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ORIGINAL ARTICLE

Microbial nitrate-dependent cyclohexane degradation coupled with anaerobic ammonium oxidation

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An anaerobic nitrate-reducing enrichment culture was established with a cyclic saturated petroleum hydrocarbon, cyclohexane, the fate of which in anoxic environments has been scarcely investigated. GC–MS showed cyclohexylsuccinate as a metabolite, in accordance with an anaerobic enzymatic activation of cyclohexane by carbon–carbon addition to fumarate. Furthermore, long-chain cyclohexyl-substituted cell fatty acids apparently derived from cyclohexane were detected. Nitrate reduction was not only associated with cyclohexane utilization but also with striking depletion of added ammonium ions. Significantly more ammonium was consumed than could be accounted for by assimilation. This indicated the occurrence of anaerobic ammonium oxidation (anammox) with nitrite from cyclohexane-dependent nitrate reduction. Indeed, nitrite depletion was stimulated upon further addition of ammonium. Analysis of 16S rRNA genes and subsequent cell hybridization with specific probes showed that approximately 75% of the bacterial cells affiliated with the *Geobacteraceae* and approximately 18% with *Candidatus* 'Brocadia anammoxidans' (member of the Planctomycetales), an anaerobic ammonium oxidizer. These results and additional quantitative growth experiments indicated that the member of the *Geobacteraceae* reduced nitrate with cyclohexane to nitrite and some ammonium; the latter two and ammonium added to the medium were scavenged by anammox bacteria to yield dinitrogen. A model was established to quantify the partition of each microorganism in the overall process. Such hydrocarbon oxidation by an alleged 'denitrification' ('pseudo-denitrification'), which in reality is a dissimilatory loop through anammox, can in principle also occur in other microbial systems with nitrate-dependent hydrocarbon attenuation.
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Introduction

Cyclic saturated hydrocarbons (cycloalkanes) with or without alkyl substituents are common in petroleum (Tissot and Welte, 1984) and refined petroleum products. There are no explicit reports about cycloalkanes produced by living organisms, whereas unsaturated alkyl-substituted cyclic hydrocarbons such as monoterpenes are abundant and structurally diverse plant metabolites (Langenheim, 1994).

Biodegradation of cycloalkanes has been studied to a lesser extent than the biodegradation of *n*-alkanes and aromatic hydrocarbons (Prince, 2002; Van Hamme *et al.*, 2003). Axenic bacterial strains hitherto reported to grow with cycloalkanes were aerobic. Most microbiological studies with cycloalkanes used cyclohexane as a model substrate (Stirling and Watkinson, 1977; Anderson *et al.*, 1980; Trower *et al.*, 1985; Rouvière and Chen, 2003). Cyclohexane is aerobically activated by cyclohexane monooxygenase yielding cyclohexanol, and further oxidized through cyclohexanone, caprolactone and adipate (Perry, 1984; Cheng *et al.*, 2002).

Information about anaerobic biodegradation of cycloalkanes is scarce, whereas anaerobic degradation of *n*-alkanes has become well-established (for overview see reference Widdel *et al.*, 2009). Complete degradation of ethylcyclopentane was shown with a sulfate-reducing enrichment culture (Rios-Hernandez *et al.*, 2003). In addition, anaerobic biodegradation of alkylcyclopentanes, cyclohexane and alkylcyclohexanes under conditions of sulfate reduction or methanogenesis was observed in sediment samples amended with gasoline and gas condensate (Townsend *et al.*, 2004). Anaerobic growth of pure cultures with cycloalkanes has not been observed (for example, Wilkes *et al.*, 2003). An activation of alicyclic hydrocarbons by a

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radical-catalyzed addition to fumarate, analogous to *n*-alkane activation (Kropp *et al.*, 2000; Rabus *et al.*, 2001), was postulated on the basis of metabolite analysis in a sulfate-reducing enrichment culture with ethylcyclopentane (Rios-Hernandez *et al.*, 2003). A denitrifying bacterium growing anaerobically with *n*-alkanes from petroleum co-metabolized cyclopentane and formed cyclopentylsuccinate (Wilkes *et al.*, 2003); cyclopentane did not allow productive growth.

This study was undertaken to show bacteria potentially involved in cycloalkane degradation with nitrate as electron acceptor in anoxic surroundings. Cyclohexane (C_6H_{12}) was used as a prominent unsubstituted representative of cycloalkanes. Cyclohexane is a slightly water-soluble (saturation, 0.68 mM at 25 °C) and relatively volatile (bp, 80.7 °C) hydrocarbon (Eastcott *et al.*, 1988; Dean, 1992). Containing exclusively apolar σ-bonds, cyclohexane is chemically rather unreactive. Still, from a thermodynamic point of view, cyclohexane is a slightly endergonic compound ($\Delta G_f^\circ = +26.7\,\mathrm{kJ\,mol^{-1}}$; for comparison: *n*-hexane, $\Delta G_f^\circ = -3.8\,\mathrm{kJ\,mol^{-1}}$; benzene, $\Delta G_f^\circ = +124.4\,\mathrm{kJ\,mol^{-1}}$). Cyclohexane is less toxic than many aromatic hydrocarbons, but slightly more toxic than volatile *n*-alkanes (Sikkema *et al.*, 1994, 1995). Cyclohexane may enter the environment through petroleum and fuel spills, and due to its use as a solvent and raw chemical (Perry, 1984). It is expected that cyclohexane volatilizes easily in oxic environments at the air, whereas it may tend to persist in 'closed' anoxic environments. While straight-chain saturated and aromatic petroleum hydrocarbons under anoxic conditions in the presence of nitrate often promote enrichment of Betaproteobacteria (Rabus *et al.*, 1999; Ehrenreich *et al.*, 2000; Widdel *et al.*, 2009), the presently obtained enrichment with cyclohexane and nitrate was dominated by a deltaproteobacterium. Growth of this bacterium, the likely degrader of cyclohexane, was accompanied by a member of the Planctomycetales, which was apparently responsible for scavenging nitrite by anaerobic ammonium oxidation (Musat, 2005). Here we present a detailed analysis of the enrichment culture and quantitative model of the microbial interaction.

Materials and methods

Source of organisms, media and cultivation techniques
Anoxic sediment from a lake (Bad Zwischenahner Meer, Germany) was used as a source of bacteria. A 5-ml volume of homogenized sediment was added per 50 ml of defined $NaHCO_3/CO_2$-buffered freshwater medium containing 5 mM NO_3^- and 4.7 mM NH_4^+ (Rabus and Widdel, 1995; Widdel, 2009) in a flat 100 ml bottle. The medium was overlaid with 3 ml of 2,2,4,4,6,8,8-heptamethylnonane (HMN; serving as inert carrier phase) containing 0.8% (v/v) cyclohexane. The bottles were sealed under an N_2–CO_2 mixture (9:1, v/v) with rubber stoppers and incubated at 28°C nearly horizontally so as to maximize the contact area between the medium and the hydrocarbon phase; orifices were kept below the medium surface to avoid contact between the hydrocarbon phase and the stopper (Rabus and Widdel, 1995). Sediment-containing cultures were briefly (few seconds) shaken once per day, whereas sediment-free subcultures were incubated with constant horizontal shaking (50–70 r.p.m.). The inoculum size for all subcultures was 10% (v/v). Growth experiments with highly enriched cultures were performed in flat 120-ml bottles with 100 ml culture medium overlaid with 5 ml HMN containing 0.2 or 0.5% (v/v) cyclohexane. In a growth experiment for determination of the electron balance, a limiting amount of 0.098 mmol (10.6 µl, at 20 °C; 0.21%, v/v) cyclohexane in the carrier phase (5 ml), 0.98 mmol nitrate and 0.95 mmol ammonium were added per 100 ml culture medium. Sterile bottles with cyclohexane and inoculated bottles lacking cyclohexane or nitrate were used as controls.

