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Characterization of the Acclimation Period before Anaerobic Dehalogenation of Halobenzoates†

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The acclimation periods prior to detectable dehalogenation of halogenated benzoates in anaerobic lake sediments ranged from 3 weeks to 6 months. These acclimation periods were reproducible over time and among sampling sites and were characteristic of the chemical tested. The lengthy acclimation period appears to represent an induction phase in which little or no aryl dehalogenation is observed, followed by an exponential increase in activity typical of an enrichment response. Continuous growth from the time of the first exposure to the chemical is inconsistent with the extremely low per-cell activities estimated for the early days of the acclimation period and the fact that the dehalogenation yields no carbon to support microbial growth. The finding of a characteristic acclimation time for each chemical argues against nutritional deficiency, inhibition, or predation as an explanation for this phase of metabolism, while the reproducibility of the findings with time and space and among replicates argues against genetic changes as the explanation. The acclimation times did correlate with the eventual dehalogenation rates. This may reflect the general energy limitations in the anaerobic communities and suggests that those chemicals with faster dehalogenation rates provide more energy for the induction and growth phases of the active population.

Reliable evaluation of the risks of xenobiotic compounds in nature requires a mechanistic and quantitative understanding of the fate of those compounds. Microbial biodegradation is one of the most important fates affecting the concentration of xenobiotic compounds; in quantitative models, this is usually described by a rate constant. However, rate constants do not accurately describe the acclimation period prior to the onset of detectable degradation after a community is exposed to a new chemical. In aerobic microbial communities, the acclimation periods for xenobiotic compounds typically range from several hours to several days (10, 18, 23), but for anaerobic communities these periods are much longer—often from 2 weeks to 6 months or longer (8, 21). Because of its length and broad range, the acclimation period is probably more important in predicting chemical exposure concentrations in habitats than is the shorter biodegradation period. In particular, it would seem difficult to ignore the acclimation period in predictive models of environmental transport and fate, especially if anaerobic microbial communities play important roles in this process.

Several hypotheses have been offered by investigators studying aerobic biodegradation to explain the observed acclimation periods (10, 18, 23). The most likely hypotheses include the time for microbial populations to: (i) grow to a size sufficient to achieve detectable bioconversion rates (18, 22, 23); (ii) induce new enzymes (18, 19); (iii) undergo genetic changes, e.g., mutation, gene exchange, or rearrangement (9, 17); and (iv) exhaust preferential substrates before switching to the xenobiotic substrate, i.e., a diauxie pattern (10). Other explanations for a delay in biodegradation include the lack of nutrients (10), temporarily inhibitory environmental conditions (e.g., unfavorable pH or temperature or a toxin), and predation by protozoa or other microbial grazers (23). Furthermore, the concentration and structure of the xenobiotic compound itself probably influence the acclimation period. In particular, low concentrations (3, 14, 20) and structure-biodegradability relationships (1, 2, 6, 12) are known to affect aerobic biodegradation rates, and in some cases they have been shown to affect the acclimation period as well (10, 18, 21, 23). The hypotheses posed for aerobic acclimation periods should be equally valid for the anaerobic ones, although some are less likely because of the composition of anaerobic microbial communities (e.g., the lack of nutrients and of predation).

The objectives of this study were to: (i) characterize acclimation periods preceding the reductive dechlorination of halobenzoates by assessing their length and reproducibility, (ii) determine the influence of chemical structure and concentration on the length of the acclimation period, and (iii) determine the cause of these lengthy acclimation periods. Halogenated benzoates were chosen as model compounds for study because previous work had shown that they were dehalogenated in sediments and that they had acclimation periods that were lengthy but still in a range reasonable for study (8, 21).

MATERIALS AND METHODS

Sediment was taken from the profundal zone of hypereutrophic Wintergreen Lake, Kalamazoo County, Mich., by using an Eckman dredge. Dilute sediment slurries (99 ml each) (70 to 90 mg [dry weight] per ml) were transferred to 160-ml serum bottles as previously described (15). Sludge was collected from the primary anaerobic digestor at the municipal treatment plant in Holt, Mich. Raw sludge (10 ml) was transferred to serum bottles and subsequently diluted with 89 ml of 14.3 mM Na_2CO_3 solution. The bottles were sealed with 1-cm-thick butyl rubber stoppers and aluminum crimp seals. All transfers were carried out inside an anaerobic glove box under a 90% N_2–10% H_2 atmosphere. However, to maintain a bicarbonate-carbonate buffering system

