris hydrochloride (pH 7.5), whereupon enzyme activity was revealed by the appearance of white bands of the insoluble dodecanol product of alkylsulfatase action. For each stone sample, the number of alkylsulfatase-producing (AS⁺) strains was expressed as a fraction of the total number of isolates screened for the AS⁺ phenotype.

Statistical analysis. To identify the factors affecting the

prevalence of AS⁺ strains and any interaction between them, we analyzed the data by a three-way factorial analysis of variance (ANOVA). Factors examined were sampling site, sampling time, and composition of the growth medium (i.e., \pm SDS). Raw data (proportions of AS⁺ isolates in each epilithic sample) were transformed ($\arcsin \times \sqrt{\text{proportion}}$) to weight the variances. Computation was performed by using the SPSS-X package on a Vax mainframe computer. When appropriate, 95% confidence limits for mean values were estimated assuming a normal distribution.

RESULTS AND DISCUSSION

ANOVA for site, time, and medium (\pm SDS) effects (ANOVA 1). The results of the three-way ANOVA for epilithic isolates are shown in Table 1. An effect or interaction was regarded as significant at the 95% level of confidence. The analysis indicates that the proportions of alkylsulfatase-positive (AS⁺) isolates varied significantly with site and sampling time, but were not significantly affected by SDS in the growth medium. There was a significant interaction between site and time, but the site-medium and time-medium interactions were not significant.

Geographical distribution. The variation in prevalence of AS⁺ isolates with sample site (A to E), averaged over sampling times 1 to 5 and over values obtained after growth in the presence and absence of SDS, is shown in Fig. 1a. Proportions of AS⁺ isolates increased from consistently low values (<2%) at the source to much higher values (>10%) at polluted sites downstream. This was true irrespective of SDS supplements in the growth medium, as shown by the insignificant site-medium interaction in ANOVA 1 (Table 1).

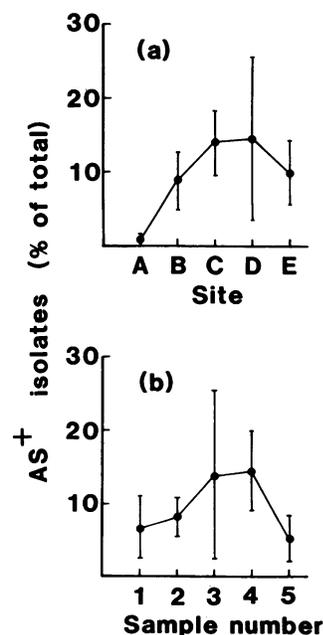


FIG. 1. Mean temporal and geographical distributions of epilithic AS⁺ isolates in the River Ely. Prevalence of AS⁺ isolates is shown at various sites, averaged over 1 year (a), and at different times of the year, averaged over all sites (b). Each point is the mean of 10 values (five samplings in 1 year for each site in panel a, and five sites for each sampling time in panel b; each isolate was analyzed after growth in the presence and absence of SDS). Bars represent 95% confidence limits. See the text for further details.

