Evidence for degradation of 2-chlorophenol by enrichment cultures under denitrifying conditions

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Although chlorophenol (CP) degradation has been studied, no bacterium responsible for degradation of CP under denitrifying conditions has been isolated. Moreover, little substantial evidence for anaerobic degradation of CPs coupled with denitrification is available even for mixed cultures. Degradation of CP [2-CP, 3-CP, 4-CP, 2,4-dichlorophenol (DCP) or 2,6-DCP] under denitrifying conditions was examined in anaerobic batch culture inoculated with activated sludge. Although 3-CP, 4-CP, 2,4-DCP and 2,6-DCP were not stably degraded, 2-CP was degraded and its degradation capability was sustained in a subculture. However, the rate of 2-CP degradation was not significantly enhanced by subculturing. In 2-CP-degrading cultures, nitrate was consumed stoichiometrically and concomitantly during 2-CP degradation, and a dechlorination intermediate was not detected, suggesting that 2-CP degradation was coupled with nitrate reduction. A 2-CP-degrading enrichment culture degraded 2-CP in the presence of nitrate, but did not in the absence of nitrate or the presence of sulfate. This suggests that the enrichment culture strictly requires nitrate for degradation of 2-CP. The apparent specific growth rate of the 2-CP degrading species was 0.0139 d^{-1} . Thus the apparent doubling time of the 2-CP-degrading population in the enrichment culture was greater than 50 d, which may explain difficulty in enrichment and isolation of micro-organisms responsible for CP degradation under denitrifying conditions.

Keywords: anaerobic degradation, chlorophenols, denitrification, enrichment culture

INTRODUCTION

Anaerobic processes are beneficial for eliminating pollutants from contaminated sites, in which oxygen is often unavailable due to its quick depletion with easily utilizable substrates, low solubility in water and low rate of transportation in saturated porous matrices such as soils and sediments. Denitrifying bacteria, which are basically categorized as aerobes, have received attention because they could be active under anoxic conditions. Their facultative trait allows them to have a more extensive range of habitats with different oxygen concentrations than other microbial groups. Moreover, their potential to degrade hazardous pollutants such as

aromatic hydrocarbons, phenols and benzoate derivatives has been reported (Braun & Gibson, 1984; Nozawa & Maruyama, 1988; Dolfing *et al.*, 1990; Fries *et al.*, 1994; Frazer *et al.*, 1995; Rabus & Widdel, 1995; Heider & Fuchs, 1997; van Schie & Young, 1998; Rockne *et al.*, 2000). Thus, it has sometimes been considered that denitrifying processes would be an alternative clean-up strategy in sites contaminated with pollutants.

Chlorophenols (CPs) are common environmental pollutants arising from the extensive use of wood preservatives, herbicides and fungicides (Valo *et al.*, 1985), and are also found in pulp bleaching effluents and industrial wastewater (Kringstad & Lindström, 1984; Valo *et al.*, 1984). Anaerobic degradation of CPs has mainly been studied under methanogenic conditions in anaerobic sediments (Zhang & Wiegel, 1990) with microbial consortia obtained from various environments (Boyd & Shelton, 1984; Gibson & Suflita, 1986; Mikesell & Boyd, 1986; Genthner *et al.*, 1989a; Madsen & Aamand, 1992;

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Abbreviations: CP, chlorophenol; DCP, dichlorophenol.

