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EXPERIMENTAL SYSTEMS FOR THE STUDY OF BACTERIAL DEGRADATION OF POLLUTANTS FROM THE OIL INDUSTRY

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ABSTRACT - The release of pollutants into the environment, either during normal operations or due to accidental discharges, has focussed attention on the fate and effects of xenobiotic compounds in the marine environment. This interest has been heightened by the development of the off-shore industry with the subsequent discharge of biocides from platforms. This paper describes the results obtained from investigations into the fate of biocides in the marine environment using continuous-culture techniques.

Key words : biodegradation, continuous-culture, biocides.

RÉSUMÉ : La décharge de polluants, qu'elle soit chronique ou relative à des rejets accidentels, a attiré l'attention sur le devenir et les effets des composés xenobiotiques dans le milieu marin. Cet intérêt a été croissant avec le développement de l'industrie off-shore et les rejets de biocides à partir des plateformes qui ont suivi. Ce papier décrit les résultats obtenus lors de travaux menés en milieu marin sur le devenir de biocides et utilisant les techniques de cultures en continu.

Mots clés : biodégradation, culture en continu, biocides.

INTRODUCTION

An increasing number of pollutants are released into the environment by industry either through normal operations or as a result of accidental discharges. The development of the off-shore industry with the subsequent discharge of a number of oil based pollutants and, in particular, biocides from platforms has heightened interest in the environmental impact of these compounds and their subsequent transformation or degradation.
Substantial quantities of biocides find their way into the marine environment as a result of off-shore operations. Biocides are used extensively by the oil industry in two main areas ; to inhibit biological fouling of storage tanks, pipelines and off-shore structures, secondly, to inhibit microbial growth in injection water during secondary oil recovery (Crouch 1982, Wilkinson 1982).
In recent years, the fate of examples of biocides have been investigated in our laboratory using continuous-culture systems.
The rationale behind this approach is that while direct plating methods and batch culture enrichments are obviously useful, they each have drawbacks for this kind of investigation. Where the microbial population is sparse and rates of activity low, such techniques are useful to give an indication of the number of organisms present, however, one cannot be sure that the population isolated is truly representative of the natural autochthonous population. Similarly, experiments carried out under batch culture condi-

tions can be misleading in that a succession of predominant organisms will occur as the conditions change during the culture. These disadvantages have been discussed by Jannasch (1967).

A continuous culture system on the other hand allows bacteria to be cultured under controlled conditions at very low nutrient levels — more akin to those encountered in the natural environment — and at low growth rates which can be varied independently of the temperature. Perhaps more importantly for this type of work, it is possible using continuous cultures to isolate defined communities or associations of organisms growing on complex organic substrates (Cossar *et al.* 1981, Harder 1981, Senior *et al.* 1976, Slater 1978). By varying the conditions of the enrichment it is possible to isolate different associations from the same environment, giving some indication of both the spectrum of organisms present and their inter-relationships (Dunn *et al.* 1980).

Although the experimental systems used often appear complex they are simple to operate and we have found that if mounted on a gimbal table such apparatus can be run effectively on board ships while at sea (Brown and Wardell 1983).

EXPERIMENTAL

The experiments described are enrichments of water and sediment from a marine environment carried out in the presence of the biocide Dodigen (Hoescht Ltd) — chemical name *coco-propylene diamine guanidinium acetate* — which has properties very similar to those of a quaternary ammonium compound. Four chemostats were employed using a carbon-limited artificial sea water medium. The four experimental regimes were :

A No carbon source provided - as a control
B Acetate at 5mM
C Acetate at 5mM + 10 ppm Dodigen 181-1
D 100 ppm Dodigen 181-1 as sole source of carbon and energy.

The culture vessels were inoculated with a slurry of sediment and water collected from a depth of 33 m on the continental shelf between Malin Head and the Outer Hebrides. After inoculation, medium was metered in to give a dilution rate of 0.05 h^{-1}. The cultures were continuously stirred and aerated during the course of the experiments and the culture temperature maintained at 5° Celsius. The cultures were sampled daily, serial dilutions prepared and inoculated onto 3 different media. These were Artificial Sea Water Agar containing 5mM acetate as carbon source (ASWA), ASWA + 10 ppm Dodigen and ASWA + 10 ppm Dodigen. Plates were incubated at 5°C until mature colonies had developed after about 6-10 days. Viable counts were performed and the results are given in Table 1.

The control experiment (A) provided useful information, it will be noted that there was an increase in the population during the course of the enrichment although no carbon and energy source was provided. This must be attributed to traces of carbon compounds present in the other chemicals used to prepare the medium and gives some indication of the background population to be expected. Note also that the presence of the biocide in the plating medium had a depressing effect on the population, although not as great as might have been expected. Also, although at no time exposed to the biocide during culture, organisms tolerant to 100 ppm Dodigen were always recovered suggesting that this was an inherent property of the heterotrophic microbial population. These results are confirmed by the second enrichment (B Table 1) with 5mM acetate, the essential difference here being the elevated populations due to the provision of a carbon and energy source.

