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A comparison of organochlorine removal from bleached kraft pulp and paper-mill effluents by dehalogenating *Pseudomonas*, *Ancylobacter* and *Methylobacterium* strains

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Abstract The relative importance of each of three dechlorinating species to overall organochlorine removal from bleached kraft-mill effluents (BKME) was assessed. *Ancylobacter aquaticus* A7, *Pseudomonas* P1, and *Methylobacterium* CP13, strains indigenous to a BKME treatment system, were tested for growth on chlorinated acetic acids and alcohols, and for adsorbable organic halogen (AOX) reduction in batch cultures of sterile BKME from three sources. *A. aquaticus* A7 exhibited the broadest substrate range, but could only affect significant AOX reduction in softwood effluents. *Methylobacterium* CP13 exhibited a limited substrate range, but was capable of removing significant amounts of AOX from both hardwood and softwood effluents. By contrast, *Pseudomonas* sp. P1 exhibited a limited substrate range and poor to negligible reductions in AOX levels from both effluent types. Mixed inocula of all three species combined and inocula of sludge from mill treatment systems removed as much AOX from softwood effluents as did pure populations of *Methylobacterium* CP13. When BKME was hydrolysed prior to AOX analysis, the subsequent estimates of recalcitrant, or non-hydrolysable, AOX levels were far less variable than their counterpart total AOX measures. It is suggested that this is a relevant and useful measure of AOX for biodegradation studies.

Introduction

Waste water from the production of bleached kraft pulp contains wood-derived sugars, carbohydrates, organic acids, resin acids and lignin-transformation products and a variety of chlorinated derivatives, only 200 of which have been identified (Sunito et al. 1988; McKague and Kringstad 1989). Data on the chronic toxicity and environmental hazards posed by the bulk of this chlorinated material are conflicting, but it is generally agreed that less organochlorine is better, and government regulations in Canada and other nations have consistently demanded lower discharge limits over time.

Aerated stabilization basins or lagoons are often employed to reduce the levels of suspended solids and biological oxygen demand of bleached kraft-mill effluent (BKME). The same technology is relied on to reduce the toxicity of the effluent to fish and the overall levels of adsorbable organic halide (AOX, an estimate of the total chlorinated organics). Both toxicity and AOX reductions are highly variable (Ontario Ministry of the Environment 1991; Tomar and Allen 1991). An understanding of the microbiology of these systems could lead to improved process controls, but there are few studies on the bacteria indigenous to these systems and their impact on AOX levels in BKME.

The purpose of this study was to determine which bacterial species had the greatest potential to reduce AOX in BKME. In a recent study, three species able to dehalogenate simple aliphatic compounds were found to be indigenous to a BKME treatment system (Fulthorpe et al. 1993). *Ancylobacter aquaticus* and *Methylobacterium* species dominate the culturable flora of the latter stages, while *Pseudomonas* species peak in the initial clarification stage. This study examines the ability of each strain to dechlorinate a range of chlorinated acids and alcohols and their relative effectiveness in reducing AOX levels in actual BKME.

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Materials and methods

Organisms

Ancylobacter aquaticus strain A7, and *Methylobacterium* strain CP13 were isolated directly from the aerated lagoon and the settling pond of a Canadian Pacific Forest Products treatment system for bleached kraft pulp and are described in Fulthorpe et al. (1993). *Pseudomonas* species strain P1 is a non-fluorescent pseudomonad isolated from lagoon flora and is capable of growth on vanillin, phenol and chloroacetate. All strains were tentatively identified using Biolog GN microplates, and identifications were confirmed with Gram stain, oxidase, catalase, motility and cell-morphology tests. Strains were maintained on chloroacetate plates made from a mineral medium base (medium A; Wyndham 1986) supplemented with 100 ppm yeast extract and 2 mM chloroacetate.

