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# Anaerobic Degradation of Pristane in Nitrate-Reducing Microcosms and Enrichment Cultures

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**Microcosm studies were conducted under nitrate-reducing conditions with diesel fuel-contaminated aquifer material from a site treated by in situ bioremediation. In the microcosms, the consumption of nitrate and the production of inorganic carbon were strongly stimulated by the addition of the isoprenoid alkane pristane (2,6,10,14-tetramethylpentadecane). Within 102 days enrichment cultures degraded more than 90% of the pristane supplied as coatings on reticulated sinter glass rings. The study demonstrates that pristane can no longer be regarded as recalcitrant under anaerobic conditions.**

Pristane (2,6,10,14-tetramethylpentadecane) is a naturally occurring isoprenoid alkane that is probably derived from the phytyl moiety of chlorophyll, from thermal degradation of tocopherols, and/or from the catagenic decomposition of methyltrimethyltridecylchromans (26). Pristane has been detected in bacteria, algae, higher plants, various tissues of fish and mammals, sediments, coal, and mineral oil (29). During bioremediation of mineral oil-contaminated sites, it is commonly observed that *n*-alkanes are biodegraded more rapidly than isoprenoid alkanes (7, 24). The biodegradation of pristane is therefore of particular interest, since it has often been used as a relatively inert biomarker in studies of oil degradation (4, 36).

Under aerobic conditions, mixed as well as pure microbial cultures can utilize pristane as the sole carbon and energy source (29, 33, 35). The soil bacterium *Rhodococcus* sp. strain BPM 1613 (33, 34) was found to produce the monoterminal oxidation metabolites pristanol, pristanic acid, pristyl pristanate, pristyl aldehyde, and other pristane-derived products, which are further biodegraded through β- or ω-oxidation (35). Other isoprenoid alkanes, like phytane (2,6,10,14-tetramethylhexadecane), norpristane (2,6,10-trimethylpentadecane), and farnesane (2,6,10-trimethyldodecane), were also reported to be degraded under aerobic conditions (9, 20, 34).

However, no evidence for the microbial degradation of isoprenoid alkanes under anaerobic conditions has been presented so far. Hydrocarbons which are known to be degraded in the absence of $O_2$ include *n*-alkanes (1, 40), alkenes (17), and aromatic hydrocarbons such as benzene (11, 19, 28), toluene (19, 27, 38, 46), *o*-xylene (12), *m*-xylene (42, 46), *p*-xylene (21, 22), ethyl- and propylbenzene (31, 39), trimethylbenzenes (23), naphthalene (2, 8, 30), phenanthrene (8), and acenaphtene (30). For the monoaromatic hydrocarbons, the anaerobic biodegradation pathways have been partially elucidated (15, 41). For other hydrocarbons, such as *n*-alkanes, the degradation pathways and, in particular, the first step of degradation are still unknown (32).

Recently we reported on the biological and chemical processes in a diesel fuel-contaminated aquifer in Menziken, Switzerland, which was in situ bioremediated by adding $O_2$, $NO_3^-$, $PO_4^{3-}$, and $NH_4^+$ through an infiltration well (25). Material excavated from this aquifer contained $>10^6$ microbial cells $g^{-1}$ that were able to degrade hydrocarbons and $1.15 \pm 0.15$ mg of weathered diesel fuel $g^{-1}$ (5). Samples of the aquifer material were used to enrich microbial cultures which could grow on *p*-xylene (22), 1,2,4-trimethylbenzene (23), or 1,3,5-trimethylbenzene (23) as the sole carbon and energy source under denitrifying conditions. Samples of this aquifer material were also used to study the degradation of the weathered diesel fuel in microcosms (5). It was found that the concentration of the isoprenoid alkane pristane decreased under both aerobic and denitrifying conditions. However, solid evidence for a biological degradation of pristane in the absence of $O_2$ was lacking. In this study, we report the biological degradation of pristane under $NO_3^-$-reducing conditions in cultures enriched from the contaminated aquifer material. Electron balances demonstrate that the degradation of pristane is coupled to the consumption of $NO_3^-$ and the production of inorganic carbon.