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in the sludge (15), the headspace gas was replaced with 80% N_2–20% CO_2 by using O_2-free gases and Hungate-type gassing probes. Since the sediment has a high marl content, no additional buffering was required to maintain a neutral pH; the gassing probes were used to achieve O_2-free N_2 as the final gas phase. The bottles were incubated for 7 days prior to substrate addition to ensure that they were anaerobic and rapidly producing CH_4. The incubation temperature was 20°C. The incubation and sampling systems have been tested extensively and do not allow significant O_2 intrusion over the long incubation periods of this study.

The test substrates—3-fluorobenzoate (3-F-BZ), 3-chlorobenzoate (3-Cl-BZ), 2-bromobenzoate (2-Br-BZ), 3-bromobenzoate (3-Br-BZ), 4-bromobenzoate (4-Br-BZ), 3-iodobenzoate (3-I-BZ), 3,5-dichlorobenzoate (3,5-diCl-BZ), and 4-amino-3,5-dichlorobenzoate (4-NH_2-3,5-diCl-BZ)—were obtained from Aldrich Chemical Company, Milwaukee, Wis., and Frinton Laboratories, S. Vineland, N.J., and were used without further purification. The substrates were added as the Na^+ salt to achieve final concentrations of 20, 40, 400, or 800 μM. The serum bottle contents were periodically sampled by using a syringe and needle. The aqueous samples were filtered and analyzed for substrate depletion and product formation. Analysis was by reversed-phase high-pressure liquid chromatography (HPLC) as previously described (8). The lower detection limit for the parent substrates and their dehalogenated metabolites was 5 μM.

The acclimation period was defined as the period until measurable substrate depletion was noted. For most substrates, the acclimation time was confirmed by the appearance of a dehalogenated product that was representative of quantities greater than 3% of the initial substrate. Experiments were performed in duplicate the first year. Because of the relative inactivity in the sludge blottles, only the sediment portion of the experiment was repeated the second year. Bottles were prepared in triplicate for those repeated experiments. All data are means of results from the replicate bottles and are presented in the figures as the natural log of the concentration.

At the termination of the initial biodegradation reaction, the remaining acclimated sediments for all substrate concentrations were consolidated and combined with unacclimated sediment in a large flask under strict anaerobic conditions. These flasks of 3-Br-BZ, 3-I-BZ, and 3,5-diCl-BZ were enriched by feeding 800 μM (three times), followed by a 100 μM feeding (once) of their respective substrates. After biodegradation of the latter quantity, each enriched sediment was redistributed among six serum bottles, three prepared with 800 μM of the respective substrates and three prepared with 400 μM. The higher concentrations were used for the degradation rate determinations to enable a greater sampling duration. A similar procedure was used for 4-NH_2-3,5-diCl-BZ and 3-Cl-BZ, except that they were added less frequently and at lower concentrations to sediment enrichments because of their slower rate of degradation.

To determine the consistency of the acclimation periods, separate sediment samples were taken after the spring overturn at three sampling stations located in the deeper portions of Wintergreen Lake. The second sampling station corresponded to that used in the earlier experiments. A 2-year-old refrigerated (70 to 90 mg [dry weight] per ml) sample was also used. These samples were divided among 10 replicate serum bottles as described above. After all 40 bottles were actively producing CH_4, 3-Br-BZ was added to each one to achieve a final concentration of 800 μM, and the acclimation period in each bottle was determined by measuring product appearance.

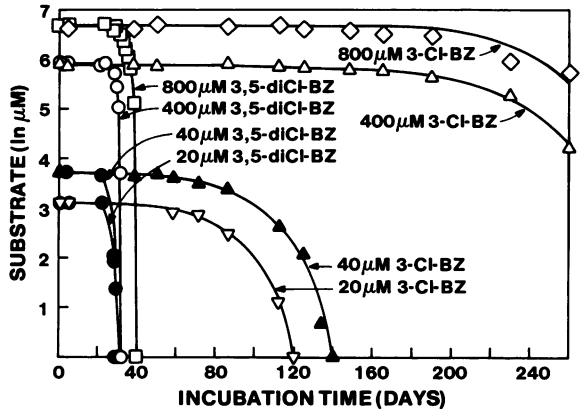

FIG. 1. Effect of substrate concentration on the length of the acclimation period prior to dehalogenation of 3,5-diCl-BZ and 3-Cl-BZ. Data points in all figures are the means of triplicate bottles.