Determination of the cell mass ratio of two bacteria by microscopy
If in a mixed culture of three microbial types their microscopically counted numbers are N_1, N_2, and N_3, the fraction by numbers of each type is $N_1/(N_1+N_2+N_3)$, and so on (index in nominator from 1 to 3). Designating the average cell volume of each type V_1^{cell}, V_2^{cell} and V_3^{cell}, and assuming the same dry mass/volume conversion factor for each cell type, we obtain for the fraction by mass (dry mass share, biomass share) of each type:

$$[m_1/m_{\mathrm{tot}}]_{\mathrm{micr}} = \frac{V_1^{\mathrm{cell}} N_1}{V_1^{\mathrm{cell}} N_1 + V_2^{\mathrm{cell}} N_2 + V_3^{\mathrm{cell}} N_3}, \text{etc.} \quad (1)$$

Symbol m designates mass, m_{tot} total biomass and index micr the microscopic approach (for distinction from other approaches). If bacterial type-1 and type-2 dominate by biovolume (minor biovolume contribution by the 'accompanying' type-3), we can write for the two dominant types

$$[m_1/m_{\mathrm{tot}}]_{\mathrm{micr}} \approx [m_1/(m_1+m_2)]_{\mathrm{micr}} = \frac{V_1^{\mathrm{cell}} f_1^{\mathrm{num}}}{V_1^{\mathrm{cell}} f_1^{\mathrm{num}} + V_2^{\mathrm{cell}} f_2^{\mathrm{num}}} \quad (2)$$

and

$$[m_2/m_{\mathrm{tot}}]_{\mathrm{micr}} \approx [m_2/(m_1+m_2)]_{\mathrm{micr}} = 1 - [m_1/(m_1+m_2)]_{\mathrm{micr}}. \quad (3)$$

The volume of a spherical cell with diameter d is $V_{\mathrm{sphere}}^{\mathrm{cell}} = \pi d^3/6$. The volume of a rod-shaped cell with rounded (hemispherical) ends, which has diameter d and total length l, is the sum of the volume of the cylindrical part (length, $l-d$) and the volume of a whole sphere, that is, $V_{\mathrm{rounded\ rod}}^{\mathrm{cell}} = \pi d^2(3l-d)/12$.

Chemical analyses
Nitrate and nitrite were measured by high-pressure liquid chromatography with UV (220 nm) detection as described by Rabus and Widdel (1995).

Ammonium was determined colorimetrically by the indophenol formation reaction (Boltz and Taras, 1978).

Cyclohexane concentrations in the carrier phase (HMN) were measured by headspace analysis. The measurement is based on comparison of the cyclohexane content in gas phase samples from the culture and from calibrated samples in bottles, after gas phase equilibration with the carrier phase at constant temperature (28 °C). A 0.1-ml volume of gas phase was withdrawn with an N_2-flushed gas-tight syringe and injected into a Shimadzu GC14B gas chromatograph (Shimadzu, Duisburg, Germany) equipped with a flame ionization detector and a 30 m Supel-Q PLOT fused silica capillary column (inner diameter 0.53 mm; Sigma Aldrich GmbH, Taufkirchen, Germany). Nitrogen was used as a carrier gas. The oven was operated isothermally at 140 °C, the detector at 280 °C and the injector at 150 °C.

Before metabolite extraction, the HMN phase was removed with a separatory funnel. Metabolites were extracted from an acidified culture volume of 400 ml as described previously (Wilkes *et al.*, 2000; Rabus *et al.*, 2001). Inoculated cultures without cyclohexane and sterile cultures containing cyclohexane were used as controls. Methylated culture extracts were analyzed by gas chromatography–mass spectrometry (GC–MS) using a type 5890 gas chromatograph (Hewlett Packard, Waldbronn, Germany) connected to a type 95SQ mass spectrometer (Finnigan MAT/ThermoFinnigan, Egelsbach, Germany) as described by Wilkes *et al.* (2000) and Rabus *et al.* (2001). Cyclohexylsuccinic acid as a standard was purchased from Sigma-Aldrich (Deisenhofen, Germany).

Clone library construction, sequencing and phylogenetic analysis

The nearly complete 16S rRNA gene was amplified by PCR using bacteria-specific primers (Musat *et al.*, 2006). The 16S rRNA gene of the anammox bacteria was amplified with the primer pairs AMX368F—Univ1392R and Pla46F—Univ1392R (Schmid *et al.*, 2005). PCR products were purified using the QIAquick Purification kit (Qiagen, Hilden, Germany) and cloned into the pCR4 vector (Invitrogen, Groningen, The Netherlands) according to the manufacturer's recommendation. The recombinant vectors were transformed into competent *Escherichia coli* Top10 cells (Invitrogen). The 16S rRNA gene libraries were screened by PCR using the primers M13F and M13R. Positive clones were sequenced using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequences were aligned to those in the SILVA rRNA database (Pruesse *et al.*, 2007), and phylogenetic trees were constructed in ARB (Ludwig *et al.*, 2004) by neighbor joining, maximum likelihood and maximum parsimony, and by applying different sets of filters. In addition, maximum likelihood phylogenetic trees were constructed using the RAxML algorithm (Stamatakis *et al.*, 2008; http://phylobench.vital-it.ch/raxml-bb). The sequence data have been deposited in the DDBJ, EMBL and GenBank databases under accession numbers GU230600 and GU230601.

Fluorescence in situ *hybridization*

For fluorescence *in situ* hybridization (FISH), 1.0 ml from the enrichment culture was fixed with paraformaldehyde (final concentration, 20 g l^{-1}) for 3 h at 4 °C, washed twice with 1 × phosphate-buffered saline (10 mM sodium phosphate of pH 7.2, 130 mM NaCl) and stored in 1 × phosphate-buffered saline–ethanol (1:1) at −20 °C. For FISH, aliquots were filtered onto 0.2-μm pore GTTP polycarbonate filters (Millipore, Eschborn, Germany). Cells were hybridized with 16S rRNA-targeted probes, additionally stained with 4′,6′-diamidino-2-phenylindole (DAPI) and microscopically counted as described previously (Snaidr *et al.*, 1997). FISH with catalyzed reporter deposition (CARD-FISH) using the probe AMX820 specific for the anammox bacteria (Schmid *et al.*, 2001) was performed as described by Pernthaler *et al.* (2002). Values were corrected for the signals counted using the nonsense probe NON338. The oligonucleotide probes used in this study, GEO825 (30% formamide; Lowe *et al.*, 2000), EUB338 (Amann *et al.*, 1990), AMX368 (Schmid *et al.*, 2000) and AMX820 (Schmid *et al.*, 2001), were purchased from Biomers GmbH (Germany; www.biomers.net).

