Chlorinated solvents cometabolism by an enriched nitrifying bacterial consortium

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Abstract The biodegradability of three of the most frequently halogenated aliphatics (trichloroethene, chloroform and 1.1.1.-trichloroethane) found in drinking water aquifers by a nitrifying enriched mixed biomass was investigated during batch tests. Within this mixed biomass, ammonia oxidisers were the effective degraders. The presence of ammonia stimulated chlorocarbon biodegradation, and the presence of chlorocarbon inhibited ammonia oxidation. This contrasted phenomenon was explained by a balance between electron supply from ammonia necessary to sustain the chlorocarbon oxidation and competitive inhibition for the ammonia monooxygenase active site between both substrates. About 0.03 to 0.2% of the electrons generated by ammonia oxidation were used for chlorocarbon degradation. Trichloroethene and chloroform oxidation induced a biomass inactivation (around 30 to 40 mg of proteins inactivated per µmol of chlorocarbon oxidised). Biomass re-activation due to exergonic ammonia catabolism was estimated to 24±6 mg of proteins reactivated per mmol of ammonia oxidised in both cases. No inactivation of re-activation was observed in the case of 1.1.1-trichloroethane.

Keywords Ammonia; biodegradation; chlorocarbon; co-metabolism; monooxygenase; Nitrosomonas

Introduction
Trichloroethene (TCE), chloroform (CF) and 1.1.1-trichloroethane (TCA) are of the most frequently halogenated aliphatics found in drinking water aquifers, as a consequence of illegal or uncontrolled spillage of industrial wates (Westrick et al., 1984). Because of their widespread distribution and their suspected carcinogenicity (Infante and Tsongas, 1982), they belong to the 129 priority pollutants list established by the US EPA (1979). From economical, environmental or technical considerations, aerobic cometabolic biodegradation constitutes an interesting, possible or complementary alternative to other physical, chemical or biological elimination pathways.

Cometabolism is defined by the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound (Dalton and Stirling, 1982). Aerobic cometabolism of halogenated aliphatics is catalysed by non-specific mono- or di-oxygenase enzymes which are usually designed for the first step of a growth substrate oxidation. This kind of reaction has been demonstrated for methane (Oldenhuis et al., 1989; Alvarez-Cohen and McCarty, 1991a, b; Henry and Grbic-Galic, 1991; Chang and Alvarez-Cohen, 1995, 1996), propane (Wackett et al., 1989 and Malachowsky et al., 1994), phenol (Nelson et al., 1988), toluene (Landa et al., 1994 and McClay et al., 1996) and ammonia (Arciero et al., 1989; Vannelli et al., 1990 and Melin et al., 1996) oxidising bacteria.

Ammonia oxidisers are of particular interest because of their autotrophy (i.e. ammonia and carbonate as sole energy and carbon sources, respectively) which allows for enrichment through mineral feeding and their ubiquity (presence in almost all environments, and particularly in drinking water or wastewater treatment plants). Ammonia oxidisers, e.g. Nitrosomonas, which transform ammonia to nitrite as well as nitrite oxidisers, e.g. Nitrobacter, which transform nitrite to nitrate, belong to nitrifying bacteria (Hooper, 1989).
In *Nitrosomonas europaea* (ATCC 19718), the degradation of chlorocarbon is catalysed by the ammonia monoxygenase (AMO) and corresponds to the incorporation of one oxygen atom from O₂ in the molecule (Vanneli *et al.*, 1990). Ammonia (growth substrate) and the chlorocarbon (non-growth substrate or cometabolic substrate) are in competition for the enzyme active site. In addition, chlorocarbon cometabolic biodegradation generates unstable reactive and toxic intermediates which may irreversibly inactivate the AMO (Rasche *et al.*, 1991). This phenomenon has been expressed as the maximal transformation capacity, i.e. the maximal amount of chlorocarbon degraded per unit of biomass, before its complete inactivation (Alvarez-Cohen and McCarty, 1991a). This corresponds to the inverse of an inactivation yield (amount of biomass inactivated per mole of chlorocarbon degraded). Ammonia oxidisers may recover the activity by the energy dependent AMO neosynthesis process, i.e. through ammonia catabolism (Rasche *et al.*, 1991 and Hyman *et al.*, 1995). However, little information exists about the electron supply: during the chlorocarbon oxidation, the second oxygen atom is reduced to water, requiring two electrons which may come from either endogenous reserves of reducing power or further oxidation steps of ammonia (via hydroxylamine oxidoreductase (HAO)). Since ammonia transformation to nitrite liberates two electrons, and chlorocarbon degradation consumes two electrons, one mole of ammonia oxidised allows theoretically for the degradation of one mole of chlorocarbon.