Mohn & Kennedy, 1992; Nicholson *et al.*, 1992; Takeuchi *et al.*, 2000) and anaerobic reactor systems (Woods *et al.*, 1989; Juteau *et al.*, 1995). These studies have indicated that CPs are initially dechlorinated to less chlorinated phenols via a reductive dechlorination, and, then, ultimately mineralization to methane and CO₂ in some cases. Recently, some anaerobic bacteria capable of aryl-dechlorination have been obtained from methanogenic enrichments (Madsen & Licht, 1992; Cole *et al.*, 1994; Utkin *et al.*, 1995; Sanford *et al.*, 1996). A few studies have been conducted to investigate the anaerobic degradation of chloroaromatics under reducing conditions other than methanogenic conditions such as denitrifying conditions, sulfate-reducing conditions and iron reducing conditions (Genthner *et al.*, 1989b; Häggblom & Young, 1990, 1995; Häggblom *et al.*, 1993; Kazumi *et al.*, 1995). These studies established sulfate- and iron-reducing enrichments capable of degrading CPs, and demonstrated that the anaerobic degradation of CPs was coupled with sulfate reduction (Genthner *et al.*, 1989b; Häggblom & Young, 1990, 1995; Häggblom *et al.*, 1993) or iron reduction (Kazumi *et al.*, 1995). Anaerobic degradation of 2-CP was observed in a sediment or a water sample incubated with nitrate (Genthner *et al.*, 1989b; Häggblom *et al.*, 1993), but the capability could not be sustained by re-feeding with 2-CP or by transferring the sample culture to fresh medium. Thus, there has been no substantial evidence for anaerobic degradation of CPs coupled with denitrification; thereby CPs have been regarded as recalcitrant under denitrifying conditions until now. Obtaining evidence of CP degradation under denitrifying conditions was the aim of this study. First, we attempted to establish enrichment cultures with sustained capability to degrade CPs under denitrifying conditions. Second, the kinetics and physiological characteristics of the enrichment cultures were quantitatively evaluated with respect to CP degradation under denitrifying conditions.

METHODS

Sludge samples. Five activated sludge samples were collected from different wastewater treatment plants. To deplete indigenous carbons, an aliquot (0.5 g dry wt) of sludge sample was incubated in a tightly sealed 68 ml serum bottle containing 50 ml mineral salt medium (MSM) with 5 mM nitrate at 25 °C. After 1 week incubation, the supernatant was discarded after centrifugation and a freshly prepared medium of the same composition was added to the precipitate. This procedure was performed twice. All the biomass in the bottle was harvested by centrifugation (7000 g for 10 min) and used as an inoculum for enrichment cultures. The harvested biomass was transferred into an anaerobic chamber (COY), in which anaerobic handling was conducted, and suspended in a fresh medium anaerobically prepared as described below. The anaerobic chamber was filled with a gas mixture of 80% N₂/10% CO₂/10% H₂.

Media. The culture medium was prepared by strictly anaerobic techniques and used throughout the study. The MSM used in this study was composed of 2 g Na₂HPO₄, 1.0 g KH₂PO₄, 0.5 g NH₄Cl and 0.043 g NaNO₃ in 1 l distilled water. After autoclaving, the medium in the reservoir was quickly chilled

with running water under flushing N₂ that is passed through an oxygen-trap filter (Chemical Research Supplies), and then 1 ml EDTA-chelated trace element mixture, 1 ml alkaline-earth-metal solution (100 g MgCl₂·6H₂O, 25 g CaCl₂·2H₂O in 1 l distilled water), 1 ml Na₂SeO₃/Na₂WO₄ solution (Widdel & Bak, 1992), vitamin solutions (Widdel & Bak, 1992) and 5 ml bicarbonate solution (84 g NaHCO₃ in 1 l distilled water) were added to 1 l MSM in an anaerobic chamber. The trace element solution was composed of 5.2 g EDTA, 0.45 g FeCl₂·4H₂O, 52 mg ZnCl₂, 0.19 g CoCl₂·6H₂O, 0.1 g MnSO₄·5H₂O, 24 mg NiCl₂·6H₂O, 29 mg CuCl₂·2H₂O, 36 mg Na₂MoO₄·2H₂O and 30 mg H₃BO₃ in 1 l distilled water. As a reducing agent, Na₂S·9H₂O was added to the medium as a stock solution (0.2 M) to a final concentration of 0.2 mM. The pH of the media was adjusted to 7.2. The stock solutions (0.5 M) of CPs were prepared by dissolving them in 0.2 M NaOH.