Substrate range experiments

The substrate ranges of the strains were tested in three ways: (a) using Biolog MT substrate-utilization test kits; (b) growth on the substrate as a sole carbon source; (c) dechlorination activity by actively growing cells.

(a) Biolog MT test kits are 96-well microtitre plates that exploit the colour change associated with tetrazolium dye reduction occurring as a by-product of active bacterial respiration. Strains were grown on standard plate-count agar (SPA, Difco) for 18–24 h, resuspended in sterile distilled water to a density of 10^8 cells/ml and inoculated into the test wells supplemented with 2 mM test compound. Dye reduction in the test wells, indicative of metabolic activity, was read after 48–36 h as the absorbance at 570 nm with a Dynatech Laboratory MR700 automatic plate reader and compared with that of control wells containing no carbon source.

(b) For growth tests, the strains were grown for 2–3 days on 2 mM chloroacetate/agar plates containing 50 ppm bromothymol blue as an indicator of dechlorination activity. Dechlorinating cultures were inoculated (100 μ l resuspended solution of 10^8 cells/ml) into 20 ml defined media (medium A supplemented with 100 ppm yeast extract) containing 2.5 mM 2-chloroethanol (ClEtOH), monochloroacetate (ClAc), dichloroacetate (Cl₂Ac), trichloroacetate (Cl₃Ac), 1,1,1-trichloroethanol or 1,2-dichloroethane. Cultures were incubated in 30-ml screw-cap culture tubes at 150 rpm at 30°C for 7 days. Cell growth was monitored as the absorbance at 560 nm, and chloride ion concentrations were measured with an Orion 94.17B combination chloride electrode and an Accumet 50 ion meter, using 1-ml subsamples adjusted to 100 mM KNO₃. These values were compared to standards and to uninoculated controls containing the same media and substrate.

(c) To test for the dechlorination of compounds other than those that can be used for growth substrates once the strain has been induced on a chlorinated substrate, strains were grown at 30°C in 250 ml 5 mM ClAc or ClEtOH in medium A supplemented with yeast extract. Absorbance and chloride release were measured daily as above. When the culture reached an absorbance at 560 nm of 0.100, it was subdivided into six 20-ml portions in sterile 30-ml screw-capped tubes and supplemented with 2.5 mM test chemicals (as above). Absorbance and chloride release were monitored for 7 days and compared to values in uninoculated chemical controls and in the unsupplemented inoculated subculture.

Growth in bleached kraft-mill effluents

Effluents were obtained from two sources. The Noranda James MacLaren Mill in Thurso, Quebec, shipped effluent from hardwood pulping (50% maple, 19% poplar, 6% elm, 2% basswood, 5% beech

and 8% birch, the remainder softwood), which was bleached using chlorine, oxygen and chlorine dioxide (C.E.D.E.D.). The Canadian Pacific Forests Mill in Dryden, Ontario, sent 20 l of each of hardwood (90% trembling aspen, 10% balsam poplar) and softwood (65% jack pine, 30% black spruce, 5% balsam fir) pulping effluent. Both of these had been bleached with chlorine dioxide and chlorine (D.W.E.D.E.D.). Effluents were subdivided and stored in nalgene containers at –20°C on arrival. Subsamples were prepared for batch studies by centrifuging at 2000 *g* for 20 min in a Sorvall 2B centrifuge, and filtration through a 1.2-mm glass-fibre filter and a 0.45-mm Gelman supor membrane followed by a sterilization filtration through a 0.2-mm supor membrane. Effluents were supplemented with 0.1 mM phosphate and 2.5 mM ammonium sulphate prior to inoculation with the strains.