This work aimed to: (i) study the biodegradability of TCE, CF and TCA by ammonia oxidisers within a mixed culture; and (ii) evaluate four phenomena likely to occur during cometabolism: competitive inhibition (between growth- and non-growth substrate for the AMO active site), biomass inactivation (after chlorocarbon degradation) and re-activation (through ammonia catabolism) and electron supply from endogenous reserves or exogenous substrate (ammonia). Linked to the study of these four phenomena, the complex role of ammonia was deeply investigated by the mean of batch kinetic experiments carried out with various initial ammonia concentrations.

**Material and methods**

**Biomass cultivation and measurements**

An enriched nitrifying biomass obtained from an initial activated sludge inoculum (Morainvilliers, France) was used in these experiments. Culture of microorganisms was carried out in a continuously aerated (80 l/h) and stirred 1.5 litre bioreactor (Biolaffite, France) over several months by daily ammonia and carbonate additions (10.7 and 0.5 mmol/d, respectively) pH was adjusted daily and maintained between 6.9 and 7.8. Mineral growth medium was the one used by Sato *et al.* (1985), except that phosphate buffer concentration was raised to 50 mM and ammonia and carbonate removed from the composition. Biomass concentration was estimated by protein dosage (micro-BCA, Pierce). Preliminary results showed proteins/COD and proteins/VSS ratios of 0.39 and 0.57 mg/mg, respectively (data not shown). Biomass activity (particularly ammonia oxidisers) was determined by differential respirometry, using allylthiourea and azide as selective inhibitors of ammonia- and nitrite-oxidisers, respectively (Ginestet *et al.*, 1998). Results expressed in mgO₂/h/g prot (at pH 7.6 and 20°C, in the presence of 0.71 µM ammonia or nitrite). Endogenous, ammonia dependent- and nitrite dependent oxygen uptake rates were estimated between 9 and 23, 71 and 260 and 30 and 102 mgO₂/h/g prot for experiments reported here, respectively (data not shown).

**Analytic**

Chlorocarbon analysis were performed by gas-chromatography (GC, Carlo Erba GC6000 series coupled to a HP automatic sampler) in a 12 ml vial (2 to 3.5 ml of liquid) and hermetically closed by a silicone-PTFE septum and an aluminium cap. 1.25 ml of the gas phase were sampled and directly injected in the column (JW, megabore DB624, 60 m length, 0.54 mm
section, film 2 μm). Split in the head of the column was 1/20th. Compounds were detected by electron capture (ECF40, 63Ni). Results were interpreted by comparison of peak areas with a standard curve realised under the same conditions with EPA standard solutions (Interchrom, France). Concentrations were reported to the liquid phase (as if the whole compound was present in the liquid phase). pH measurements were done with a WTW pH meter (WTW, Germany). Ammonia, nitrite and nitrate concentration were determined spectrophotometrically using a DU-64 spectrophotometer (Beckman Instruments, USA) (Standard Methods for the Examination of Water and Wastewater, 1976).

Degradation experiments
Batch test for the study of individual cometabolic biodegradation of TCE, CF and TCA by an enriched nitrifying biomass freshly sampled from the fermentor and washed twice in the ammonia-free growth medium by centrifugation (10 min, 2,500 g, 4°C). The last pellet was finally resuspended in the growth medium and distributed in three series of vials and eventually supplemented with ammonia (0 to 15 mM). Some control samples were prepared in the same way but with allylthiourea (86 μM), used as a selective inhibitor of AMO. Reactions were initiated by injection of 10 μl of chlorocarbon (TCE, 98.1%, Prolabo, France; CF, 99%, Prolabo, France; TCA, 99%, Carlo Erba, Italy) with a gas-tight syringe (Hamilton, from a freshly prepared concentrated stock solution, in order to obtain a 4 mM final concentration, except for the third series which did not receive any chlorocarbon (control). Vials were closed immediately and incubated at 21°C in an inverted position on an orbital shaking table (400 rpm). Oxygen was supplied by diffusion from the gas phase to the liquid phase during shaking.

One vial of each series was sampled at different times of incubation. The vials of the first series were placed 30 minutes at 80°C in order to stop the reaction and conserve the samples until used for chlorocarbon analysis. Suspensions of series 2 and 3 were centrifuged twice (3 minutes, 2,500 g). Ammonia, nitrite, nitrate and pH were analysed on the 0.45 μm filtered supernatant. The residual activity of the washed biomass by differential respirometry (10). The third series corresponded therefore as the control with no chlorocarbon. Ammonia oxidisers activities after incubation with (series 2) or without (series 3) chlorocarbon were compared yielding a percent of residual activity, which was related to the biomass concentration in the reaction vial in order to obtain the active biomass concentration (X_{act}). Experimental conditions are summarised in Table 1.