Enrichment culture. An aliquot of the sludge biomass (20 mg dry wt), pre-treated as mentioned above, was transferred to a 68 ml serum bottle containing 50 ml MSM with 5 mM nitrate in an anaerobic chamber. The bottle was tightly sealed with a butyl rubber stopper and an aluminium seal before exposure to ambient air. Thus, the resultant headspace of the bottle was nominally the same as that of the anaerobic chamber. A filter-sterilized stock solution of either 2-CP, 3-CP, 4-CP, 2,4-dichlorophenol (DCP) or 2,6-DCP was injected using a syringe through the butyl rubber stopper to provide a final concentration of 0.1 mM in the anaerobic chamber. To avoid oxygen contamination during the withdrawal of culture samples, the headspaces of the bottles were kept under positive pressure by an additional supply of oxygen-free nitrogen up to 20–30 KPa. All cultures were incubated in the dark at 25 °C. When all the CP supplied to the bottle was degraded, 10 ml of the CP-degrading culture in the bottle was transferred into 68 ml bottles containing 40 ml freshly prepared MSM with 5 mM nitrate. The same CP species was added to bottles inoculated with a CP-degrading culture to a final concentration of 0.1 mM, and no CP was added to the other bottles to serve as a control.

Degradation of 2-CP under other anaerobic conditions. Biomass from the enrichment culture that degraded 2-CP in the once-transferred cultures was collected by centrifugation (7000 g for 10 min) from a bottle containing 50 ml culture broth and washed three times with nitrate-free MSM to remove nitrate. The washed biomass pellet was placed in the anaerobic chamber and suspended in nitrate-free MSM. Fifty millilitres of the biomass suspension was transferred to each of six 68 ml serum bottles and 2-CP added to a final concentration of 70 µM. Of the six bottles, two were anaerobically spiked with nitrate (5 mM), two with sulfate (5 mM) and the remaining two with neither nitrate nor sulfate.

Analytical procedures. During incubation, 1 ml of each culture fluid was anaerobically collected several times using a syringe injected through a butyl rubber stopper. Sampling was carried out in the anaerobic chamber. Samples were filtered through a PVDF 0.22 µm pore-size Millipore filter and stored at –20 °C until use. CPs were analysed using a high-performance liquid chromatograph (Hewlett Packard) equipped with a C-18 column and a diode-array detector for monitoring substances at 280 nm. A mixture of water/methanol/acetic acid (40:60:1, by vol.) was used as a running buffer for HPLC analysis, the flow rate of which was 1 ml min^{–1}. Nitrate and nitrite were analysed using an ion chromatograph (IC7000, Yokogawa) equipped with a conductivity detector and an anion exchange column, with 3 mM sodium bicarbonate

buffer at a flow rate of 1 ml min⁻¹. Nitrous oxide was quantitatively sampled using a gas-tight syringe and analysed using a gas chromatograph (HP5890, Hewlett Packard) equipped with ECD and 3 m stainless-steel column (3.2 mm external diameter) packed with Porapak Q50/80. A gas mixture comprising 95 % argon and 5 % methane was used as the carrier gas, the flow rate of which was 30 ml min⁻¹.

RESULTS AND DISCUSSION

Sustained 2-CP degradation capability under denitrifying conditions

The activated sludge samples collected from five different wastewater treatment plants were anaerobically incubated with nitrate and one of the CPs 2-CP, 3-CP, 4-CP, 2,4-DCP or 2,6-DCP. Of the five CPs examined in this study using five sludge samples, 2-CP and 3-CP were degraded in 207 d, but the others (4-CP, 2,4-DCP and 2,6-DCP) were not. As shown in Table 1, 3-CP was degraded faster, but by fewer sample cultures than 2-CP. It appears that the number of substituted chlorine molecules on an aromatic ring, as well as their positions, could be factors affecting CP degradation under the given conditions. All the activated sludge samples tested degraded monochlorophenols, but not DCPs. Among the monochlorophenols used, 2-CP was susceptible to degradation by the largest number of samples while 3-CP was most quickly degraded by two samples.