Each strain was grown in both softwood and hardwood effluents, and tested in more than one batch of each effluent type. For each batch experiment the strain was grown for 24–48 h on a chloroacetate plate, inoculated at 10^5 cells/ml into 100 ml effluent in a 250-ml conical flask, and incubated at 150 rpm in a New Brunswick shaker at 30°C for 7 days. Experiments were done in triplicate, and triplicate uninoculated controls were run for each effluent type. Biomass was estimated by reading the absorbance at 600 nm using control cultures as blanks, and by plating on both Difco's standard plate-count agar and on 2 mM chloroacetate agar. After 7 days, AOX was measured as described below in both the inoculated flasks and uninoculated controls.

Organic halogen determination

Two approaches were used to quantify the organic halogen in the cell cultures and controls. In the first, the AOX in the culture fluid and the organochlorine sorbed to the cells and precipitates (solid-bound organochlorine) were measured separately. In the second approach the entire sample was hydrolysed to solubilize precipitates and bacterial cell contents prior to measuring the remaining AOX, termed here recalcitrant or non-hydrolysable AOX.

Culture-fluid AOX was directly determined using 1:100 diluted samples in a Mitsubishi AOX analyser as described below. To measure solid-bound organochlorine, 25–40 ml well-mixed culture was centrifuged at 2000 *g* for 20 min to harvest cell biomass and associated particulates. The solid material was dried and ground with a mortar and pestle into a fine powder, which was resuspended in 20 ml ultrafiltered water and sonicated for 1 h (Branson 2200 Ultrasonicator, Branson Cleaning Equipment Co.). The material was then filtered through a pre-weighed 0.2- μ m filter and washed with 0.8 M KNO₃ to remove free chloride ions. The filter and biomass were dried at 104°C for 24 h, weighed, and placed in a plastic vial. The total chlorine in the vial was measured via neutron activation analysis using a Slowpoke nuclear reactor at the University of Toronto (Tomar and Allen 1991; Hoffman et al. 1988). Previous work by our group on municipal sludge containing inorganic chloride has demonstrated that the sonication and washing procedure is effective at removing trapped chloride ions (Tomar 1991). The mass of solid-bound organochlorine determined in this way was standardized to the total culture volume (reported as ppm).

To measure non-hydrolysable or recalcitrant AOX, a 10-ml subsample of a well-mixed cell culture was hydrolysed at pH 11 and 65°C for 1 h. The lysate was mixed well, diluted 1:100, and acidified to pH 2. Two separate dilutions were analysed from each sample for AOX content.

The AOX concentration in the culture supernatant and/or hydrolysate was measured with a total organic halogen analyser (Mitsubishi Chemical Industries Ltd., model TOX-10, Tokyo, Japan) as detailed elsewhere (Odendahl et al. 1990; SCAN-Test SCAN-W 9:89 1989; CPPA Standard H.6P 1991). The three steps involved in AOX measurement were: adsorption of organic matter onto activated carbon, organically bound chlorine mineralization through combustion and determination of chloride by microcoulometric titration.

Results

Utilization of simple chlorinated compounds

The Biolog MT microplates suggested substrate ranges for the strains that could not be confirmed by growth tests. *A. aquaticus* A7 and *Pseudomonas* P1 exhibited broad substrate ranges according to the MT plates, and *Methylobacterium* CP13 seemed to be the least versatile of the three (Table 1). In batch-growth tests using medium A supplemented with yeast extract, A7 exhibited good growth on and complete dechlorination of ClAc, Cl₂Ac, and ClEtOH (Fig. 1). It also showed scant growth on Cl₃Ac, accompanied by release of 11% of the available chlorine. When induced on ClEtOH, A7 could remove 30% of the chlorine from Cl₃Ac (Table 1). Attempts to induce dechlorination activity in A7 using methanol-grown cultures were not successful, even though a methanol dehydrogenase has been found responsible for dechlorination of 2-chloroethanol in *Xanthomonas autotrophicus* GJ10 (Jansson et al. 1989). P1 grew only on ClAc and ClEtOH (data not shown). After induction on ClEtOH it could remove chlorine from ClEtOH and ClAc (Fig. 2), but not from Cl₂Ac, Cl₃Ac, or dichloroethane even though the MT plate results suggested activity against these compounds. Growth of P1 on phenol did not enhance its dechlorination activity although phenol oxidation enzymes have been shown to be responsible for the degradation of trichloroethylene in other pseudomonads