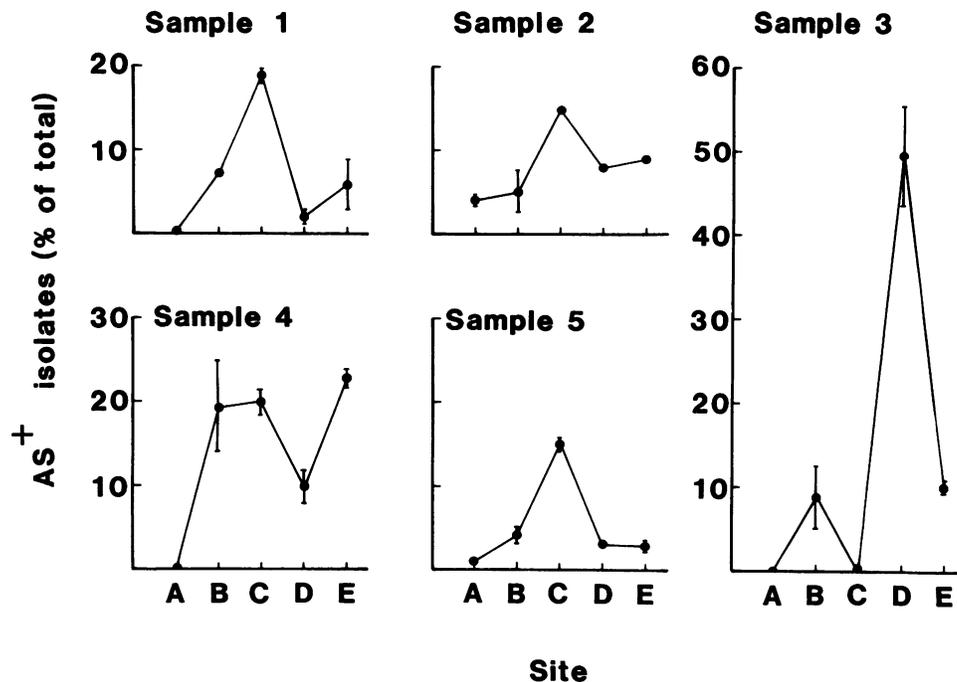


FIG. 2. Geographical distribution of epilithic AS^+ isolates at different times of the year. Each point is the mean from experiments with and without SDS in the growth media, and the bars indicate the range of the two values; where no bars are shown, the values were the same. See text for details.

Plate counts indicated that cell densities (expressed as CFU per square centimeter) were much higher in the polluted sites (10^6 to 10^8 CFU/cm² at sites B, C, and D) than at the source (10^5 to 10^6 CFU/cm²). These values are typical of those observed elsewhere for fresh river water epilithon (7, 11, 14, 16, 18). Clearly, AS^+ isolates are more abundant in the middle reaches of the river, both in percentage terms and also when expressed as AS^+ cells per cm². These sites (B to D) correspond to the most polluted reaches in the river, as shown by earlier surveys (4). Moreover, using more recent data supplied by the Welsh Water Authority, we showed that the site variation of percentage of AS^+ isolates correlates positively with summer biochemical oxygen demand ($r = 0.94$, $P < 0.02$) and winter biochemical oxygen demand ($r = 0.89$, $P < 0.05$) and negatively with dissolved oxygen concentrations in summer ($r = -0.91$, $P < 0.05$) and winter ($r = -0.91$, $P < 0.05$).

In the estuarine samples (site E), proportions of AS^+ isolates were high but total numbers of isolates were low (ca. 10^4 to 10^5 CFU/cm²), so that cell densities for AS^+ isolates were low compared with those of polluted sites and similar to those of the source. It should be noted, however, that isolations were made on a low-salinity medium. Isolations with saline medium may have resulted in higher recoveries, but it is impossible to predict what proportion of these would be AS^+ . Further work is in progress to establish the influence of salinity on the relative distributions of AS^+ and AS^- strains.

Temporal variation. Proportions of AS^+ isolates, averaged over all sites, varied significantly with sampling time, increasing during the summer months and decreasing in winter (Fig. 1b). The presence of SDS in the growth medium did not affect this pattern (Table 1). Percentages of AS^+ isolates in midwinter (samples 1 and 5) were very similar to each other ($P > 0.5$) but significantly less than the end-of-summer

sample 4 ($P = 0.05$ and $P < 0.02$, respectively). Differences between means for other sampling times were not significant ($P > 0.05$). Although the present data show the broad summer-winter variation, they do not reveal any other shorter time scale variations that may occur. Such an analysis would require a more intensive sampling regimen than was possible in the present program.