CP-degrading cultures were transferred to a freshly prepared medium of the same composition in terms of the added CPs, and incubated under denitrifying conditions. In the once-transferred cultures (first subculture), the capability of 2-CP degradation by the four en-

richment cultures originally inoculated with sludges B, C, D or E was successfully retained (Table 1). Further, 2-CP degradation was also observed in two of the four enrichment cultures (originally inoculated with sludges B and C) when they were transferred for a second time (second subculture) (Table 1). On the other hand, with the transfer of two 3-CP-degrading cultures to a freshly prepared medium, their 3-CP degradation capability was not retained.

During the degradation of 2-CP in original and transferred cultures, no UV-absorbable intermediates such as phenol or benzoate were detected by HPLC analysis. This suggests that 2-CP degradation in our enrichment cultures is distinguished from transformation of CPs to less- or non-chlorinated phenol via a reductive dechlorination usually observed under methanogenic conditions. To our knowledge, it has rarely been observed that a microbial enrichment culture capable of degrading CP is sustained under denitrifying conditions, which has limited the microbiological study of this subject. Thus, these 2-CP-degrading cultures obtained here may be good materials for further work on this subject.

Stoichiometry of 2-CP degradation and nitrate reduction

Fig. 1(a) shows that the 2-CP-degrading culture originally inoculated with sludge C, which was transferred twice to a freshly prepared medium (second subculture), degraded 0.1 mM 2-CP in 168 d. As shown in Fig. 1(b), nitrate was consumed concomitantly during 2-CP degradation, and nitrite transiently accumulated. Another intermediate in the denitrification pathway, nitrous

Table 1. Anaerobic degradation of CPs by primary and transferred cultures under denitrifying conditions

NT, Not tested.

Substrate*	Sludge used for inoculum	Time required for degrading 0.1 mM CP (d)†		
		Original enrichment	First subculture‡	Second subculture‡
2-CP	B	88–105 (2/2)	56–94 (8/8)	202 (2/2)
	C	88–105 (2/2)	56–148 (8/8)	168–214 (7/7)
	D	88–207 (2/2)	78–375 (7/7)	NT§
	E	68–207 (2/2)	56–327 (6/7)	NT
3-CP	A	17 (1/2)	(0/4)	NT
	D	17 (1/2)	11 (1/4)	(0/4)¶

* Only CP species which were degraded in this study are listed.

† No. positive cultures/total no. replicate cultures is shown in parentheses.

‡ When CP added was completely degraded, 10 ml enrichment was transferred into 40 ml fresh medium and then CP (0.1 mM) was added.

§ Replicates of first subculture were not transferred.

|| No degradation was found in 428 d.

¶ No degradation was found in 395 d.

Fig. 1. Degradation of 2-CP and consumption of nitrate in the second subculture derived from activated sludge C. Symbols: 2-CP (●), nitrate (■, □) and nitrite (▲, △) in the presence (■, ▲) and the absence (□, △) of 2-CP; electrons consumed via nitrate reduction (◇); 2-CP degraded (◆). Error bars represent standard deviations from triplicate (with 2-CP) and duplicate (without 2-CP) cultures.

oxide, was also detected in test cultures to which 2-CP was added (Table 2). It is notable that the calculated number of electrons consumed via denitrification appeared to be stoichiometric to 2-CP degradation all the way through the experiment (Fig. 1c). Nitrate cannot be assimilated as it is but should be reduced to ammonia before assimilation. Thus, the growing population of bacterial cells in a 2-CP-degrading culture would preferentially assimilate the ammonia (10 mM) added to the media. Rice & Tiedje (1989) demonstrated that ammonium salts contained in the medium at a high concentration inhibited assimilatory nitrate reduction. Therefore it is more likely that nitrate would be consumed during denitrification than during assimilation. Even though nitrate was used for assimilation of cellular materials, its level is much lower than that used as an electron acceptor in the degradation of 2-CP. Assuming all of the 2-CP (5 μmol) and nitrate could each be solely assimilated into the cell biomass composed of $\text{C}_5\text{H}_7\text{O}_2\text{N}$ (Hoover & Porges, 1952), 6 μmol nitrate could be consumed. The actual observed amount of

nitrate consumed by each 2-CP-degrading culture (Table 2) was much larger than this value. Thus, the amount of nitrate consumed was not explained in terms of assimilation, but in terms of another mechanism, i.e. denitrification.