Results and discussion

Enrichment and analysis of products

Enrichment of anaerobic cyclohexane-degrading bacteria was attempted with freshwater sediment (mud) incubated under heptamethylnonane (inert carrier phase) with 0.8% cyclohexane in the presence of nitrate as electron acceptor. Production of gas (detectable by a volumetric device; Widdel, 2009), which was presumably nitrogen, indicated bacterial activity. When gas production ceased, new nitrate was added. Gas production in these early states of enrichment always resumed upon nitrate addition. During approximately 8 weeks, gas production in cyclohexane-free controls was essentially the same as in incubations with cyclohexane, indicating the presence of nitrate-scavenging electron donors in the organic-rich inoculum. After 8–9 weeks, however, cultures with cyclohexane produced more gas than the controls. Repeated transfers to new media resulted in a mud-free, strictly cyclohexane-dependent enrichment culture consisting mainly of rod-shaped cells. These cultures consumed 5 mM nitrate within approximately 3 weeks. Growth was always accompanied by gas production. The bacterial biomass that settled in the grown cultures was pink to reddish. The

visible spectra of whole cells showed an absorption maximum at 408 nm, which was indicative of cytochromes.

GC–MS of extracts from the cyclohexane-grown culture showed a mixture of organic acids that were not detectable in controls lacking cyclohexane. A metabolite with prominent fragment ions at *m/z* 114, 146, 155 and 197 was identified as cyclohexylsuccinic acid dimethyl ester on the basis of comparison with an authentic standard (Figure 1). This suggests that cyclohexane is anaerobically activated by addition to fumarate, similar as *n*–alkanes (Kropp *et al.*, 2000; Rabus *et al.*, 2001) and ethylcyclopentane (Rios-Hernandez *et al.*, 2003). Furthermore, two other metabolites were tentatively identified (on the basis of the mass spectra) as 9-cyclohexylnonanoic acid and 11-cyclohexylundecanoic acid (Figure 2). Their formation can be explained by a pathway of cyclohexylsuccinate analogous to the suggested pathway of *n*-alkanes involving CoA-ligation, C-skeleton rearrangement and decarboxylation to 3-cyclohexylpropionyl-CoA (Wilkes *et al.*, 2002, 2003); the latter undergoes chain elongation with C_2-units during lipid synthesis (Figure 3). Such ω-cyclohexyl fatty acids have been also detected in oil samples and oil formation waters with high content of partly biodegraded oil (Rodrigues *et al.*, 2005). Hence, ω-cyclohexyl fatty acids may indicate anaerobic degradation of cycloalkanes under *in situ* conditions.

Even though nitrate was added in sub-stoichiometric amounts relative to cyclohexane, the highly enriched mud-free cultures, viz. the later stages of enrichment, often did not resume growth and gas production upon new nitrate addition. Products of nitrate reduction were therefore analyzed, in particular to detect possible accumulation of nitrite as a potentially growth-inhibiting compound. Nitrite accumulation was indeed observed after addition of new nitrate to stationary cultures. Nitrous oxide (N_2O) and ammonium as possible products of nitrate reduction were not detected. By contrast, the initially added ammonium (4.7 mmol per liter) was consumed to depletion during the reduction of nitrate (not shown). Such depletion could not be explained by ammonium assimilation into cell mass. The optical density (OD_{660}) at the time of ammonium depletion was approximately 0.12, corresponding to no more than approximately 40 mg cell dry mass per liter (estimated from correlations between biomass yields and OD_{660} given by Rabus and Widdel, 1996). According to an approximate bulk formula for cell mass ($C_4H_8O_2N$; van Dijken and Harder, 1975) with 14% N (by mass), the estimated cell synthesis in this enrichment needed only 0.4 mmol NH_4^+ per liter. This is less than 10% of the observed ammonium consumption. Such strong ammonium depletion can be only explained by a dissimilatory process, the known anaerobic ammonium oxidation (anammox) with nitrite. This process is catalyzed by specialized members of the Planctomycetales (Mulder *et al.*, 1995; Jetten *et al.*, 2009). Attempts to purify cyclohexane-degrading nitrate reducers and anammox bacteria to further verify the assumed reactions in the enrichment culture were not successful. To gain more insights into the bacterial types and reactions, we therefore analyzed the

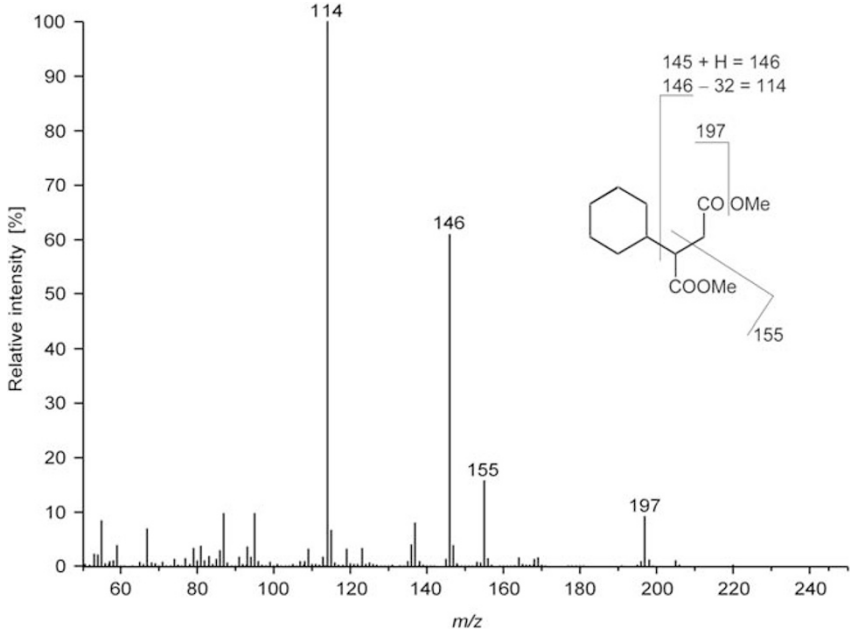

Figure 1 A mass spectrum of cyclohexylsuccinic acid dimethyl ester derived from the anaerobic enrichment culture with cyclohexane. An authentic standard showed the same mass spectrum (not shown).