Interaction of geographical and temporal factors. ANOVA 1 showed a highly significant interaction between site and time. The geographical distributions observed for epilithic AS^+ isolates at different sampling times are shown in Fig. 2. Some features were constant throughout the year. For example, the lowest value always occurred at site A, and on four of five samplings (Fig. 2, samples 1, 2, 4, and 5), there was a maximum at site C. It is also worth noting that the winter samplings in January 1985 (sample 1) and February 1986 (sample 5) produced almost identical site distributions. However, features giving rise to the significant site-time interaction, indicated by the statistical analysis, were also clearly evident. The July sampling (sample 3) appeared to be particularly unusual in that site C was devoid of AS^+ strains, whereas site D was very rich (50% of isolates were AS^+). However, when the July sampling was omitted from the ANOVA analysis, the pattern of significance (i.e., significant effects of site and time and a significant interaction between them) remained unchanged. Clearly, the significant sampling time effect did not arise solely from the apparently anomalous July sampling.

Further insight into the site-sampling time interaction was gained from a consideration of the temporal variation at each site (Fig. 3). The proportions of AS^+ isolates were considerably more time dependent at sites C and D than at the upstream sites A and B. This might indicate that the temporal variation was not directly dependent on climatic factors, which would perhaps be expected to affect all sites equally.

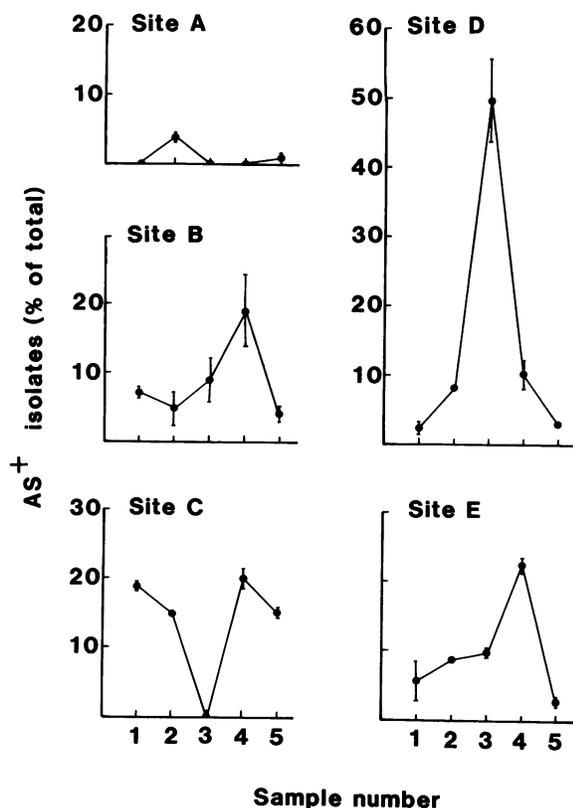


FIG. 3. Temporal variation of AS⁺ prevalence at each site. Each point is the mean from experiments with and without SDS in the growth media, and the bars indicate the range of the two values; where no bars are shown, the values were the same. See text for details.

It seemed more likely that prevalence of AS⁺ strains depended on local water quality factors (e.g., biochemical oxygen demand, oxygen concentration, see above) which, while being time dependent at a given site, would show different time dependencies at different sites. Detailed analysis of site-sampling time interactions for these and other water quality factors, together with correlation with percent AS⁺ distributions, will be published elsewhere. Meanwhile, other studies have shown that the abundance of sessile bacteria is notably higher below sewage plant discharges (18) and that there is a highly significant positive relationship between abundance of sessile bacteria and typical effluent constituents. Moreover, sulfate and nitrate reducers and strains able to utilize pollutants such as hydrocarbons and phenols are more abundant below municipal discharges than above (17).