Data analysis
The concentrations of ammonia, nitrite, nitrate, pH and chlorocarbon as well as the biomass activity (with or without chlorocarbon) were followed by sampling at different times of incubation (0 to 8–29 h, among experiments). In order to study the influence of ammonia on the chlorocarbon degradation, specific initial degradation rates (0 to 1–4 h, among experiments) were determined for ammonia and the chlorocarbon. Because ammonia oxidisers activity was variable from one experiment to another, specific degradation rates were normalised to a standard rate of 200 mgO₂/h/g prot, corresponding to the mean of control ammonia oxidisers respiration rate over all experiments. To evaluate the electron transfer efficiency from ammonia to the chlorocarbon, i.e. the percentage of electrons released by ammonia catabolism and used for chlorocarbon degradation, was calculated for each experiment. Inactivation and re-activation of the biomass were evaluated by correlating the active biomass concentration (X_{act}) with the amounts of ammonia (DS) and chlorocarbon (DCC) oxidised. Therefore inactivation- (Y_{CC}) and re-activation- (Y_{S}) yields were defined (Eq. (1)) and determined by multiple linear regression (10 points):

\[ X_{act} = X_0 - Y_{CC} \times \Delta CC + Y_S \times \Delta S \] (1)
Table 1  Individual chlorocarbon (TCE, CF and TCA) cometabolic biodegradation in the presence of various ammonia concentrations

<table>
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<tr>
<th>Chlorocarbon (CC)</th>
<th>Exp#</th>
<th>Conc. (mgprot/l)</th>
<th>Activity a (µM)</th>
<th>Specific initial oxidation rate (µmolO₂/h/gprot)</th>
<th>Electron transfer (%)</th>
<th>Inactivation (mgprot/µmolCC)</th>
<th>Re-activation (mgprot/µmolN)</th>
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<td>1.62 - 0.02</td>
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aActivity of ammonia oxidisers expressed as specific oxygen uptake rate in the presence of 0.71 µM ammonia (Ginestet et al., 1998)

bnot determined
Results and discussion

Preliminary biodegradation experiments showed that no degradation occurred in the presence of allylthiourea while more than 40% of the initial chlorocarbon were removed in its absence, strongly suggesting that AMO is involved in the biodegradation of chlorinated solvents (data not shown).

Effect of ammonia on chlorocarbon degradation rates

As a consequence of cometabolism, ammonia is expected to lead to both competitive inhibition and stimulation of the chlorocarbon degradation by electron supply. This double effect of ammonia is illustrated in Figure 1, where specific initial degradation rates were plotted versus initial ammonia concentration. Degradation rates were significantly increased when ammonia was present and maximal rates were observed for approximately 4.3 mM ammonia for TCE (24 µmol/h/gprot) and CF (10 µmol/h/gprot), and 7.6 mM for TCA (3 µmol/h/gprot) (Figure 1). At higher ammonia concentrations (4–8 mM), chlorocarbon degradation rates were lower (Figure 1).

Specific initial degradation rates of the chlorocarbons were divided by the ones found for ammonia oxidation, yielding the electron transfer efficiency, i.e. the percent of electron generated by ammonia catabolism and effectively used for the chlorocarbon degradation. On average, electron flux generated by ammonia oxidation was used at 0.2 ± 0.09%, 0.14 ± 0.08% and 0.03 ± 0.01% for the biodegradation of TCE, CF and TCA, respectively (Table 1). These very low percentages, may be dependent of the ratio between ammonia and chlorocarbon initial concentration, are in the same order of magnitude as values calculated from previous results (Vannelli et al., 1990). In these experiments (Vannelli et al., 1990), biodegradation rates were higher in the presence of 1 mM ammonia than in its absence, with an electron transfer ratio between 0.4 and 0.8%. However, with methanotrophs (Oldenhuis et al., 1989 and Henry and Grbic-Galic, 1991) and marine ammonia oxidisers (Melin et al., 1996), only a decrease in biodegradation rates was observed in the presence of the growth substrate (methane or ammonia). The hypothesis was that sufficient reducing power reserves were present in the cells and therefore, electron supply by the growth substrate was insignificant in comparison to competition phenomena. Chlorocarbon biodegradation rates are increased by intracellular (reserves) or extracellular (growth substrate oxidation) electron supply and decreased by competitive inhibition due to the growth substrate. Biodegradation rates are maximal when these two phenomena are in equilibrium. As a function of the intracellular reserves of reducing power, the growth...
substrate leads to an increase (low reserves) or a decrease (high reserves) in chlorocarbon biodegradation rates.