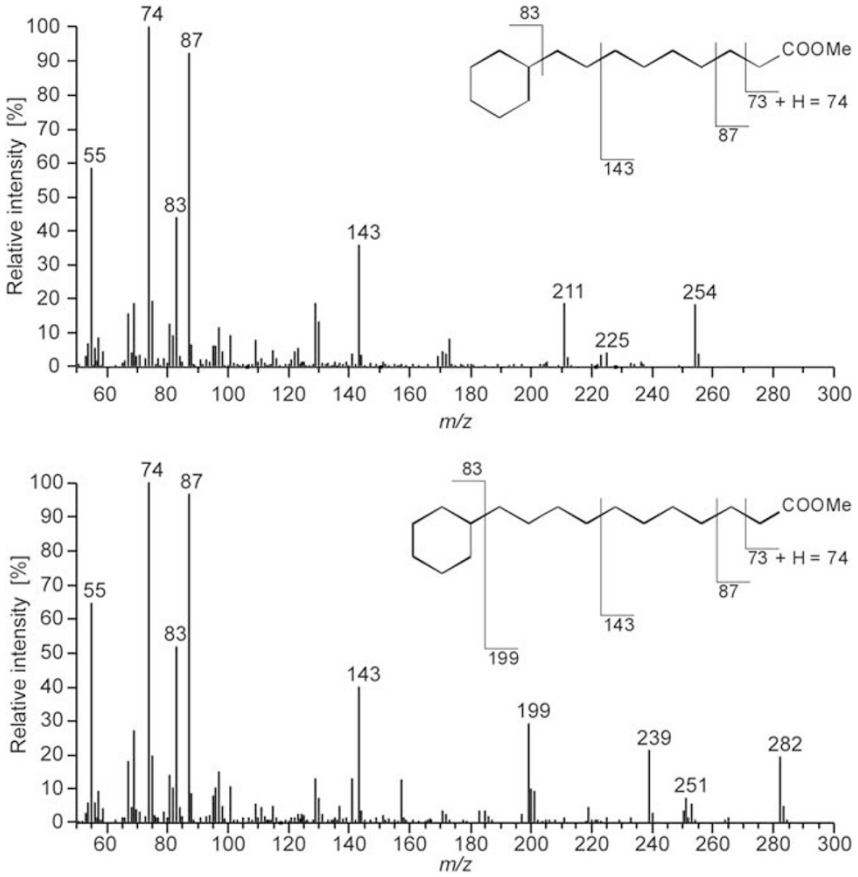

Figure 2 Mass spectra of the methyl esters of cyclohexylnonanoic acid (upper panel) and cyclohexylundecanoic acid (lower panel) derived from the anaerobic enrichment culture with cyclohexane. The specific signal at *m/z* 83 indicates the presence of a cyclohexyl moiety in both molecules.

dominant phylotypes on the basis of 16S rRNA sequences and performed physiological experiments with the enrichment culture.

Phylotypes in the enrichment culture

The 16S rRNA gene library generated using standard bacterial primers was dominated by a sequence characteristic of the family *Geobacteraceae* of the Deltaproteobacteria (clone type I; Figure 4a). The closest cultivated relatives were *Geobacter* sp. FRC-32 and *Geobacter uraniireducens* (sequence similarity, 96.0 and 95.5%, respectively). The apparent abundance of this phylotype in the enrichment culture was corroborated by FISH using a 16S rRNA-targeted fluorescent probe specific for the *Geobacteraceae*. The *Geobacter*-related phylotype accounted for approximately 75% of the (total) cell number determined by DAPI staining (Figures 5a and b). Because of the above indication of anaerobic nitrite-dependent ammonium oxidation, two primer pairs for 16S rRNA genes of anammox bacteria were also applied. A phylotype affiliating with the anammox cluster of the Planctomycetales (clone type II; Figure 4b) was indeed retrieved; it was most closely related to *Candidatus* 'Brocadia anammoxidans' (93.6% sequence similarity). Hybridization with specific probes showed that cells belonging to the anammox phylotype accounted for up to 18% of the total cell number in the enrichment culture (Figures 5c and d).

Assignment of anaerobic ammonium oxidation in our enrichment to the detected Planctomycetales would leave the assignment of cyclohexane degradation to the *Geobacter*-related phylotype. Anammox bacteria with their special nitrogen catabolism are not known to use hydrocarbons. However, there is much biodegradative potential in the genus *Geobacter* and other Deltaproteobacteria towards low-molecular-mass organic compounds. Iron(III)-reducing *Geobacter* species have been shown to anaerobically oxidize aromatic compounds (Lovley *et al.*, 1989; Wischgoll *et al.*, 2005). Several sulfate-reducing Deltaproteobacteria oxidize *n*-alkanes or aromatic hydrocarbons (for overview see reference Widdel *et al.*, 2009). *Geobacter* species and some sulfate-reducing bacteria may also reduce nitrate, even though these anaerobes are usually not directly enriched with nitrate. Enrichments with hydrocarbons (*n*-alkanes, alkylbenzenes) and nitrate very often selected true denitrifiers (viz. that reduce NO_3^- to N_2) of the Betaproteobacteria (for example, Rabus *et al.*, 1999; Ehrenreich *et al.*, 2000). Physiological and genomic

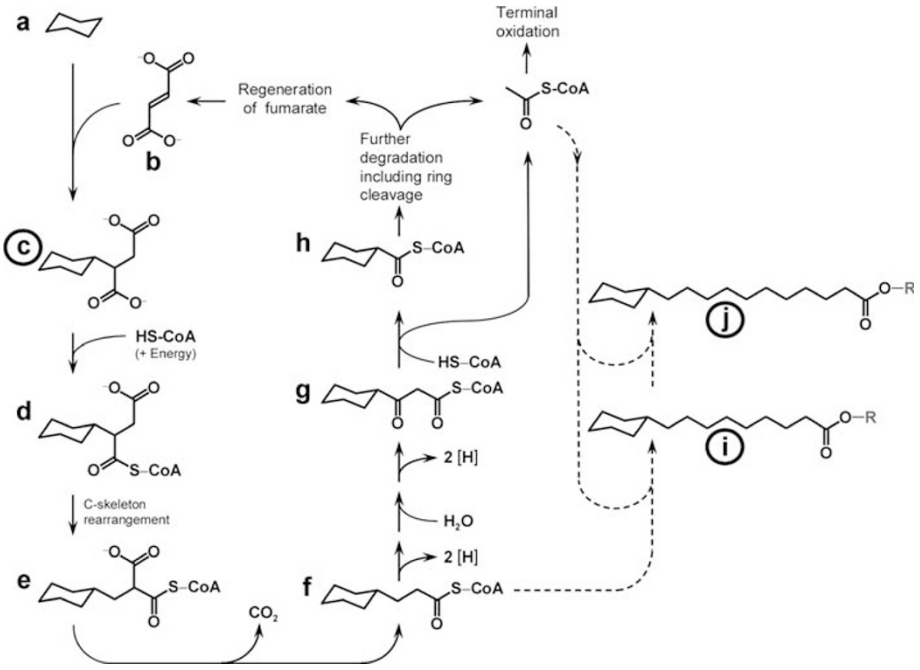

Figure 3 The proposed pathway for the anaerobic degradation of cyclohexane. The circled letters indicate compounds detected as methyl esters. Cyclohexane (**a**) is activated by addition to fumarate (**b**), yielding cyclohexylsuccinate (**c**), which may then be activated to cyclohexylsuccinyl-CoA (**d**). The latter may undergo rearrangement of the C-skeleton to (cyclohexylmethyl)malonyl-CoA (**e**), which through decarboxylation (or transcarboxylation) yields cyclohexylpropionyl-CoA (**f**). The bulk of the latter is degraded by regular β-oxidation through 3-oxo-3-cyclohexylpropionyl-CoA (**g**), cyclohexylcarboxyl-CoA (**h**), and ring cleavage to yield acetyl-CoA as well as new fumarate for the activation reaction. Some cyclohexylpropionyl-CoA (**f**) may contribute to the synthesis of cellular fatty acids by addition of C_2-units yielding 9-cyclohexylnonanoate (**i**) and 11-cyclohexylundecanoate (**j**).