RESULTS

Effect of substrate concentration and chemical structure. Acclimation periods prior to significant substrate depletion and product formation ranged from about 3 weeks to 6 months and were not correlated with complete mineralization of the substrates in this habitat (8). For those compounds that were metabolized at one or more concentrations, two biodegradation patterns were observed. One pattern was an extended acclimation period at high substrate concentrations (400 to 800 μM) compared with that at low concentrations (20 to 40 μM); this pattern occurred for both 3-Cl-BZ (Fig. 1) and 3-F-BZ (Fig. 2). The other main pattern, shown by 4-NH_2-3,5-diCl-BZ, was degradation at the high concentrations (400 μM) but not at the low concentrations (20 μM) (Fig. 2). Metabolism of this benzoate at a starting concentration of 20 μM has never been observed in repeated experiments (for example, see reference 8), not even for cultures incubated for more than a year (unpublished data). However, once induced by higher concentrations, dehalogenation of this substrate occurred at levels well below 20 μM (note that the 400 μM concentration in Fig. 2 and the 800 μM concentration in Fig. 3 go below ln 20 μM = 2.99). For the

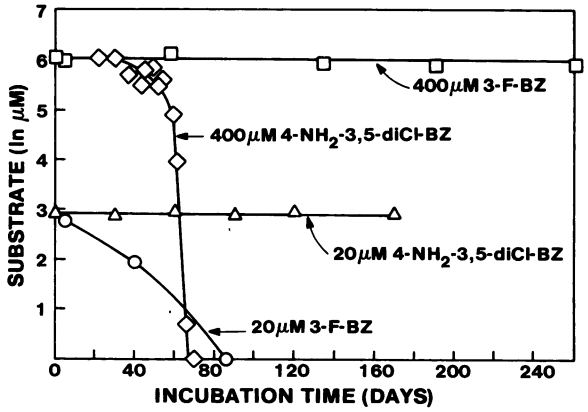

FIG. 2. Differences in acclimation period before dehalogenation of 20 μM and 400 μM 3-F-BZ and 4-NH_2-3,5-diCl-BZ.

TABLE 1. Effect of concentration and aromatic substitution on the acclimation period before onset of dehalogenation of several benzoates added to sediments

Year	Substrate concn. (μM)	Acclimation period (days) with the following substituent[a]:					
		3-F-BZ	3-Cl-BZ	3-Br-BZ	3,5-DiCl-BZ	3-I-BZ	4-NH_2-3,5-diCl-BZ
1	20	>170	150–170	29	20–25	34	>170
	40	>170	150–170	28	20–25	29	50–55
	400	>170	150–170	27	30	29	50–55
	800	>170	150–170	27	34	34	60–65
2	20	<40	86	30–34	20–28	28	>70
	40	<40	71	30–34	20–30	28	79
	400	>365	125–148	20–25	23–33	23–28	30
	800	>365	125–148	21–24	29–35	28	37

[a] Year 1 data are from duplicate bottles, and year 2 data are from triplicate bottles. Ranges shown include data from all bottles, and where a single value is shown all bottles were within ±5% of the mean.

other substrates, different concentrations did not alter the degradation patterns and did not result in any significant differences in acclimation periods (Fig. 1 [3,5-diCl-BZ]; Table 1).

Acclimation periods were also a function of chemical structure, as illustrated for dehalogenation of the substrates at a concentration of 800 μM, where the halogen type and other ring substituents altered the acclimation period (Fig. 3, Table 1). In the case of the bromobenzoates, the effect of ring position is clearly demonstrated; the *para* isomer exhibited greater acclimation periods than did either the *ortho* or the *meta* form. With an average initial concentration of approximately 900 μM, the lag periods for the *ortho*, *meta*, and *para* isomers were 20, 23, and 39 days, respectively (data not shown). This pattern was reproducible; the *ortho* and *meta* forms always exhibited lag periods 16 to 19 days shorter than that of the *para* form.