**Chemicals.** Pristane was obtained from Fluka (Buchs, Switzerland), and the purity was >96% according to the supplier. In our laboratory, we confirmed this purity by running a full-scan ion chromatogram on a gas chromatograph-mass spectrometer (GC-MS) (Hewlett-Packard Company, Avondale, Pa.). In addition, the mass spectrum of the pristane used in this study was identical to the reference spectrum found in the library of the National Institute of Standards and Technology (Gaithersberg, Md.). 2,2,4,4,6,8,8-Heptamethylnonane (HMN) was purchased from Sigma (Buchs, Switzerland), and $N_2$ (>99.999%) was from Pan Gas (Schlieren, Switzerland). All other chemicals were purchased from Fluka or Merck ABS (Dietikon, Switzerland) at the highest purity available and were used as received.

**Coating of quartz sand and sinter glass rings.** Quartz sand (Fluka) and reticulated sinter glass rings (Schott Glaswerke, Mainz, Germany) were used in the microcosms and the enrichment cultures, respectively, as surfaces to be coated with the poorly water soluble hydrocarbons. Quartz sand ($SiO_2$) was washed with water and pentane and mortared, and the sinter glass rings ($SiO_2$; external diameter, 8 mm; internal diameter, 6 mm; height, 8 mm; weight, 0.3 g) were washed with water and flamed before use. According to the manufacturer, the sinter glass rings have a total surface area of 0.4 $m^2$ $g^{-1}$.

Quartz sand was coated with pristane or *n*-alkanes by dis-

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solving either pristane (2 or 20 mg $ml^{-1}$) or a mixture of the *n*-alkanes undecane, dodecane, pentadecane, hexadecane, nonadecane, and eicosane (each at 0.35 mg $ml^{-1}$) in 20 ml of pentane. The solutions were thoroughly mixed with 10 g of quartz sand. The pentane was allowed to evaporate under a stream of $N_2$. A subsequent extraction and analysis by GC with flame ionization detection (GC-FID) revealed that at least 3.6 and 38.9 mg of pristane (for the initial 2 and 20 mg $ml^{-1}$, respectively) and 2.4 mg of *n*-alkanes (undecane, 0.12 mg; dodecane, 0.12 mg; pentadecane, 0.51 mg; hexadecane, 0.51 mg; nonadecane, 0.56 mg; and eicosane, 0.63 mg) were affixed per g of quartz sand. The rest was probably lost during the evaporation process. For control experiments, uncoated quartz sand was prepared by mixing quartz sand with pure pentane only and subsequent evaporation of the pentane.

Sinter glass rings were coated with pristane and HMN by dissolving pristane and HMN in equal amounts (0.52 mg $ml^{-1}$ each) in pentane. Ten milliliters of this solution was transferred to serum flasks containing five sinter glass rings. The pentane was allowed to evaporate for 15 h at room temperature. HMN is known to be resistant to microbial degradation (13, 39) and therefore was chosen as an inert standard for quantification. Recovery measurements revealed that volatilization during the evaporation process was <1 and 24% for pristane and HMN, respectively. For control experiments, uncoated sinter glass rings were prepared by transferring 10 ml of pure pentane to serum flasks containing five sinter glass rings followed by subsequent evaporation of pentane.

**Preparation of denitrifying microcosms.** Microcosms containing aquifer material and synthetic anaerobic ground water supplemented with 3 mM $KNO_3$ were prepared as previously described (5) and incubated at 12°C (mean value of the in situ temperature of the aquifer) without agitation. After 98 days of incubation, the butyl rubber stoppers of some selected microcosms were briefly removed, and 2.5 g of quartz sand coated with 9 or 97 mg of pristane or 6 mg of *n*-alkanes (corresponding to 0.56, 6.03, and approximately 0.44 mM, respectively) was added. After being sealed, the microcosms were flushed with $N_2$, slightly shaken, and further incubated as described above. The following controls were also incubated: (i) 2.5 g of quartz sand coated with 0.56 mM pristane plus 1 mM $HgCl_2$ (poisoned control), (ii) 2.5 g of quartz sand coated with 0.44 mM *n*-alkanes plus 1 mM $HgCl_2$, and (iii) uncoated quartz sand. $HgCl_2$ was added from a sterile anaerobic stock solution. After an incubation time of 508 days, a specific microcosm (designated microcosm P) which received 0.56 mM pristane and which had consumed $NO_3^-$ and produced inorganic carbon in considerable amounts (see "$NO_3^-$ consumption and $C_T$ production in microcosms," below) was shaken, the particles were allowed to settle, and the turbid supernatant was used as an inoculum for the enrichment cultures.