studies (Lovley and Phillips, 1988; http://img.jgi.doe.gov) suggest that reduction of nitrate by *Geobacteraceae*, if they reduce it beyond the level of nitrite, produces ammonium, as in sulfate-reducing Deltaproteobacteria (Widdel and Pfennig, 1992; Simon, 2002). The anammox bacteria may therefore be fostered by the ammonium added to the medium as well as by the ammonium and nitrite produced during cyclohexane oxidation.

Time course of substrate consumption and product formation

The fate of cyclohexane, nitrate and ammonium, as well as formation of nitrite and optical cell density, was monitored in a time-course experiment (Figures 6a and b). As long as ammonium was present, nitrite appeared only transiently and at low concentration. If new nitrate was added, nitrite accumulated to higher concentration as soon as the residual ammonium was depleted (Figure 6b). But even if ammonium was no longer detectable, more nitrate was reduced than nitrite was formed, indicating that a part of formed nitrite was further reduced by the cyclohexane-degrading bacteria, or by accompanying bacteria that may be nourished by metabolic products excreted by the cyclohexane degrader. New addition of ammonium resulted in rapid nitrite consumption (Figure 6b). Consumption of cyclohexane or ammonium was not observed in control incubations without nitrate. Also, ammonium was not consumed in inoculated cyclohexane-free controls containing nitrate (not shown).

Electron balance and proportion of reactions and cell types in the overall process

A net electron balance was determined using a culture provided with a small, defined amount of cyclohexane. The results are summarized in Table 1. In this culture, the added cyclohexane was completely consumed. Absence of N_2O and presence of anammox bacteria leave N_2 as the only end product of nitrate reduction and ammonium oxidation. Calculation showed that electrons from consumed cyclohexane and ammonium essentially matched the electrons channeled into cell synthesis and into reduction of nitrate (through nitrite).

As suggested by the time-course experiment (Figure 6), nitrite formed by the nitrate reducer (the *Geobacter* type) is not only scavenged by anammox bacteria but also further reduced by the nitrate reducer itself to yield ammonium. The proportions of nitrite entering these two branches are in principle variable and influence the extent of coupled anammox. To calculate the proportions of the reactions in the overall process, we formulate

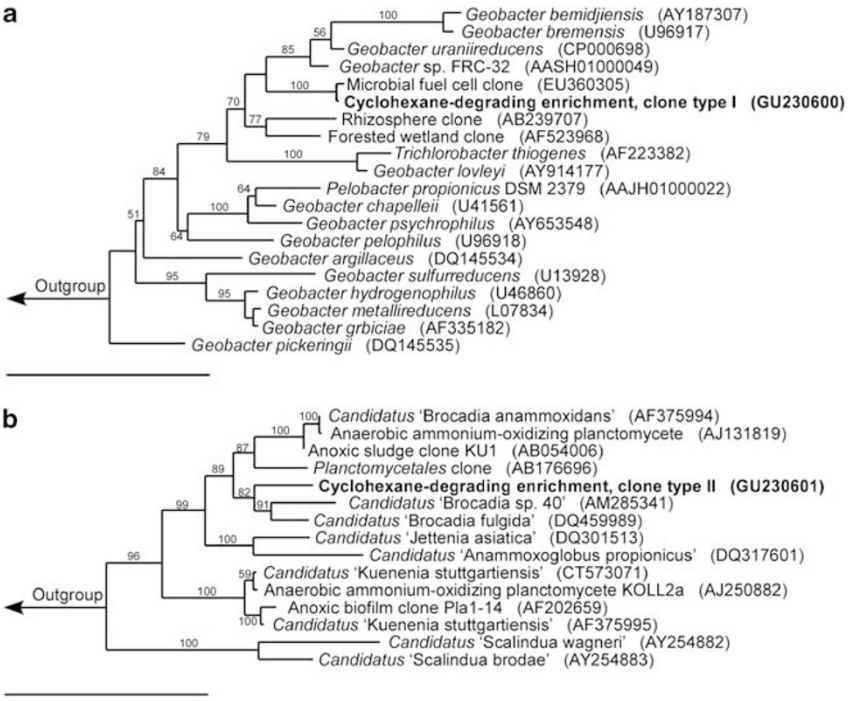

Figure 4 Phylogenetic affiliation of the dominant microorganisms in the enrichment culture with (**a**) clone type I among the *Geobacteraceae* and (**b**) clone type II among anammox bacteria (*Planctomycetales*). Only nearly full-length sequences (>1300 bp) were used for the calculation. The sequences retrieved in this study are indicated in boldface. The numbers adjacent to nodes indicate maximum likelihood bootstrap values higher than 50%. The scale bars represent 10% estimated sequence divergence.

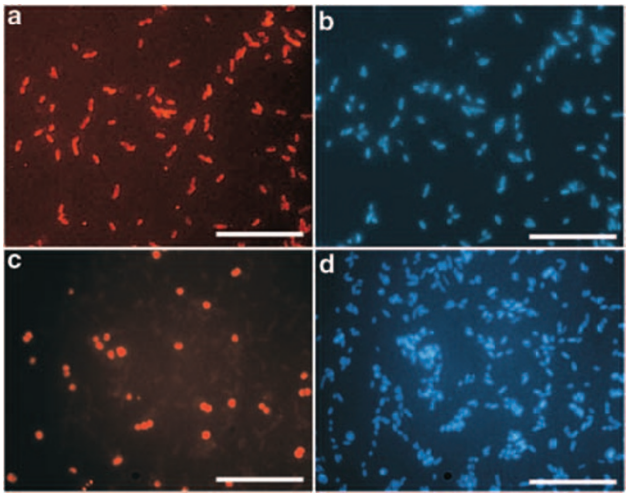

Figure 5 FISH of the cyclohexane- and nitrate-utilizing enrichment culture with the GEO825 probe (**a**) showed dominance of *Geobacteraceae* related organisms, accounting for up to 75% of the DAPI-stained cells (**b**). Hybridization with the AMX368 probe (**c**) indicated that anammox-related organisms represented up to 18% of the DAPI-stained cells (**d**). Scale bar: 10 μm (applicable to all images). DAPI, 4′,6′-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization.