(Nelson et al. 1988). CP13 exhibited growth and dechlorination intermediate between these two species. While MT plates predict an extremely narrow substrate range, CP13 grew well on and completely dechlorinated both ClAc and Cl₂Ac and showed some dechlorination of Cl₃Ac although this was not accompanied by growth (Fig. 3). Dechlorination of Cl₃Ac was not abiotic, however, since it did not occur in the uninoculated control tubes. CP13 did not show any activity against CE.

Removal of AOX from hardwood and softwood bleached kraft-mill effluents

Initial AOX levels in the three effluents used for the experiments were 21.0 ppm for the Noranda hardwood effluent, 27.9 ppm for the Dryden softwood, and 11.4 ppm for the Dryden hardwood effluent. The growth of the three species on the effluents is illustrated in Fig. 4. In all cases, viable counts of cells growing on chloroacetate media did not differ from those growing on standard plate-count agar, indicating that the ability to degrade ClAc was a stable property in these effluents. In the softwood effluents, both A7 and P1 reached maximum populations of 2×10^8 cells/ml, while CP13 reached a population of 1.4×10^8 cells/ml. Note that the absorbance of a given number of cells is quite a bit higher for A7 than for the CP13 and P1. Both A7 and CP13 seem to increase maximally

Table 1 Results of substrate utilization tests. The MT plate results record A_{570} of the test well (indicative of dye reduction) minus that of control. The chloride released during growth is expressed as a percentage of the total added in the chlorinated substrate, and the growth of ClEtOH culture records results from a culture actively

growing on chloroethanol, not performed on CP13. (+, cell biomass accumulation above that seen in control flasks where no substrate is added; — biomass change not different from control; Neg negligible response in MT plates)

Compound	Test	A7 <i>A. aquaticus</i>		CP13 <i>Methylobacterium</i>		P1 <i>Pseudomonas</i>	
		Test result	Cl (%)	Test result	Cl (%)	Test result	Cl (%)
Chloroacetate	MT plates (A_{570})	0.242		0.034		0.408	
	Growth	+	100	+	100	+	100
	Growth ClEtOH culture	+	58			+	40–100
Dichloroacetate	MT plates (A_{570})	0.430		0.100		0.213	
	Growth	+	100	+	100	—	0
	Growth ClEtOH culture	+	43			+	9–20
Trichloroacetate	MT plates (A_{570})	0.132		Neg		0.174	
	Growth	+	11	—	9	—	0
	Growth ClEtOH culture	—	30			—	0–6
Chloroethanol	MT plates (A_{570})	0.188		Neg			
	Growth	+	100	—	0	+	40–60
	Growth ClEtOH culture	+	30			—	0–24
Trichloroethanol	MT plates (A_{570})	0.113		Neg			
	Growth	—	0	—	0	—	0
	Growth ClEtOH culture	—	0			—	0
Dichloroethane	MT plates (A_{570})	0.106		Neg			
	Growth	—	2	—	0	—	0
	Growth ClEtOH culture	—	0			—	0–8

Fig. 1a, b Growth of *A. aquaticus* A7 on (a) no substrate and chloroethanol and (b) on chloroacetates. ● Absorbance at 560 nm; □ chloride concentration in inoculated cultures; ▽ chloride concentration in uninoculated controls

Fig. 2 Dechlorination of chloroethanol and chloroacetates by chloroethanol-grown cultures of *Pseudomonas* P1. ● Absorbance at 560 nm; ○ chloride concentration in inoculated cultures; ▽ chloride concentration in uninoculated controls

after 2 days, thereafter experiencing little change. In hardwood effluents, P1 experienced a dramatic rise in population from 2.4×10^7 cells/ml on day 2 to 6.0×10^8 cells/ml on day 4, thereafter decreasing to a final population of 1.3×10^8 cfu/ml. This growth and decline may be indicative of the production of toxic compounds during the rapid growth phase. A7 and CP13 exhibited behaviour similar to that in softwood effluents.