**Preparation of enrichment cultures.** Enrichment cultures were prepared and incubated in a glove box (Kleiner AG, Wohlen, Switzerland) which was permanently flushed with $N_2$ (>99.999%). Serum flasks (117 ml) containing five sinter glass rings coated with both 5.1 mg of pristane and 3.9 mg of HMN (corresponding to 0.37 and 0.33 mM, respectively) were filled with 40 ml of anaerobic growth medium, inoculated with 12 ml of the supernatant of microcosm P, and sealed with butyl rubber stoppers. The growth medium consisted of basal medium (45) supplemented with $Na_2SO_4$ (1.4 mM) and $KNO_3$ (2.0 mM). After autoclaving and cooling under an atmosphere of $N_2$, the following components were added from sterile stock solutions: nonchelated trace element mixture SL10 (final concentration in medium, 1 ml $liter^{-1}$) (45), a selenite-tungstate solution (1 ml $liter^{-1}$) (44), a seven-vitamin solution (1 ml $liter^{-1}$) (44), and $NaHCO_3$ (10 mM). The pH of each enrichment culture was individually adjusted to 7.4 ± 0.2 by adding HCl or NaOH from sterile anaerobic stock solutions. After preparation, the enrichment cultures were incubated at 25°C in a glove box without agitation. The following controls were also incubated: (i) 52 ml of medium, no inoculum, and five sinter glass rings coated with 0.37 mM pristane and 0.33 mM HMN plus 1 mM $HgCl_2$ (uninoculated, poisoned control) and (ii) 40 ml of medium, 12 ml of supernatant of microcosm P, and five uncoated sinter glass rings (i.e., not containing pristane and HMN). Enrichment cultures were performed in duplicate. Throughout this paper, the data reported are arithmetic means ± absolute deviations.

**Extraction of pristane and HMN from enrichment cultures.** At the end of the incubation, the concentrations of pristane and HMN in the enrichment cultures were determined by hexane extraction of the whole contents of the serum flasks and a part of the butyl rubber stoppers. The serum flasks were opened and supplemented with 20 ml of hexane containing 26 μg of hexadecane $ml^{-1}$ as an internal standard and sealed with mini-valve stoppers (Supelco, Buchs, Switzerland). The flasks were vigorously agitated by hand three times for 2 min, with opening of the valves each time, and then shaken for 60 h on a rotary shaker (Kühner AG, Birsfelden, Switzerland) at 240 rpm. On the shaker, the sinter glass rings were crushed to sand due to the mechanical forces. The flasks were then sonicated in an ultrasonic bath for 30 min and thereafter allowed to stand for 1 h for the separation of the water and hexane phases. A slice of 5 mm from the bottom of each butyl rubber stopper was cut and extracted with 10 ml of hexane for 60 h. The hexane extracts of the enrichment culture and the butyl rubber stopper were analyzed by GC (see below).

**Analysis of hydrocarbons, anions, gases, and $C_T$.** Pristane, HMN, and hexadecane were quantified by injecting 2 μl of the hexane extracts into a Fisons HRGC Mega II GC (Fisons Instruments, Rodano, Italy) equipped with an FID (5). The column temperature was held at 60°C for 2 min and then increased to 250°C at a rate of 7°C $min^{-1}$. External standards were used for quantification. Anions and gases were determined as described previously (5). Total inorganic carbon ($C_T$) was calculated from alkalinity (3) and $pCO_2$ (43).

**$NO_3^-$ consumption and $C_T$ production in microcosms.** Aquifer material was incubated in microcosms, and after 98 days pristane was added (9 mg used to coat 2.5 g of quartz sand to yield a final concentration of 0.56 mM pristane in the medium). Upon this addition, $NO_3^-$ consumption and $NO_2^-$ production were drastically stimulated (Fig. 1A). The concentrations recorded in Fig. 1A were measured in microcosm P, and it was found that these data represent a typical case. The initial consumption of about 0.7 mM $NO_3^-$ from day 0 to 27 (Fig. 1A) is likely to be due to the microbial metabolism of the weathered diesel fuel in the aquifer material, which was probably made bioavailable by the filling process (5). Within 508 days, 9.2 mM $NO_3^-$ was consumed and 6.7 mM $C_T$ was produced. In a microcosm which received 6.03 mM pristane and which was incubated for 890 days, $NO_3^-$ consumption and $C_T$ production of 13.2 and 12.6 mM, respectively, were determined (data not shown). No stimulation of $NO_3^-$ consumption was observed after addition of 0.56 mM pristane plus 1 mM $HgCl_2$ (Fig. 1B) or 2.5 g of uncoated quartz sand (Fig. 1C). In these controls, no significant $C_T$ production was measured. The addition of 0.44 mM *n*-alkanes (Fig. 1D) resulted in a total $NO_3^-$ consumption and $C_T$ production of 3.3 and 3.2 mM, respectively, within 508 days. In the control which received 0.44 mM *n*-alkanes plus 1 mM $HgCl_2$, $NO_3^-$ consumption and $C_T$ production were comparable to those in the control presented in