The most sensitive indication of the onset of dehalogenation was the appearance of product, since there was no background quantity of chemical. Both the rate of product accumulation (Fig. 4) and the rate of substrate disappearance (Fig. 3 and 4) are straight lines on a logarithmic axis, indicating an exponential increase in the rate of dehalogenation. For product appearance from 3-Cl-BZ and 3,5-diCl-BZ, these lines were extrapolated to time zero in order to estimate a theoretical initial rate of dehalogenation (Fig. 4 [inset]). Assuming a constant exponential increase in dehalogenation, the rate of benzoate production for the first day was 6.2×10^{-10} μM/day and the rate of 3-Cl-BZ production was 3.4×10^{-11} μM/day.

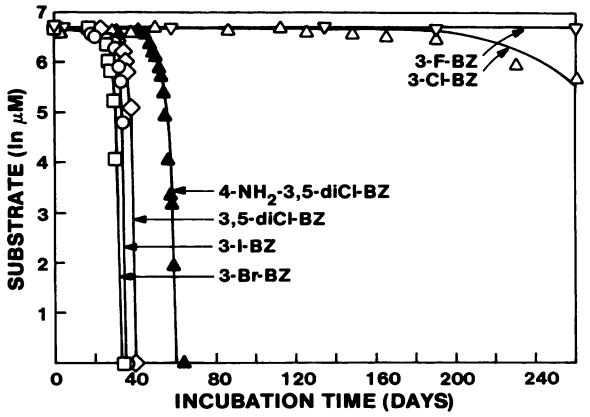

FIG. 3. Acclimation period and degradation pattern exhibited by various halobenzoates at initial concentrations of 800 μM.

The observed acclimation periods were reproducible for the studies done in two different years (Table 1), except for the lower concentrations of 3-F-BZ and the higher concentrations of 4-NH_2-3,5-diCl-BZ. Furthermore, sediment samples taken from three different sites in the lake and one sediment sample that had been stored for 2 years showed the same acclimation period for that chemical (Table 2). Thus, the acclimation period appears to be characteristic for the particular chemical used.

Comparisons of sediment and sludge. Halobenzoate metabolism differed with respect to substrate specificity and extent of degradation in the two habitats. No parent substrate was detected in sediment at the end of the experiment (300 days) with all concentrations of 3-Br-BZ, 3-I-BZ, 3,5-diCl-BZ, and 3-Cl-BZ or with the 40 to 800 μM levels of 4-NH_2-3,5-diCl-BZ. Sludge could completely degrade all concentrations of 3,5-diCl-BZ, the three lower concentrations of 3-Cl-BZ, and the two lower concentrations of 3-Br-BZ. For low concentrations (20 to 40 μM) of 3-Cl-BZ, 3-Br-BZ, and 3,5-diCl-BZ, the approximate acclimation times in sludge were 80 to 100 days, 125 days, and 30 to 122 days, respectively. For 400 μM 3-Cl-BZ and 400 to 800 μM 3,5-diCl-BZ, the acclimation time was approximately 125 to 170 days. The longer acclimation times observed for sludge than for sediment may reflect the fact that sludge is not a good source of organisms capable of halobenzoate degradation and that

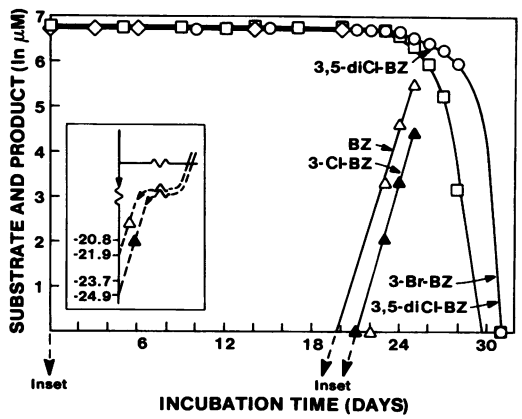

FIG. 4. Daily measurement of dehalogenation of 3-Br-BZ and 3,5-Cl-BZ to benzoate (BZ) and 3-Cl-BZ, respectively. (Inset) Extension of the benzoate and 3-Cl-BZ product lines to the interception of the ordinate. Also shown are the extrapolated concentrations at zero time and day 1, which were used to estimate the first-day rates.