AOX losses in the effluents were not due to accumulation of organic chlorine in the particulate fraction, but rather were true losses from the BKME. Table 2 compares the level of bound chloride in each of the experimental treatments to that in the uninoculated controls. Since AOX is an aqueous-phase measurement of the chlorinated organic material that binds to activated carbon, it measures most but not all of the organically bound chlorine. Therefore AOX and the solid-bound organochlorine measurements are not measuring exactly the same quantity and cannot be additive. Our work on biosorption of AOX (Yan and Allen 1994)

Fig. 3 Growth of *Methylobacterium* CP13 on chloroacetates. ● Absorbance at 560 nm; ○ chloride concentration in inoculated cultures; ▽ chloride concentration in uninoculated controls

indicates that the solid-bound organochlorine reading is only slightly higher than the AOX and therefore the solid-phase measurement will, if anything, tend to overestimate the quantity of AOX biosorbed. There is only one case where the solid-bound organochlorine is significantly higher than in the control, that is in the case of A7 growing on the first softwood batch, where the value exceeds that of the control by 0.4 ppm.

All of the control effluents lost AOX to abiotic processes (volatilization, hydrolysis) over the course of the 7-day incubations. These losses averaged 29% for the

Fig. 4 Growth of all species on hardwood and softwood effluents from the Canadian Pacific Forest Products mill in Dryden. ● *Ancylobacter aquaticus* A7; ▲ *Methylobacterium* CP13; ■ *Pseudomonas* P1

Table 2 Solid-bound organic chlorine. Results are means of triplicates performed for each effluent $\pm$ 1SD (ND not determined)

Effluent	Solid-bound organochlorine (mg/l culture fluid)			
	Control	A7	CP13	P1
Hardwood-1	2.83 $\pm$ 0.48	2.83 $\pm$ 0.48	3.03 $\pm$ 0.08	3.03 $\pm$ 0.08
Softwood-1	3.70 $\pm$ 0.10	4.12 $\pm$ 0.09*	3.91 $\pm$ 0.09	3.84 $\pm$ 0.11
Softwood-2	3.99 $\pm$ 0.60	4.36 $\pm$ 0.21	4.39 $\pm$ 0.21	3.65 $\pm$ 0.34
Softwood-3	3.43 $\pm$ 0.22	ND	3.29 $\pm$ 0.11	ND

* Significantly different from control at $P < 0.05$, Student's t -test

Dryden softwood and 31% for the Dryden hardwood. We were concerned only with AOX losses due to biological transformations, and so in Table 3 we report the differences in AOX between uninoculated controls and biologically treated effluents at the end of the 7-day incubation. Therefore all losses reported below are in addition to the abiotic AOX losses.

CP13 and A7 were superior to P1 in removing AOX from softwood effluents (Table 3). CP13 and A7 removed 14%–17% of the total AOX and 17% of the recalcitrant AOX from softwood BKME. There was

Table 3 Adsorbable organic halogen losses from hardwood and softwood effluents

Parameter	Effluent	CP13		A7		P1		Mixture	
		(mg/l) (%) ^c		(mg/l) (%)		(mg/l) (%)		(mg/l) (%)	
Total loss	Soft-1	1.10	6.1*	3.85	21.4***	2.28	12.7*		
	Soft-2	4.74	21.7**	2.94	13.5*	NS	0	4.6 ^a	22.4
	Soft-3	2.91	13.9***	—	—	—	—	3.7 ^b	19.6
Average			14.1 ± 6.2		17.6 ± 4.0		6.4 ± 6.4		
	Hard-1	0.86	13.7**	NS	—	NS	—		
Recalcitrant loss	Soft-1	2.75	14.1**	2.78	14.2**	NS	—		
	Soft-2	3.12	21.8**	2.85	19.6*	0.65	4.6**	3.4 ^a	23.8
	Soft-3	3.09	16.6**	—	—	—	—	3.7 ^b	21.3
Average			17.6 ± 3.3		17.0 ± 2.8		2.3 ± 2.3		
	Hard-1	0.57	14.8*	NS	—	NS	—		
	Hard-2	2.02	20.8**	NS	—	NS	—		