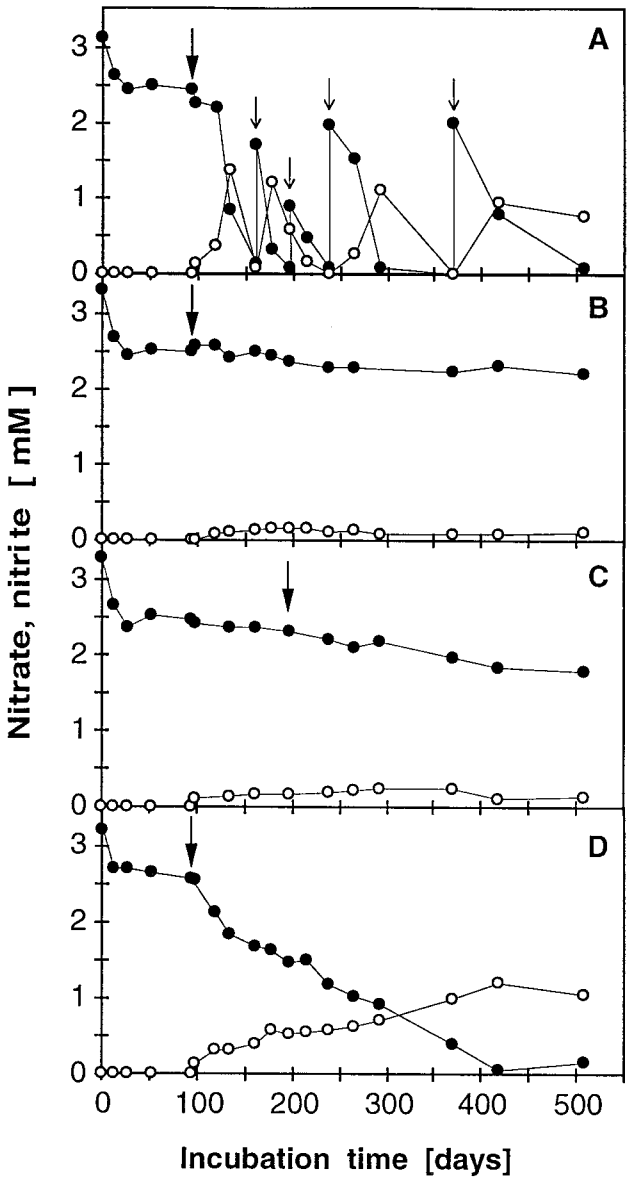


FIG. 1. $NO_3^-$ consumption (●) and $NO_2^-$ production (○) in microcosms. The heavy arrows indicate the addition of 2.5 g of quartz sand coated with 0.67 mM pristane (A), 2.5 g of quartz sand coated with 0.67 mM pristane plus 1 mM $HgCl_2$ (B), 2.5 g of uncoated quartz sand (C), and 2.5 g of quartz sand coated with 0.53 mM *n*-alkanes (D). The light arrows indicate the addition of $KNO_3$ from an anaerobic stock solution.

Fig. 1B (data not shown). The rate of $NO_3^-$ consumption was increased from 2 μM $day^{-1}$ (average rate between days 27 and 98) to >45 μM $day^{-1}$ (average rate between days 98 and 160) after addition of 0.56 mM pristane (Fig. 1A) and only to 7 μM $day^{-1}$ after addition of 0.44 mM *n*-alkanes (Fig. 1D). The weathered diesel fuel in the aquifer material used in this study consists mainly of isoprenoid alkanes and compounds of the unresolved complex mixture (comprising branched and cyclic alkanes [18]), and therefore, the aquifer microorganisms in this material may be better adapted to isoprenoid alkanes than to *n*-alkanes.