the two reactions of the nitrate reducer, both starting with nitrate (Equations 4, 5), as well as the anammox reaction (Equation 6):

$$18\,NO_3^- + C_6H_{12} \rightarrow 18\,NO_2^- + 6\,CO_2(g) + 6\,H_2O$$
$$\Delta G^\circ = -138\,kJ\,(mol\,NO_3^-)^{-1} \quad (4)$$

$$9\,NO_3^- + 2\,C_6H_{12} + 18\,H^+ \rightarrow 9\,NH_4^+ + 12\,CO_2\,(g) + 3\,H_2O$$
$$\Delta G^{\circ\prime} = -499\,kJ\,(mol\,NO_3^-)^{-1} \quad (5)$$

$$NH_4^+ + NO_2^- \rightarrow N_2\,(g) + 2\,H_2O$$
$$\Delta G^\circ = -358\,kJ\,(mol\,N_2)^{-1} \quad (6)$$

Strictly speaking, energy changes in Equations 4–6 represent changes of chemical potential. We assume that the nitrogen of NO_3^- and NH_4^+ consumed in the culture always ends up as N_2, irrespective of whether the intermediate NO_2^- enters anammox directly or indirectly after further reduction to NH_4^+. If the measured amounts (in mol) of NO_3^- and NH_4^+ consumed from the medium are termed a and b, respectively, they must yield $[(a+b)/2]$ N_2. Because equimolar amounts of nitrite and ammonia must react in anammox (Equation 6), an amount of $[(a+b)/2]$ NO_2^- derived from $[(a+b)/2]$ NO_3^- must finally react with $[(a+b)/2]$ NH_4^+. Ammonium for anammox has two sources: NH_4Cl added to the medium and NH_4^+ formed by the nitrate reducer. If from the ammonium initially present in the medium the (measured) amount b NH_4^+ has been consumed, the proportion $[(a+b)/2]-b=[(a-b)/2]$ NH_4^+ must have been provided by the cyclohexane degrader through complete reduction of $[(a-b)/2]$ NO_3^- (Figure 7). With such generally expressed amounts (in mol), the two branches of nitrate reduction and the associated free energy changes (indices: nire1, nire2) are represented by Equations (7) and (8).

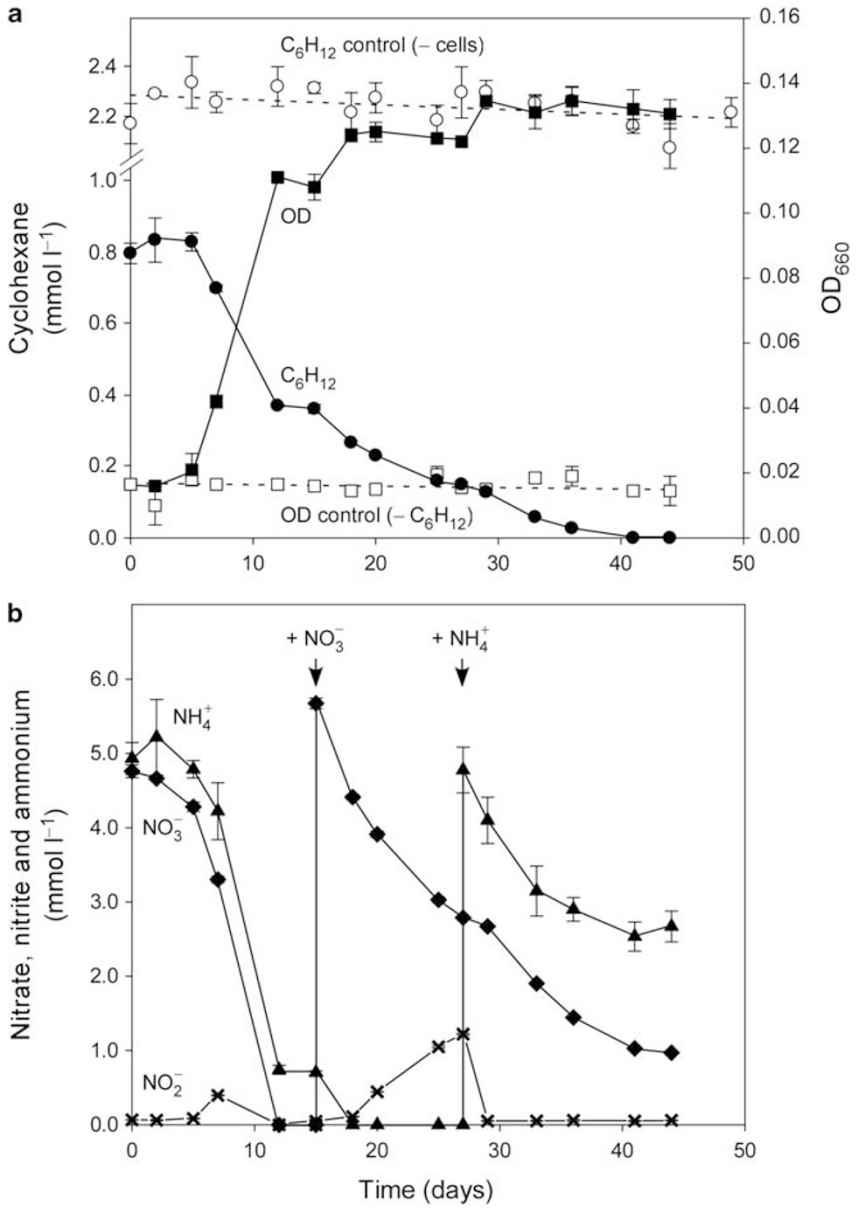

Figure 6 Anaerobic consumption of cyclohexane, nitrate and ammonium, as well as formation and consumption of nitrite (as an intermediate), in the anaerobic enrichment culture. Experiments were performed in anoxic bottles with a culture volume of 100 ml and 5 ml heptamethylnonane as inert carrier for cyclohexane (the given values are relative to the aqueous phase). (**a**) Cell density (OD, ■) increased only if cyclohexane (●) was present. In a cyclohexane-free control the cell density (□) remained constant. The main increase in OD occurred during the initial incubation period. Cyclohexane in a sterile control (○; tested at higher concentration) did not show significant disappearance. (**b**) In the same experiment, nitrate (◆) consumption was accompanied by ammonium (▲) depletion. Addition of new nitrate after 15 days resulted in its partial reduction and some accumulation of nitrite (×). No growth was observed during this period. New ammonium addition after 27 days caused consumption of nitrite and some growth.

$$\frac{a+b}{2}NO_3^- + \frac{a+b}{36}C_6H_{12} \rightarrow \frac{a+b}{2}NO_2^- + \frac{a+b}{6}CO_2 + \frac{a+b}{6}H_2O \qquad \Delta G^{\circ}_{nire1} = -69(a+b)\,kJ \quad (7)$$

$$\frac{a-b}{2}NO_3^- + \frac{a-b}{2}H^+ + \frac{a-b}{9}C_6H_{12} \rightarrow \frac{a-b}{2}NH_4^+ + \frac{2(a-b)}{3}CO_2 + \frac{a-b}{6}H_2O \qquad \Delta G^{\circ\prime}_{nire2} = -249(a-b)\,kJ \quad (8)$$

Total nitrate reduction (index: nire) is their sum given by Equation (9).

$$a\,NO_3^- + (a-b)\,H^+ + \frac{5a-3b}{36}C_6H_{12} \rightarrow \frac{a+b}{2}NO_2^- + \frac{a-b}{2}NH_4^+ + \frac{5a-3b}{6}CO_2 + \frac{a}{3}H_2O \qquad \Delta G^{\circ\prime}_{nire} = -6(53a-30b)\,kJ \quad (9)$$

The anammox reaction (index: anam) is represented by Equation (10).