Biomass inactivation and re-activation

Since chlorocarbon and ammonia-oxidation are susceptible to influence the activity of ammonia oxidisers, the evolution of active biomass along with time was related to the amounts of ammonia and chlorocarbon oxidised by multiple linear regression, according to Eq. (1) (Figure 2). Thus, for TCE and CF, inactivation and re-activation yields could be determined. For TCA experiment, because of the absence of experimental evidence for inactivation and/or re-activation of the biomass, yields could not be calculated. Among all experiments carried out on TCE and CF, biomass inactivation yields were found to be $29 \pm 7$ (n = 9) mg prot/µmol TCE and $43 \pm 4$ (n = 3) mg prot/µmol CF, and biomass re-activation yields $24 \pm 6$ (n = 6) mg prot/µmol N and $23 \pm 5$ (n=2) mg prot/µmol N, respectively (Table 1).

As a result of respirometric measurement, neither nitrite oxidation nor endogenous respiration were affected during chlorocarbon degradation (data not shown).

The biodegradation of CF and TCE led to an inactivation of ammonia oxidisers. No change in nitrite oxidisers or endogenous activity was detected. The decrease in ammonia oxidisers activity was assimilated to a biomass inactivation and estimated by inactivation yields, corresponding in fact to the inverse function of the transformation capacity used in methanotrophic studies (Alvarez-Cohen and McCarty, 1991a, b; Chang and Alvarez-Cohen, 1995, 1996). Inactivation yields found for methanotrophs are in general 5 to 50 times lower than those we found in our study. This fact has been discussed previously by Ely et al. (1997) because of a double biological and experimental bias: high reducing power reserves in methanotrophs may allow them to use this energy for reactivation of the biomass, i.e. endogenous re-activation of the biomass, and secondly, the use of formate as additional electron supply in these organisms could allow for energy production and thus for MMO neosynthesis, resulting in an additional re-activation of the biomass. In our case, the limited reducing power reserves and the inability of ammonia oxidisers to use an external electron donor (like formate for methanotrophs) could partially explain the apparent higher sensitivity of these organisms to chlorocarbon degradation.

For CF and TCE, re-activation of the biomass was also shown in our results and estimated in terms of re-activation yields, which were found to be very close for both compounds.
(about 25 mg prot/µmolN). This suggests that re-activation is independent of the inactivating compound and that its mechanism is related to the usual turnover rate of enzymes (AMO and possibly HAO) in cells. In its mathematical expression, reactivation yield is similar to a growth yield. Values of re-activation yields were about 20 times higher than theoretical growth yield of ammonia oxidisers (Sharma and Ahlert, 1977). Thus, in our case, re-activation of the biomass is not able to be assimilated to growth, a previously assumed for methanotrophs (Chang and Alvarez-Cohen, 1995).

Neither inactivation nor re-activation of the biomass was detected in TCA experiments. TCA degradation intermediates were toxic for *Nitrosomonas europaea* (Rasche et al., 1991) but not for *Methylosinus trichosporium* OB3b (Oldenhuis et al., 1989). It is possible that in our experiments TCA mediated inactivation (provided that TCA intermediates are toxic) was systematically compensated by re-activation due to ammonia oxidation. Further experiments should be carried out to check this eventuality, e.g. working with higher TCA concentrations.

Conclusion

This study showed that a non-marine nitrifying enrichment is able to biodegrade three among the most frequent chlorinated aliphatics, with the following biodegradability scale: TCE>CF>TCA.

Chlorocarbon biodegradation tests led to the evidence and the quantification for four phenomena occurring during cometabolism: (i) the competitive inhibition between ammonia and chlorocarbon for the AMO active site; (ii) the need of electron supply through growth substrate oxidation and/or intracellular reducing power mobilisation; (iii) the inactivation of the biomass through chlorocarbon toxic intermediates which may be compensated by (iv) ammonia dependent energy supply for biomass reactivation.

This tends to increase the need to further understand and characterise the role of ammonia in cometabolism (competition, electron donor for biodegradation and re-activation), one way being modelling. Particularly, these results show that the electron supply for the biodegradation via ammonia oxidation is an important phenomenon, which should be taken into account in future model developments.

Acknowledgements

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References