Table 2 shows that 23.5–41.5 μmol nitrate was consumed, although 2-CP was not added to the cultivation vials, and significantly transformed to denitrification intermediates, such as nitrite (50.3–95.7%) and nitrous oxide (0.4–8.8%). Cultures to which 2-CP was added consumed more nitrate; however, percentages of denitrification intermediates formed from nitrate, such as nitrite (0%) and nitrous oxide (0.4–10.5%), were much smaller than those found in vials to which 2-CP was not added. These results indicate that each enrichment culture has an endogenous denitrification activity that was apparently enhanced by the addition of 2-CP. The number of electrons consumed via nitrate reduction was calculated for each culture using the amount of consumed nitrate and accumulated intermediates (nitrite and nitrous oxide) after the 2-CP was completely degraded (Table 2). In the calculation, it was presumed that nitrate was completely consumed via dissimilatory denitrification.

Regarding results obtained for a 2-CP-degrading denitrifying culture originally inoculated with sludge C, it was estimated that a mean of 143.8 μmol electrons was consumed by denitrification (Table 2). The theoretical amount of electrons produced via 2-CP oxidation was also calculated for each 2-CP degrading culture under denitrifying conditions (Table 2). In theory, complete oxidation of 1 μmol 2-CP would provide 26 μmol electrons. For the 2-CP-degrading culture originally inoculated with sludge C, 127.4 μmol electrons would theoretically be produced from 4.9 μmol 2-CP consumed, which is similar to the estimated amount of electrons consumed (143.8 μmol). As shown in Table 2, the amount of electrons consumed via dissimilatory nitrate reduction was similar to that theoretically produced by 2-CP degradation for other cultures, suggesting that nitrate reduction was apparently accompanied by 2-CP degradation. Other previous studies showed anaerobic degradation of 2-CP in denitrifying enrichment cultures (Genthner *et al.*, 1989b; Häggblom *et al.*, 1993), however, these studies did not demonstrate the consumption of nitrate during degradation of 2-CP, which may suggest that the substrate used was coupled to denitrification. The present study would provide information on this aspect.

Apparent specific growth rate of micro-organisms responsible for 2-CP degradation

The shape of the 2-CP degradation curve shown in Fig. 1(a) indicates that the 2-CP degradation rate increased with incubation time, possibly as a result of growth of micro-organisms responsible for 2-CP degradation and/or release from substrate inhibition. The experimentally obtained time course data for 2-CP degradation by the enrichment culture originally inoculated

Table 2. Estimation of electrons consumed by denitrification in denitrifying 2-CP-degrading consortia, and theoretical amount of electrons produced from 2-CP degradation

Data used were obtained from first subcultures in inocula D and E, and second subcultures in inocula B and C. All parameters were obtained after 2-CP was completely degraded.