**** Statistical significance of difference from control, one-tailed *t*-test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. NS no statistically significant difference

^a Mixture of CP13, A7, P1

^b Inoculum of non-sterile lagoon fluid

^c ((Final AOX of Control – Final AOX of Treated Sample)/Final AOX of Control) × 100

considerable variation in the total AOX losses from batch to batch, but much less variability in the recalcitrant AOX losses. CP13 absolute removals of total AOX varied by a factor of 4, absolute removals of recalcitrant AOX varied by only 7% (3.0 mg ± 0.2 mg). P1 reduced the total AOX in one effluent by 12.7%, but had no impact on the recalcitrant AOX levels (a phenomenon that would occur if it only removed labile AOX). In a second test no significant total AOX reduction was detected and only 4.6% of the recalcitrant AOX was removed.

Significant removals from hardwood effluent were effected only by *Methylobacterium* CP13. Growing in Dryden hardwood effluents it removed about 14% of both the total and the recalcitrant AOX. Only recalcitrant AOX measurements were made on the Noranda hardwood effluents, but these gave the same results—i.e. no losses occurred with A7 and P1, but there was 20% removal by CP13. These apparently low percentage removal levels must be judged in relation to the percentage losses achievable by a mixed inoculum from the lagoon, and also by a mixture of all three species under the same experimental conditions. In these cases the best percentage loss achievable was 23.8% from softwood effluent (Table 3).

Discussion

Removal of AOX from effluents treated in aerated lagoons or activated-sludge treatment systems averages about 25%–60% or greater (Tomar and Allen 1991). In these 7-day batch experiments, when both abiotic and biotic losses are summed, total AOX removals lay between 35% and 51% of the initial AOX. Cell

biomass increased over a period of 7 days to a maximum of 10⁸ cells/ml, whereas in aerated lagoons cell concentrations are at 10⁹ cells/ml (Liss and Allen 1992) and in activated-sludge systems they may be even higher. The loss rates presented are not intended to be indicative of the maximum AOX removals achievable, but of the differences between the three species. It is possible that at high cell densities of individual species, much higher AOX biotic removal may be achieved.

Of the three species investigated in this study, it is clear that *Methylobacterium* CP13 is superior to the *Ancylobacter aquaticus* A7 and to *Pseudomonas* P1 with respect to its ability to remove total and recalcitrant AOX from both softwood and hardwood effluents. This conclusion could not be predicted from simple assays of the substrate range of the bacteria, in which CP13 appeared to be less versatile than the other two species. It may be that the action of the bacteria on the chlorinated acetic acids alone is relevant to their ability to remove AOX from the effluents. Chlorinated acetic acids do form a major portion of the AOX of BKME (Lindstrom and Mohamed 1988). CP13 and A7 did grow equally well on ClAc and Cl₂Ac and both dechlorinated equivalent amounts of Cl₃Ac. It may be that abilities to degrade the alcohols and dichloroethane are not relevant to the BKME situation. However, it is more likely that the species have differential abilities to degrade other chlorinated substrates that could not be tested in our experiments. The fact that both CP13 and A7 were able to remove more recalcitrant AOX from softwood effluents than P1 is consistent with the observation that *Ancylobacter* and *Methylobacterium* species dominate in the latter stages of an aerated-lagoon treatment system—they are probably better able to utilize the less labile compounds that persist in the system. AOX removals correlate to Total