Absence of medium (\pm SDS) effects. The results of ANOVA 1 show that the media, with or without SDS, exert no selective pressure on the number of AS⁺ isolates obtained. Two factors may account for this observation. First, the alkylsulfatase enzymes present may be predominantly constitutive, so that the presence or absence of SDS does not affect their production. However, previous experience with isolates studied in detail in Cardiff over the past 15 years has shown that many alkylsulfatases in soil bacteria are inducible (6); dominance by constitutives is therefore probably not the only factor involved. Second, some enzymes observed in the present study were repressible by the addition of SDS, i.e., zymogram bands were present in cells grown on me-

dium M alone but absent for the same isolate grown on medium M containing SDS. Such repression by readily assimilable carbon sources including dodecanol has been observed previously for the P1 SDS-sulfatase in *Pseudomonas* strain C12B (T. J. Bateman, Ph.D. thesis, University of Wales, Cardiff, 1985). In the present study, the disappearance of the repressible enzymes could compensate for the appearance of inducible enzymes. To resolve this problem, we performed a second ANOVA in which the AS⁺ isolates were subclassified as constitutive, inducible, or repressible.

ANOVA for site-time-enzyme type (ANOVA 2). A three-way ANOVA involving the factors site, time, and enzyme type (constitutive, appearing on both medium M and M plus SDS; inducible, appearing on M plus SDS only; and repressible, appearing on M but not M plus SDS) was performed on epilithic data as before (Table 2).

Site and enzyme type, but not time, were significant main effects. However, it should be noted that it is difficult to interpret meaningfully the site and time effects, because these particular data are generated by averaging three types of enzyme regulation. The overall (site- and time-averaged) proportions for each enzyme type were as follows: constitutive, $7.2 \pm 3.2\%$ ($P = 0.05$); inducible, $4.5 \pm 1.7\%$; and repressible, $1.7 \pm 0.8\%$. Clearly, the constitutive enzymes were quantitatively the most important, but together the inducible plus repressible enzymes represent an almost equivalent proportion. The site distributions averaged over the five sampling times are shown separately for each enzyme type in Fig. 4a to c. Similarity of the trend within each enzyme type is responsible for the insignificant interaction ($P > 0.5$; Table 2). Inducible strains were absent from the source but present in roughly similar proportions (5 to 6%) at downstream sites. Similarly, repressible strains were absent from the source but were found in constant proportions downstream (1 to 4%). Thus, the absence of an effect of SDS in ANOVA 1 arose, at least partly, from the similar distributions of inducible and repressible AS⁺ isolates; presence of the surfactant would eliminate the repressibles but give rise to a roughly parallel distribution of inducible isolates. The overall excess of inducibles over repressibles was only 2% of all isolates, and because constitutives accounted for 7% of all isolates, the addition of SDS had only a small effect on the overall pattern. This conclusion is supported by the almost identical geographical distributions for all isolates (Fig. 1a) compared with the constitutive isolates only (Fig. 4a). Moreover, it now emerges that the

TABLE 2. Three-way ANOVA 2 of AS⁺ prevalence for the factors sample site, sample number (time), and enzyme regulation (constitutive, inducible, or repressible)

Factor and interaction	df	Mean square	F	Significance level (P)
Main effects				
Site	4	0.091	11.38	<0.001
Sample number (time)	4	0.012	1.50	<0.25
Enzyme regulation	2	0.105	13.13	<0.001
Interactions				
Two-way				
Site-time	16	0.033	4.13	0.001
Site-enzyme	8	0.006	0.75	>0.5
Enzyme-time	8	0.020	2.50	0.025
Three-way (site-enzyme-time) ^a	32	0.008		

^a In the absence of replicate samples for each site on each occasion, the three-way interaction was used as the residual variance.