	A control			B Acetate lim			C Acetate lim + 10 ppm 181-1			D 100 ppm 181-1		
T_h	1	2	3	1	2	3	1	2	3	1	2	3
0	1.7×10^3	NA	1.1×10^2	1.1×10^2	NA	3.9×10^2	1.9×10^3	NA	2.1×10^2	3.0×10^3	NA	3.5×10^2
24	3.0×10^4	1.9×10^4	1.6×10^3	4.2×10^3	3.2×10^4	3.8×10^3	4.3×10^4	3.3×10^4	1.5×10^3	3.1×10^4	3.5×10^4	4.6×10^3
48	1.2×10^5	9.0×10^4	9.6×10^4	4.9×10^4	2.9×10^4	4.1×10^3	1.6×10^5	1.7×10^5	1.4×10^4	2.9×10^4	3.3×10^4	5.2×10^3
72	4.2×10^5	3.5×10^5	3.5×10^4	1.3×10^6	1.5×10^6	5.5×10^4	8.3×10^5	7.2×10^5	7.8×10^4	4.8×10^5	7.2×10^5	4.6×10^5
96	9.1×10^5	6.0×10^5	1.4×10^5	4.8×10^5	3.8×10^5	9.8×10^4	8.1×10^5	7.4×10^5	3.8×10^5	6.6×10^5	5.4×10^5	3.5×10^5
120	2.3×10^5	3.5×10^5	2.5×10^5	1.4×10^6	1.8×10^6	2.5×10^5	1.9×10^6	2.0×10^6	2.8×10^6	1.2×10^6	2.6×10^6	2.8×10^6
132	2.7×10^5	1.5×10^5	2.8×10^4	2.1×10^6	1.4×10^6	3.9×10^5	2.3×10^6	1.0×10^6	1.6×10^6	5.3×10^5	5.5×10^5	4.3×10^6
144	1.8×10^5	2.7×10^5	8.4×10^4	1.8×10^6	2.3×10^6	6.9×10^5	1.4×10^6	1.6×10^6	1.7×10^6	1.8×10^6	9.4×10^5	6.6×10^6

Viable counts cfu ml^{-1}
NA = Not available.

1 : Artificial Sea Water Agar (ASWA)
2 : ASWA + 10 ppm 181-1
3 : ASWA + 100 ppm 181-1

Table 1 : Enrichment cultures of bacteria from a Marine environment in the presence of the biocide Dodigen 181-1.

In the enrichment with 10mM Dodigen present in addition to acetate (C, Table 1) two factors became apparent as the experiment progressed. The heterotrophic population as counted as ASWA increased in a similar manner to that of the acetate enrichment (B). After five days the populations were numerically similar (B_1 C_1). Secondly, although the Dodigen tolerant population (column C_3) did not increase initially as rapidly as the heterotrophic population by the end of the experiment the two populations were numerically very similar. One interpretation of these results in that during the initial stages of the enrichment the population utilized the acetate for growth, however, as this became depleted and limiting, the proportion of the bacterial population able to utilize the biocide increased so that at the end of the enrichment both substrates were fully utilized and growth limiting. The final experiment (D) with Dodigen as the sole source of carbon and energy provides a relatively simple picture. During the first few days the heterotrophic population predominates at a level similar to that of the control. As the enrichment progresses however, there is a gradual increase in the population of organisms able to tolerate or degrade Dodigen at 100 ppm until at the end of the experiment these organisms outnumbered the heterotrophic population by a factor of 3x suggesting a selection in favour of organisms with the ability to degrade the biocide rather than merely tolerate it.

Similar experiments have been conducted to isolate organisms tolerant to Dodigen from an estuarine environment (see Table 2). The same acetate-limiting artificial sea water medium was used with either : — A no biocide — the control. C plus 1 ppm and D plus 10 ppm Dodigen.

The culture vessels were inoculated with 50 gm wet weight of sediment collected from the Forth estuary near Edinburgh and filled to over-flowing with water from the same site. The dilution rate was fixed at 0.03 h^{-1} otherwise the experimental conditions were similar to the previous enrichments. The experiment was run for 24 days at which time the final populations were enumerated by serial dilution and plating onto Tryptone-soya agar + 0.4 M NaCl followed by incubation at 10° C for 10 days. A differential count was performed and representative colony types isolated and characterized. Both quantitative and qualitative differences between the three cultures became apparent (Tab. 2). The increase in the population density of 7x between the control and the enrichment with 10 ppm Dodigen (D) must be due to an increase in the concentration of the limiting substrate. Since this is not the case, it must be attributed to the utilization of the biocide as a source of carbon and energy. The intermediate population level achieved in the second enrichment (C) with 1 ppm biocide would seem to support this view. Qualitatively the

A Control acetate lim	% Popn	C acetate lim + 1ppm	% Popn	D acetate + 10ppm	% Popn
A4 PLO	50	C1 Flavo	45	D2 Acineto	44
A1 Aer/Vib	22	C2 PLO	30	D3 Flavo	25
A2 PLO	13	C3 PLO	7	D1 Aer/Vib	22
A3 PLO	5	4 PLO	4		
Others to 100%		Others to 100%		Others to 100%.	
Total count cfu/ml	1.24×10^4		8.9×10^4		9.8×10^4

PLO, pseudomonas like organism; Acineto; *Acinetobacter* sp.
Flavo, *Flavobacterium* sp.; Aer/Vib *Aeromonas/Vibrio* sp.

Table 2 : Enrichment of bacteria from an estuarine environment in the presence of the biocide Dodigen 181-1.

populations are quite different. Although similar organisms are present in each of the enrichments the predominant organisms are quite different for each culture. A pseudomonad predominates in the control with a *Flavobacterium* spp. at 45 % of the population in the second enrichment. The predominant community of organisms in the third enrichment is a mixture of an *Acinetobacter/Flavobacterium* and *Aeromonas-Vibrio* together accounting for 91 % of the population. The absence of a typical pseudomonad in this community is interesting since these were present in appreciable numbers in the first two enrichments and traditionally pseudomonads are noted for their tolerance to and ability to degrade and utilize recalcitrant compounds. The predominance of the *Flavobacterium* and *Acinetobacter* must be attributed to a selection pressure imposed by the biocide indicating tolerance and, on the evidence of population densities, the ability to utilize the compound.

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