Sludge used for inoculum	2-CP added (μmol)	No. replicates	2-CP degraded (μmol)	Nitrate consumed (μmol)	Nitrite produced (μmol)	Nitrous oxide produced (μmol)	Electrons theoretically produced from 2-CP (μmol) [*]	Electrons consumed by nitrate reduction (μmol) [†]		Electrons consumed/produced (%)
								Overall	Net [‡]	
B	20.5§	2	20.5	147.0 $\pm$ 67.2	0	15.47 $\pm$ 0.1	533.0	727.3 $\pm$ 403.0	654.4	122.7
	None	1	None	23.5	22.5	0.1	None	72.9	0	0
C	4.9	3	4.9	51.0	0	2.9 $\pm$ 0.1	127.4	249.3 $\pm$ 11	143.8	112.8
	None	2	None	30.8 $\pm$ 0.4	15.5 $\pm$ 2.1	0.9 $\pm$ 0	None	105.5 $\pm$ 8.1	0	0
D	5.0	3	5.0	56.3 $\pm$ 5.3	0	0.2 $\pm$ 0.2	130.0	281.2 $\pm$ 26.9	143.6	110.5
	None	1	None	41.5	22.0	1.94	None	137.6	0	0
E	4.8	2	4.8	53.5 $\pm$ 2.1	0	3.2 $\pm$ 1.0	125.8	256.1 $\pm$ 12.7	158.5	126.1
	None	1	None	41.0	34.0	0.2	None	102.5	0	0

^{*} Amounts of electrons produced from 2-CP were calculated by assuming complete oxidation of 2-CP to CO_2 . One micromole of 2-CP presumably provides 26 μmol electrons.

[†] Values were calculated as: $(\text{NO}_3^- \text{ consumed} - \text{NO}_2^- \text{ accumulated} - 2 \times \text{N}_2\text{O accumulated}) \times 5 + (\text{NO}_2^- \text{ accumulated}) \times 2 + \text{N}_2\text{O accumulated} \times 8$.

[‡] Values were calculated as (overall electrons consumed by nitrate reduction with 2-CP) – (overall electrons consumed by nitrate reduction without 2-CP).

§ When 5 μmol 2-CP was completely degraded, 15 μmol 2-CP was additionally supplied to the culture.

Fig. 2. Anaerobic degradation of 2-CP by a microbial consortium inoculated with sludge E (a), and with sludge D (b) with nitrate (●) or sulfate (○), or without nitrate or sulfate (□). Bars represent the standard deviation from duplicate cultures.

with sludge C could be fitted with equation 1, which assumes exponential growth of 2-CP-degrading microbial population:

$$(S_0 - S) = k(e^{\mu t} - 1) \quad (1)$$

where S_0 is the initial substrate concentration, S is the substrate concentration at the incubation time period t , k is the initial 2-CP degradation rate at time 0 and μ is the specific growth rate of micro-organisms responsible for 2-CP degradation. It is notable that the apparent specific growth rate of the 2-CP-degrading microbial population was extremely slow, 0.0139 d^{-1} , representing an apparent doubling time of 50.2 d for the population. This result indicates that the 2-CP degrading microbial population has substantially multiplied no more than three times by incubating the enrichment culture for 150 d, which increased the biomass of micro-organisms responsible for 2-CP degradation by only eight times. This may explain why the incubation period required for degrading 0.1 mM 2-CP was not reduced when 20 %

of the culture was transferred to a freshly prepared medium (Table 1). Thus, a longer incubation of subcultures would be required to obtain an enrichment culture adequate for isolating the putative 2-CP-degrading organisms, and further microbiological studies on this subject would be difficult because of such a slow rate of 2-CP degradation.

Degradation of 2-CP under other anaerobic conditions

To examine whether 2-CP utilizing enrichment cultures degrade 2-CP under other anaerobic conditions, four first subcultures originally inoculated with sludges B, C, D and E were incubated in an anaerobically prepared medium with nitrate (5 mM) or sulfate (5 mM), or without nitrate or sulfate. The culture originally inoculated with sludge E demonstrated that 2-CP was degraded in the presence of nitrate in 147 d, but was not degraded under other conditions (Fig. 2a). This indicates

that the enrichment culture required nitrate for anaerobic degradation of 2-CP. Based on this result and the stoichiometry of 2-CP degradation and nitrate consumption (Table 2), it is likely that the anaerobic degradation of 2-CP in this enrichment culture was strictly dependent on denitrification.