Table 1 Quantification of the anaerobic consumption of cyclohexane, ammonium and nitrate in the nitrate-reducing enrichment culture; the medium volume was 100 ml

Electron source or sink (mmol)	*Culture with C_6H_{12}*	*Culture without C_6H_{12}*	*Culture without NO_3^-*
C_6H_{12} added	0.098		0.240
C_6H_{12} consumed	0.098		0.009
Electrons in C_6H_{12} consumed[a]	3.53		0.324
NH_4^+ added	0.95	0.47	
NH_4^+ consumed (from medium)	0.67	0	
Electrons in NH_4^+ consumed[b]	2.01	0	
Electrons in C_6H_{12}+NH_4^+ (sum) consumed	5.54	0	0.324
Electrons in cell mass[c]	0.92	0	
Electrons for dissimilation	4.6	0	
NO_3^- added	0.98	0.47	
NO_3^- consumed	0.94	0.01	
NO_2^- formed	0	0	
Electrons for NO_3^- converted to N_2[d]	4.7	0.05	
Electron balance[e]	0.98/1		

[a]Calculated considering complete oxidation of cyclohexane ($C_6H_{12} + 12H_2O \rightarrow 6CO_2 + 36H^+ + 36e^-$).
[b]Calculated considering oxidation of NH_4^+ to N_2 ($NH_4^+ \rightarrow 0.5N_2 + 4H^+ + 3e^-$).
[c]The cell dry mass produced was estimated to 4.7 mg (in 100 ml medium), using the OD_{660} difference between the culture with cyclohexane and the control culture and the biomass/OD ratio from other nitrate-reducing bacteria using hydrocarbons (Rabus and Widdel, 1996). The electrons in the biomass were calculated assuming a simplified bulk oxidation state, $C_4H_8O_2N$ (van Dijken and Harder, 1975), and the equation $C_4H_8O_2N + 6H_2O \rightarrow 4CO_2 + 20H^+ + 0.5N_2 + 20e^-$. Hence, 1.0 mg cell dry mass is equivalent to 9.8×10^{-3} mmol $C_4H_8O_2N$ or 0.196 mmol e^-.
[d]If there is no final nitrite and if all nitrate ends up as N_2, the formal net reduction is according to $NO_3^- + 5e^- + 6H^+ \rightarrow 0.5N_2 + 3H_2O$ (which does not indicate the pathway).
[e]Electrons available for dissimilation divided by electrons for nitrate reduction.

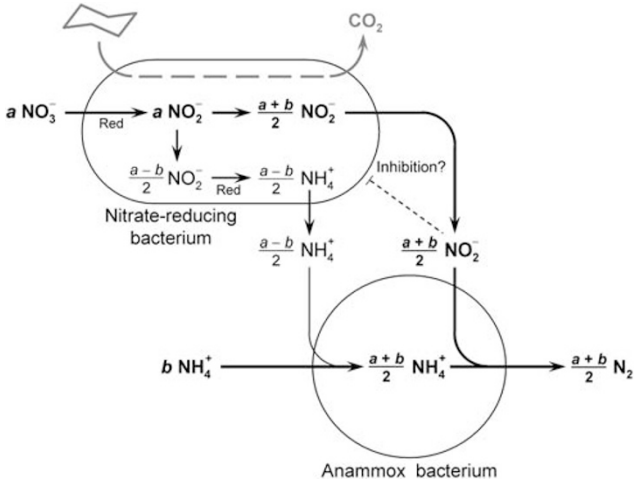

Figure 7 A functional model of the cyclohexane-degrading nitrate-reducing enrichment culture. The factors *a* and *b* denote amounts in mol. The *Geobacter* type oxidizes cyclohexane and reduces nitrate mainly to nitrite, and to a lesser extent to ammonium. Anammox bacteria use the produced nitrite for oxidation of ammonium from further nitrate reduction and from the surroundings to N_2. Nitrite may inhibit the *Geobacter* type. For convenience, the scheme does not depict the stoichiometry of cyclohexane oxidation to CO_2 (see text, Equation (9)). Red, reduction.

$$\frac{a+b}{2}NH_4^+ + \frac{a+b}{2}NO_2^- \rightarrow \frac{a+b}{2}N_2 + (a+b)\,H_2O \qquad \Delta G^{\circ}_{anam} = -179(a+b)\,kJ \quad (10)$$

Addition of Equations (9) and (10) yield Equation (11) for the total reaction in the culture (formed and consumed nitrite cancel each other; the amounts of ammonium are combined on the reactant side).

$$a\,NO_3^- + b\,NH_4^+ + (a-b)\,H^+ + \frac{5a-3b}{36}C_6H_{12} \rightarrow \frac{a+b}{2}N_2 + \frac{5a-3b}{6}CO_2 + \frac{4a+3b}{3}H_2O \qquad \Delta G^{\circ\prime}_{tot} = -(497a-b)\,kJ \quad (11)$$

In the incubation experiment using a culture volume of 100 ml, a consumption of $a = 0.94$ mmol NO_3^- and $b = 0.67$ mmol NH_4^+ was measured (Table 1). Of the 0.94 mmol NO_2^- formed as an intermediate, $(0.94 + 0.67)/2 = 0.805$ mmol NO_2^- were thus directly used by anammox to oxidize 0.805 mmol NH_4^+, whereas 0.135 mmol NO_2^- were further reduced to 0.135 mmol NH_4^+. Hence, the bulk (86%) of the NO_2^- produced was scavenged by anammox bacteria.

The free energies available from nitrate reduction and anammox in the incubation experiment were $\Delta G^{\circ}_{nire} = -178$ kJ and $\Delta G^{\circ\prime}_{anam} = -288$ kJ, respectively. The free energy change of the total process was $\Delta G^{\circ\prime}_{tot} = -466$ kJ. Hence, the free energy share between nitrate reduction and anammox is as follows:

$$\Delta G^{\circ\prime}_{nire}/\Delta G^{\circ\prime}_{tot} = 0.38\,(38\%) \quad (12)$$

$$\Delta G^{\circ}_{anam}/\Delta G^{\circ\prime}_{tot} = 0.62\,(62\%) \quad (13)$$

The biomass share of the two bacteria in the culture is not necessarily according to their free energy

share. The bacteria may differ with respect to their efficiencies of energy conservation and use of conserved energy for biosynthesis. The biomass share can be estimated from the presently consumed amounts of the reactants (substrates) and from growth yields (cell dry mass per mol substrate) reported in the literature. In addition, the biomass share is derived independently from cell numbers and their biovolumes determined microscopically. For comparison, we use both approaches.