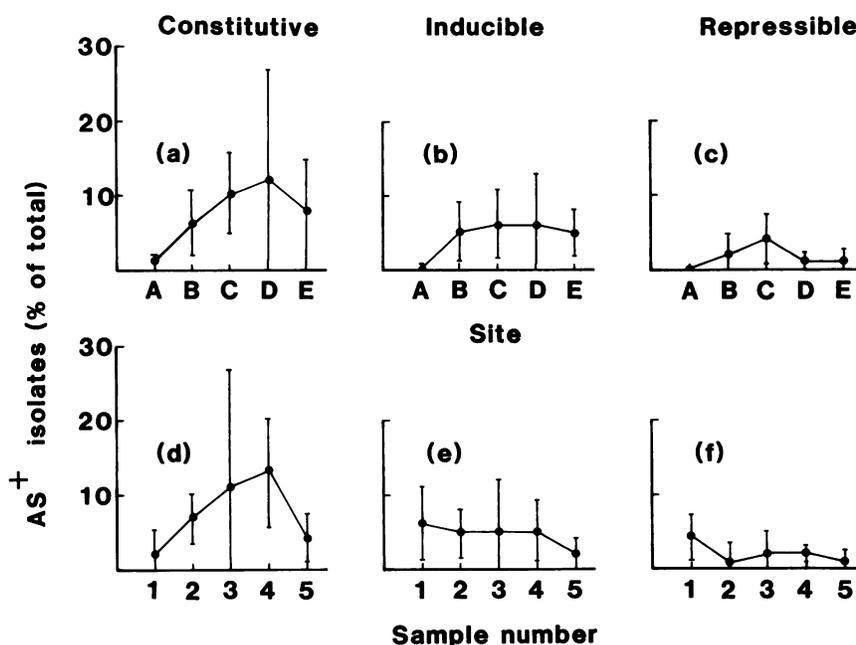


FIG. 4. Distributions of isolates containing constitutive (a and d), inducible (b and e), and repressible (c and f) alkylsulfatases. (a to c) Prevalence of AS⁺ isolates at various sites; each point is the mean of five values obtained over 1 year. (d to f) Prevalence of AS⁺ isolates at different times of the year; each point is the mean of values from the five sites. Bars represent 95% confidence limits. See the text for further details.

overall variability at site D evident in Fig. 1a is attributable largely to variation in constitutive isolates (Fig. 4a) rather than to isolates with inducible (Fig. 4b) or repressible (Fig. 4c) enzymes.

In contrast, there was a significant time-enzyme type interaction in ANOVA 2 ($P = 0.025$; Table 2). The percentage of isolates with constitutive enzymes (Fig. 4d) increased during the summer months, whereas isolates with inducible (Fig. 4e) and repressible (Fig. 4f) enzymes showed less temporal variation. As before, however, partial mutual compensation of inducible and repressible isolates together with abundant constitutive isolates gave rise to the apparent insensitivity of the overall distribution to the effects of SDS ($P = 0.09$; Table 1). The overall temporal distribution in Fig. 1b is again very similar to the constitutive pattern (Fig. 4d), and when wide variability occurs (e.g., in sample 3, Fig. 1b), it is attributable once again to constitutive strains.

The factors site and sampling time will themselves embrace numerous parameters that may affect AS⁺ distributions (e.g., water temperature, flow rate, discharges). Consequently, it is not surprising that in several instances in Fig. 1 and 3 the confidence limits are wide and often overlap. Nevertheless, several significant points emerged. First, the number of epilithic AS⁺ bacteria (expressed either in absolute numbers or as a percentage) is lower at the source than at downstream sites, and this correlated with biochemical oxygen demand and dissolved oxygen concentrations. Second, the percentage of AS⁺ isolates averaged over all sites is significantly higher at the end-of-summer sampling compared with either the previous or following winter samples. Third, the present work confirmed our earlier conclusion that the greater abundance of epilithic AS⁺ isolates in polluted downstream sites compared with the clean source site is due mainly to greater numbers of constitutive isolates. Furthermore, variations in AS⁺ prevalence with site and sampling time are dominated by changes in the abundance of

constitutive isolates rather than of those with inducible or repressible enzymes. This means that even if the SDS concentration in the river does fluctuate, the epilithon does not lose its capacity to degrade SDS. In this regard, the SDS-degrading capacity of the River Ely epilithon conforms to the conclusion of Kaplan and Bott (11) that to utilize pulses of carbon and energy, epilithic heterotrophs must already be enzymatically prepared when the pulse occurs.

ACKNOWLEDGMENTS

We thank T. Iles and M. Bale for assistance with the statistical analysis and D. Williams (Welsh Water Authority) for providing water quality data for the River Ely. We thank the Natural Environment Research Council for financial support.

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