**$NO_3^-$ consumption, $C_T$ production, and pristane degradation in enrichment cultures.** The $NO_3^-$ consumption and $NO_2^-$ production in enrichment cultures supplemented with pristane and HMN are recorded in Fig. 2A. Enrichment cultures consumed 6.0 ± 1.0 mM $NO_3^-$ and produced 6.8 ± 0.6 mM $C_T$ within an incubation time of 102 days. Controls containing 12 ml of the supernatant of microcosm P and five uncoated sinter glass rings (Fig. 2B) and uninoculated, poisoned controls (data not shown) did not consume $NO_3^-$ or produce $C_T$ in significant amounts within the same time period. At the end of the incubation, the ratio of pristane to HMN was significantly reduced in enrichment cultures compared to the poisoned controls (data not shown), indicating that the decrease in pristane concentration was due to biological processes. Pristane recovery in the uninoculated, poisoned controls was 0.35 ± 0.01 mM (Table 1). In enrichment cultures, recovery was 0.03 ± 0.01 mM, suggesting that 0.32 ± 0.02 mM pristane (>90%) was degraded within 102 days of incubation under $NO_3^-$-reducing conditions. The $NO_3^-$ consumption rate was 192 μM $day^{-1}$ (Fig. 2A) between days 0 and 20, whereas the controls showed no $NO_3^-$-reducing activity (Fig. 2B). $NO_3^-$ consumption started immediately, without a lag phase, indicating that the supernatant of microcosm P contained active pristane-degrading microorganisms.

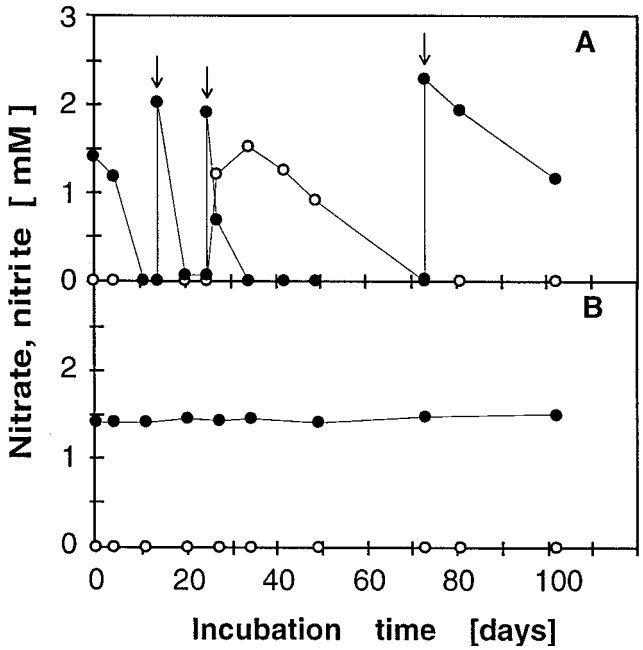


FIG. 2. $NO_3^-$ consumption (●) and $NO_2^-$ production (○) in enrichment cultures (A) and controls (B). Enrichment cultures contained 40 ml of medium, 12 ml of supernatant of microcosm P (see text), and five sinter glass rings coated with both 0.37 mM pristane and 0.33 mM HMN. Controls contained medium, supernatant, and five uncoated sinter glass rings. The arrows indicate the addition of $KNO_3$ from an anaerobic stock solution.

**Exclusion of $O_2$ involvement.** To exclude the possibility that $O_2$ was involved in the oxidation of pristane in the enrichment cultures, $O_2$ concentrations in the headspace were determined by GC (detection limit, <0.1%) during and after incubation. Moreover, $O_2$ concentrations in the medium were determined with the azide modification of the Winkler method (14) (detection limit, 0.003 mM) at the end of the incubation. $O_2$ concentrations were always below the detection limits. The oxidation of 0.32 mM pristane to pristanol by oxygenases according to the equation $C_{19}H_{40} + O_2 \rightarrow C_{19}H_{38}O + H_2O$ would require 0.32 mM $O_2$. However, our measurements indicated $O_2$ concentrations of <0.003 mM, and therefore, involvement of $O_2$ in the metabolism of pristane can be excluded.