Growth and growth yields of a nitrate reducer with cyclohexane have not been reported. As a rough estimate, we used a reported growth yield of a *Geobacter* species relative to the electrons channeled from benzoate oxidation into Fe(III) reduction, the value being 1.56 g dry mass per mol electrons (Champine *et al.*, 2000); for convenience, we further assume that the yield relative to substrate-derived electrons is the same for nitrate reduction to nitrite and nitrate reduction to ammonium. The examined culture dissimilated a total of 4.6 mmol electrons of which 2.0 mmol electrons were from added ammonium (Table 1). The amount of electrons derived from cyclohexane for dissimilation was thus $4.6-2.0=2.6$ mmol. Essentially the same value is obtained from the term $[(5a-3b)/36]\ C_6H_{12}$ in Equation (9), using the above values for a and b, and 36 mol electrons derived per mol C_6H_{12}. The resulting dry mass of the nitrate reducer in the culture (100 ml) was thus $m_{\mathrm{nire}}=4$ mg. The growth yield of anammox bacteria is 1.59 g dry mass per mol ammonium (calculated from Kuenen, 2008). Anammox cells in the culture consumed a calculated amount of 0.805 mmol NH_4^+, which would allow a biomass synthesis of $m_{\mathrm{anam}}=1.3$ mg. The resulting total biomass in the culture was $m_{\mathrm{tot}}=5.3$ mg. The predicted (index: pred) cell dry mass share between the nitrate-reducing and the anammox bacterium is thus as follows:

$$[m_{\mathrm{nire}}/m_{\mathrm{tot}}]_{\mathrm{pred}}=0.75\,(75\%) \tag{14}$$

$$[m_{\mathrm{anam}}/m_{\mathrm{tot}}]_{\mathrm{pred}}=0.25\,(25\%) \tag{15}$$

To calculate the biomass share from the fractions of microscopically determined cell numbers, we estimated the average cell volume of the nitrate reducer ($V_{\mathrm{nire}}^{\mathrm{cell}}=0.29\,\mu\mathrm{m}^3$) and the anammox bacterium ($V_{\mathrm{anam}}^{\mathrm{cell}}=0.41\,\mu\mathrm{m}^3$) by examining 20 cells of each type. These were the cell volumes after fixation and staining, which causes shrinking of the cells. However, if we assume that the cell volumes shrink by the same factor, Equation (2) can be applied. This yields the following biomass share:

$$[m_{\mathrm{nire}}/m_{\mathrm{tot}}]_{\mathrm{micr}}\approx 0.75\,(75\%) \tag{16}$$

$$[m_{\mathrm{anam}}/m_{\mathrm{tot}}]_{\mathrm{micr}}\approx 0.25\,(25\%) \tag{17}$$

These values are in good agreement with the above ones predicted from substrate consumption.

Results show that the *Geobacter* type dominates by mass, even though it shares the smaller proportion of the total free energy. The reason lies in a more efficient use of the free energy for cell synthesis in comparison with the energy use in the special lithoautotrophic metabolism of anammox bacteria.

Conclusions

The catabolic processes suggested to occur in our enrichment culture (Figure 7) possibly represent a delicate interaction. On the one hand, the nitrate-reducing *Geobacter* type and anammox bacteria may compete for nitrite. On the other hand, nitrite accumulation may inhibit the nitrate reducer (as the time course experiment suggests; Figure 6). It is therefore possible that the nitrate reducer benefits from or depends on nitrite removal. This would offer an explanation for the relatively slow growth of the present enrichment culture in comparison with that of other hydrocarbon-utilizing denitrifiers; with similar inoculum sizes, the latter grew within 8 days or less (Rabus and Widdel, 1995; Ehrenreich *et al.*, 2000). The cyclohexane-degrading nitrate reducer may thus develop only if nitrite-scavenging anammox bacteria, which generally grow slowly (Kuenen, 2008), are co-enriched. In other words, their co-enrichment would be compulsory. A different explanation would be that the cyclohexane-degrading nitrate reducer is also a slowly growing organism, like anammox bacteria. In this case, enrichment of the latter would be coincidental and due to the long incubation time needed to grow the cyclohexane degrader. Only isolation and a physiological study of the *Geobacter* type would clarify its properties and the mode of interaction in the enrichment culture.

One may expect that also *in situ* degradation of petroleum hydrocarbons with nitrate in contaminated anoxic soil or sediment is associated with anammox under certain conditions. A prerequisite for such association would be that anammox bacteria are not inhibited by petroleum, viz. that they tolerate various hydrophobic organic compounds acting on biomembranes. This is likely because anammox bacteria were shown to tolerate phenolic waste water (Toh and Ashbolt, 2002).

This study shows that anaerobic biodegradation of organic compounds coupled to NO_3^- reduction may, at first glance, pretend denitrification, but in reality yield N_2 through a 'loop' through anammox ('pseudo-denitrification'). Such dissimilatory conversion of NO_3^- to N_2 without involvement of true denitrification has been shown by isotope labeling studies using highly enriched cells of anammox bacteria alone (Kartal *et al.*, 2007). If formate was provided as electron donor, cells catalyzed the reduction of NO_3^- to NO_2^- and NH_4^+, which were further converted to N_2.

Finally, a generalized net process may be formulated for the anaerobic oxidation of an organic compound, $C_cH_hO_o$ (for convenience without N and

electrical charge), coupled to non-denitrifying nitrate reduction in combination with anammox:

$$a\,NO_3^- + b\,NH_4^+ + (a-b)\,H^+ + \frac{5a-3b}{4c+h-2o}C_cH_hO_o \rightarrow \frac{a+b}{2}N_2 + \frac{(5a-3b)c}{4c+h-2o}CO_2 + \frac{a(2c+3h-o)+3b(2c-o)}{4c+h-2o}H_2O \quad (18)$$

A special situation would be a reaction without dissimilatory consumption of external ammonium ($b=0$; see also Equation (11) and Figure 7), which as a bulk reaction is indistinguishable from true denitrification. In this case, the nitrate reducer forms equimolar amounts of nitrite and ammonium. This is analogous to the reaction shown in incubations with anammox cells (Kartal *et al.* 2007). If there is net production rather than net consumption of ammonium (formally $-a<b<0$ in Equation (18), or NH_4^+ appearing on the product side with $a>b>0$), the nitrate reducer produces more NH_4^+ than NO_2^-, and anammox is involved to a lesser extent. If NO_3^- is completely reduced to NH_4^+ ($b=-a$, or NH_4^+ on the product side with $b=a$), there is no longer a possibility for anammox (and N_2 production) to occur.

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