TABLE 2. Average acclimation period for 3-Br-BZ dehalogenation in sediments taken from three different locations and from one stored sample

Sediment site	Avg acclimation period (wks)[a]
1	5
2	4–5
3	4–5
2 (stored for 2 yrs)	4–6

[a] Data are from 10 replicate bottles. The acclimation period was estimated as the time until first appearance of dehalogenated product. A few samples had a longer acclimation period, but none had shorter ones; the longer periods are probably the result of the common problem of establishing activity in all replicate bottles after transfer.

sludge benzoate degraders may not readily adapt to the 20°C incubation temperature.

Correlation of acclimation time with biodegradation rate. Estimates of the initial dehalogenation rate (immediately after the acclimation period) ranged from about 2 to 5 μM/day for 3-Cl-BZ decay to 110 μM/day for 3-Br-BZ disappearance (Table 3). The dehalogenation rates achieved after enrichment by refeeding four times were about three to four times greater than the initial degradation rate estimates. A comparison of the rate data (initial and enriched) with the mean acclimation times for the various chemicals (Table 3) shows that the biodegradation rates were highly correlated (inversely) to the length of the acclimation period (Fig. 5).

DISCUSSION

The extensive temporal data in this study provide documentation of the earlier indications (8, 21) that lengthy acclimation periods are typical before the onset of halobenzoate degradation in anaerobic lake sediments. This acclimation period was characteristic for the chemical tested and was a function of concentration for three of the six chemicals studied. Two of these chemicals (chlorobenzoate and fluorobenzoate) showed that longer acclimation periods corresponded to higher halobenzoate concentrations. This may be attributable to the toxicity of the halobenzoates, since benzoates are known to be bacteriostatic. Increased acclimation periods as a function of high substrate concentration have also been detected during the anaerobic microbial metabolism of one- and two-carbon halogenated solvents (4, 5).

TABLE 3. Rates of biodegradation in fresh and enriched sediment compared with mean acclimation times before the onset of degradation

Substrate	Concn (μM)	Initial rate (μM/day)[a]	Enriched rate (μM/day)[b]	Mean acclimation period (days)[c]
3-Br-BZ	800	110	477	23
	400	84	499	23
3-I-BZ	800	78	331	28
	400	73	337	25
3,5-DiCl-BZ	800	80	198	31
	400	87	208	30
4-NH_2-3,5-diCl-BZ	800	56	124	37
	400	33	79	30
3-Cl-BZ	800	5	14	137
	400	3	24	137

[a] Regression coefficients > 0.95.
[b] Regression coefficients > 0.97; measured after four consecutive refeeding periods.
[c] Acclimation times are averaged from replicates of the second-year experiment.

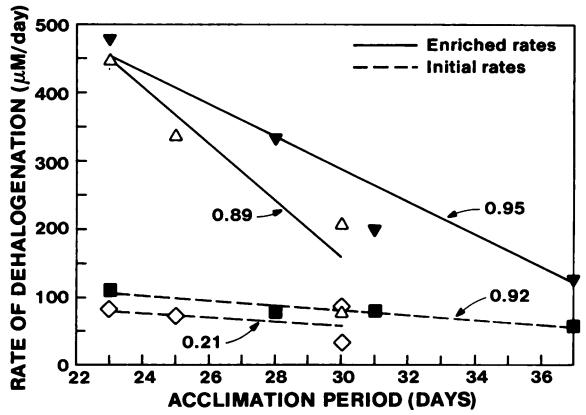

FIG. 5. Correlation of the mean acclimation period with the initial and enriched rates of dehalogenation of 800 μM (solid symbols) and 400 μM (open symbols) for 3-Br-, 3-I-, 3,5-Cl-, and 4-NH_2-3,5-diCl-BZ added to Wintergreen Lake sediment. The r^2 value is shown with each line.

The infinitely long acclimation period seen for the low concentration of 4-NH_2-3,5-diCl-BZ is more intriguing. Since this concentration is rapidly metabolized in sediments amended with higher concentrations of this substrate, the low concentration itself was not limiting for dehalogenation. This suggests that there may be a threshold concentration (≈20 μM) required for induction of the dehalogenating activity. Reber has shown that the induction of aerobic, benzoate-oxidizing activity by chlorobenzoates also has a threshold value (13). The observed threshold suggests that some type of concentration-dependent receptor is necessary to activate dehalogenation.