Organic Carbon (TOC) removals in these systems (Tomar and Allen 1991; Bryant et al. 1987), so microorganisms removing recalcitrant TOC are probably also removing recalcitrant chlorinated organics. CP13 performs better than A7 on hardwood effluents, which is consistent with the fact that CP13 is quite different from A7 in the suite of non-chlorinated substrates that it can use (Fulthorpe et al. 1993); it is better able to utilize organic acids and amino acids and less able to attack sugars and alcohols. CP13 is clearly able to dechlorinate some portion of the hardwood effluent that cannot be attacked by A7. It would be beneficial to maintain populations of CP13-like strains in BKME treatment systems, given that mills frequently change between softwood and hardwood furnishes. Further work on the ecological factors that favour this species and on the enzymology of its dechlorination activity is certainly merited.

The enzymology of *A. aquaticus* A7 is also worth exploring further. A7 was only one of four *Ancylobacter* species tested. Preliminary work showed it had the greatest activity against Cl_3Ac and so it was used for these comparative studies, but it was clear from this early work that considerable variation exists in this species. We initially thought that *Ancylobacter* species would be similar to *Xanthobacter autotrophicus* GJ10—a species that shares alkane-dechlorinating genes with *A. aquaticus* strains isolated from the River Rhine (van den Winnigaard et al. 1992). In GJ10 a pyroloquinoline-quinone-dependent methanol dehydrogenase is responsible for 2-chloroethanol utilization (Janssen et al. 1989). However the inability of A7 and other *Ancylobacter* strains to dechlorinate chloroethanol after growth on methanol suggests that another enzyme is responsible. Cell-free-extract activity gels show that the proteins responsible for the dechlorination of chloroacetate in GJ10 and three *Ancylobacter* strains differ substantially (Fulthorpe, unpublished data).

A simple assay of the substrate range of individual species is insufficient to predict their performance in a complex mixture such as BKME, particularly given that the substrates present in BKME are not readily available for testing. Particularly disturbing are the results of the MT plate assays, which suggest broad substrate ranges that cannot be duplicated using classical growth media. It may be that particular conditions of the MT plates allow broader activity than do shake flask experiments—i.e. a greater concentration of oxygen, a protective effect of the hydrophobic polyethylene or the presence of a greater range of growth co-factors. Nevertheless, P1, with a broad predicted substrate range, did not prove to be an effective dechlorinating species in BKME.

There is high variation in the AOX removals between the softwood experiments. One explanation for this is that the composition of the effluents changes from thawed batch to thawed batch and therefore the

amount of chlorinated substrate that can be acted on by the bacteria. It is noteworthy that there is far less intra-effluent variation in AOX removals when the non-hydrolysable fraction is considered. AOX may be much more variable prior to chemical hydrolysis because it is colloidal in nature, and hydrolysis helps to solubilize the colloids after dilution, in addition to removing labile and volatile components. Given these observations, the method of hydrolysing the AOX prior to analysis is a valuable way of analysing organochlorine for three reasons. It eliminates the problem of considering the organochlorine bound to cell biomass or in the particulate fraction separately from the AOX found "solubilized" in the liquid fraction. It reduces the variability in the measurement that can obscure the differences in experimental treatments. Finally, since the fraction of AOX that is of most concern to environmental regulators is that fraction that will not readily degrade in the environment, measurement of recalcitrant AOX is a more relevant parameter. To illustrate this, consider the contrasting cases of treatment with P1, which removes 2.28 mg AOX (Table 3, softwood run 1)—all of which proves to be easily hydrolysable—as contrasted with CP13 in the same effluent, which removes 1.10 mg total AOX but actually effects the loss of 2.75 mg recalcitrant AOX. This apparently conflicting result is explained by CP13 converting a portion of AOX to a hydrolysable form rather than directly removing it.

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