TABLE 1. Balance of pristane degradation in enrichment cultures incubated under $NO_3^-$-reducing conditions

| Culture | Concn (mM)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Initial conditions | | | Recovered after 102 days | | |
| | Pristane | $NO_3^-$ | $C_T$ | Pristane | $NO_3^-$ | $C_T$ |
| Inoculated | 0.37 | 6.8 ± 0.7 | 11.3 ± 0.2 | 0.03 ± 0.01[b] | 0.8 ± 0.3 | 18.1 ± 0.4 |
| | 0 | 1.5 ± 0.0 | 10.8 ± 0.3 | | 1.5 ± 0.0 | 11.4 ± 0.2 |
| Uninoculated, poisoned[c] | 0.37 | 1.6 ± 0.0 | 9.3 ± 0.1 | 0.35 ± 0.01[d] | 1.7 ± 0.0 | 9.8 ± 0.1 |

[a] Data are arithmetic means ± absolute deviations (number of incubations = 2).
[b] >99% was found in the butyl rubber stopper.
[c] Receiving 1 mM $HgCl_2$.
[d] Approximately 30% was found in the butyl rubber stopper.

**Bioavailability of pristane.** The solubility of pristane in water can be estimated to be <0.1 μg liter$^{-1}$ at 25°C from data provided in reference 10. At these concentrations, microbial growth is usually limited by the substrate availability (37). Principally, the bioavailability can be enhanced by increasing the interface area between cells and the hydrophobic substrate either by using the substrate to coat surfaces (6) or by application of large amounts of substrate followed by mechanically or chemically induced emulsification (16). The addition of large amounts of substrate, however, often leads to experimental artifacts, since commercially available chemicals like pristane may contain organic impurities which may be used by microorganisms for growth. Moreover, it is difficult to detect slow pristane degradation in microcosms containing large amounts of pristane. Therefore, pristane additions should be kept rather low. The quantification of pristane degradation by using $^{14}$C-labelled pristane would overcome analytical difficulties. However, this would not exclude the evolution of $^{14}CO_2$ from $^{14}$C-labelled impurities (1) which may be present in $^{14}$C-labelled pristane preparations and which cannot be detected by GC-MS. The coating of quartz sand or, even better, sinter glass rings with pristane (i.e., application of relatively small amounts of pristane on a relatively large surface) allows analytical and bioavailability problems to be overcome.

**Pristane degradation and carbon and electron balances.** During the degradation of pristane in enrichment cultures, low-molecular-weight polar compounds such as acetate or propionate were not detectable (by using ion chromatography for analysis) (data not shown), and at the end of the incubation, accumulation of metabolites was not observed (by using GC-FID for analysis) (data not shown). These results suggest that pristane was completely mineralized.

A carbon balance showed that 0.32 ± 0.02 mM pristane, corresponding to 6.08 ± 0.38 mM C, was degraded in the inoculated enrichment culture (Table 1) (recovery in the poisoned controls minus recovery in the enrichment cultures). After 102 days, 6.3 ± 0.6 mM C was recovered as $C_T$, indicating that (i) the pristane, and not only impurities within the pristane, was degraded and (ii) complete mineralization of pristane took place. However, in addition to complete pristane mineralization, formation of microbial biomass and degradation of compounds introduced by the inoculum must also be considered. Data from the enrichment culture receiving the inoculum but no pristane (Table 1) show that the latter process contributed to about 0.6 mM $C_T$.

An electron balance was determined by considering the amounts of pristane degraded (0.32 ± 0.02 mM) under $NO_3^-$-reducing conditions (Table 1). Since $N_2O$ was not detectable (data not shown) and $NO_2^-$ was produced only transiently, $N_2$ was assumed to be the major product of $NO_3^-$ reduction. Assuming, furthermore, that 25% of the pristane carbon serves for the production of bacterial biomass (22), the stoichiometry of pristane mineralization can be described as

$$C_{19}H_{40} + 19.4NO_3^- + 5.15H^+ \rightarrow 4.75CH_2O + 14.25HCO_3^- + 9.7N_2 + 10.7H_2O \quad (1)$$

According to this equation, the total mineralization of 0.32 mM pristane would produce 4.6 mM $C_T$ and reduce 6.2 mM $NO_3^-$ to $N_2$. The measured production of $C_T$ was 6.8 ± 0.6 mM and the consumption of $NO_3^-$ was 6.0 ± 1.0 mM, suggesting that more $C_T$ and therefore less biomass were produced as estimated by using equation 1. Assuming no production of bacterial biomass, the complete mineralization of 0.32 mM pristane would produce 6.08 mM $C_T$ and consume 7.46 mM $NO_3^-$.

This study demonstrates that pristane is degraded in the absence of molecular oxygen. Therefore, pristane can no longer be regarded as a recalcitrant biomarker to quantify the degradation of mineral oil in laboratory (24) and field (4, 36) studies. However, the pathways for degradation of pristane in the absence of $O_2$ remain unknown.

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