The acclimation period observed in this study could be due to either a "true" acclimation, i.e., a period of no biodegradation followed by initiation and acceleration of degradation, or an "apparent" acclimation, in which biodegradation would proceed from the time of addition but at rates so slow as to be nondetectable. A true acclimation could be caused by the time required for genetic changes, induction of new protein synthesis, or exhaustion of preferential substrates. An apparent acclimation would probably be the case if the initial population was very small but grew continuously until, at some point, it was large enough to result in detectable biodegradation. This was the explanation for acclimation periods in a recent study concerning the aerobic biodegradation of two aromatic compounds in lake water and sewage (23).

Insufficient analytical sensitivity is a particular problem when measuring substrate disappearance, since detecting small losses against a large background is difficult. To partially overcome this analytical limitation, we were able to measure product appearance and avoid background quantities. Even with this approach we could still not directly measure rates of dehalogenation during the acclimation period, but we were able to extrapolate rates in this period to evaluate whether growth from time zero was a reasonable interpretation. If the exponential rate measured was constant from the time of addition, the highest possible per-cell

activities (assuming one organism per 100 ml of sediment studied) would be 3.6×10^{-12} and 6.2×10^{-11} μmol per cell per day for 3-Cl-BZ and BZ production, respectively (Fig. 4). This activity is 28,000 and 1,600 times, respectively, less than the 0.1 pmol per cell per day found for strain DCB-1, the chlorobenzoate-dechlorinating organism that we have studied in pure culture (16). On this basis, we reason that there was probably no initial activity; therefore, a "true" acclimation occurred, which was followed at some later time by the induction of a more substantial per-cell dehalogenating activity. The latter event then led to the measured exponential increase in activity. Further evidence against a continuous growth response is the fact that the dehalogenation reactions studied led to the accumulation of the dehalogenated product. Thus, no carbon was produced to support a typical growth response. The fact that benzoate accumulated (Fig. 4), which is a more readily degradable anaerobic substrate (8), suggests that a benzoate-degrading population had not been enriched during the acclimation period. It is only after dehalogenation is complete that benzoate begins to be consumed (8), and thus it serves as a carbon source. Thus, an explanation that entails continuous benzoate production throughout the acclimation period is unlikely.

As indicated above, a true acclimation could be caused by genetic change, induction, or exhaustion of preferential substrates. If a single genetic change were responsible, a greater randomization of the acclimation times would have occurred, as has been documented for other mutations (11). Since the acclimation was constant with time and site and among replicates a single genetic change is ruled out. However, the occurrence of multiple genetic changes could arguably direct events towards a mean yielding a more reproducible acclimation period. We feel that the apparent simplicity of the dehalogenation step (16) precludes the necessity for multiple genetic changes. A delay until preferential substrates are exhausted is also an unlikely explanation since the stored sediment, which should have less available substrates than fresh sediments, had the same acclimation time as freshly collected sediment. Furthermore, samples collected at different times would not be likely to have the same nutrient status (23), and thus a more variable acclimation period would be noted if substrate switching was the main explanation. Thus, an induction phenomenon remains as the most reasonable explanation. The specificity between chemical structure and acclimation time, and in some cases between concentration and acclimation time, is the type of response expected from inducing substrates. This specificity also argues against environmental explanations for the delay in dehalogenation, such as lack of nutrients and the presence of inhibitors or predators.

While the points stated above support induction as the explanation for at least part of the acclimation time, the correlation of these periods with the degradation rate would be expected for a continuous growth response. The correlation, however, may reflect the relative energy available to accomplish the induction of new protein synthesis as well as to initiate growth, which is consistent with the induction-followed-by-growth interpretation. The very lengthy acclimation periods are understandable when one considers the extremely limited energy available to anaerobes, especially when restricted to natural sediment substrates. Dechlorination itself, however, might provide some energy, as was recently suggested for the dechlorination of 3-Cl-BZ by strain DCB-1 (7). This opportunity to use a previously unrecognized electron acceptor may provide the stimulus for induction of the appropriate enzymes and then growth that eventually results in an enriched dehalogenating community.

While an induction period followed by an increase in activity appeared to explain the lengthy acclimation period for dehalogenation of benzoates in these sediments, other explanations for delays in biodegradation are probably important in other situations. Because of its lengthy nature, especially in anaerobic environments, the acclimation period cannot be ignored in understanding and predicting the transport and fate of these and probably other pollutant chemicals which may reside in anaerobic environments. In addition, short-term evaluations of anaerobic biodegradation potential can lead to erroneous conclusions as to whether particular chemicals can be metabolized.

ACKNOWLEDGMENTS

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