Sanford & Tiedje (1997) reported a degradation pathway of 2-CP in anaerobic microcosms supplemented with nitrate. They demonstrated that 2-CP was transformed to phenol by a reductive dechlorination, and the resulting phenol was subsequently degraded coupled with denitrification. They observed that dechlorination was inhibited by 5 mM or higher nitrate, and the reaction proceeded without nitrate. On the other hand, we have observed 2-CP degradation in the presence of 5 mM nitrate, and no accumulation of dechlorination intermediates in enrichment cultures. Furthermore, 2-CP degradation and putative electron consumption via denitrification proceeded concomitantly and stoichiometrically (Fig. 1), unlike Sanford & Tiedje (1997) observed. Thus, it is unclear that reductive dechlorination activity was involved in the degradation of 2-CP in our enrichment culture especially derived from sludges C and E, and it appears that the mechanism of 2-CP degradation which we observed in this study could be different from what Sanford & Tiedje (1997) observed. These observations suggest the presence of a denitrifying population capable of degrading CP by a mechanism not involving reductive dechlorination. As for enriching conditions, ours retained nitrate during incubation, but theirs did not and was in a more reduced condition during most of the incubation period. Thus, denitrifying conditions may have been well maintained in our samples.

The culture originally inoculated with sludge D degraded 2-CP in the absence of nitrate or sulfate and in the presence of nitrate (Fig. 2b). However, this culture did not degrade 2-CP in the presence of sulfate (Fig. 2b). When 62 μ M 2-CP was degraded in the absence of an electron acceptor, 59 μ M phenol was detected, indicating that 2-CP was reductively dechlorinated. One possible explanation for the observation is that this enrichment culture may have retained two pathways for anaerobic degradation of 2-CP: one dependent on denitrification and the other independent of it. Further examination would be necessary to clarify whether the reductive dechlorination, which is basically a denitrification-independent pathway, occurs in the presence of nitrate. However, since phenol was not detected in the culture supplied with nitrate, it is not really evident whether the enrichment culture in the bottle concomitantly underwent two reactions, reductive dechlorination and denitrifying degradation of 2-CP.

Cultures originally inoculated with sludges B and C did not degrade 2-CP in the presence of nitrate or sulfate or in the absence of exogenous electron acceptors (data not shown), although these cultures successfully degraded 2-CP when they were transferred into a fresh medium with nitrate without washing as described in Table 1. The

reason why 2-CP was not degraded even in the nitrate-added medium is unclear, but one possible explanation may be the inadequate preparation of the inocula. The sludge samples used as inocula were aerobically washed three times with a nitrate-free medium. This procedure may have unintentionally damaged the degradation capability of the micro-organisms or discharged factors required for degradation.

Conclusion

We have demonstrated that denitrifying conditions supported an anaerobic degradation of CPs in enrichment cultures derived from activated sludge samples. Concomitant consumption of nitrate was observed during 2-CP degradation, the amount of which was stoichiometric, assuming that 1) 2-CP was completely oxidized to CO₂ and 2) nitrate was solely consumed as an electron acceptor in denitrification (Fig. 1 and Table 2). This would be evidence demonstrating that 2-CP degradation is coupled to respiratory denitrification. The strict requirement of nitrate for degradation of 2-CP was observed in a 2-CP-degrading enrichment culture under denitrifying conditions (Fig. 2a), which provides support for the involvement of the denitrification process in anaerobic degradation of 2-CP. Although the 2-CP-degrading enrichment cultures under denitrifying conditions were subcultured repeatedly, the degradation rate could not significantly be increased, possibly because the apparent growth rate of the 2-CP-degrading microbial population was extremely slow, the apparent doubling time of which was 50.2 d. The degradation capability of the culture once enriched was easily lost, particularly when the biomass of the enrichment culture was washed under aerobic conditions. Thus, we do not yet know the conditions for properly and stably maintaining a 2-CP-degrading enrichment culture under denitrifying conditions. Studies are still needed to determine the contribution of the denitrification process in the anaerobic degradation of CPs in contaminated environments, however, the extremely slow degradation rate may imply that this process could easily be predominated by other anaerobic processes.

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