Oxidation of various potential energy sources by the methanotrophic endosymbionts of *Siboglinum poseidoni* (Pogonophora)

Rolf Schmaljohann

Institut für Meereskunde an der Universität Kiel, Düsternbrooker Weg 20, W-2300 Kiel, Germany

ABSTRACT: The small pogonophore *Siboglinum poseidoni*, which lives in symbiosis with intracellular methanotrophic bacteria, was investigated with respect to its oxidation of higher homologues of methane. Ethane and propane were readily consumed if supplied together with methane and, at a considerably lower rate, in the absence of methane. Consumption of butane could not be detected. The addition of ethane or propane in equal amounts to methane always resulted in a decrease in methane consumption rate. Like all methanotrophic bacteria the endosymbionts of *S. poseidoni* are also capable of ammonia oxidation. Homogenates of bacteria-containing posterior parts of this organism incubated with ammonia or hydroxylamine oxidized these compounds to nitrite, while symbiont-free anterior parts were nearly inactive in this respect. Ammonia in high concentrations (>0.05 %) had an inhibitory effect on methane oxidation rates of *S. poseidoni*. The relevance of these activities in the natural habitat of this pogonophore is discussed. Since ethane and propane are present only as constituents of gas adsorbed to sediment particles and only in low concentrations compared to methane, the in situ utilization of these compounds is unlikely. Ammonia, however, being present at concentrations about 2 orders of magnitude higher than those of methane, could well serve as an additional energy source, especially in periods of low methane supply.

INTRODUCTION

The small pogonophore *Siboglinum poseidoni* has been shown to live in symbiosis with intracellular methanotrophic bacteria. This was established by \(^{14}\text{CH}_4\) uptake experiments and electron micrographs showing extensive intracellular membrane stacks typical of type I methanotrophs (Schmaljohann & Flügel 1987). These results were confirmed by stable carbon isotope analyses of pogonophoran tissues indicating that most of the cell carbon originated from biogenic methane, and by enzyme assays showing the presence of methanol dehydrogenase and hexulosephosphate synthetase (Schmaljohann et al. 1990).

Free-living obligate methanotrophic bacteria are known to co-oxidize a variety of other compounds during growth on methane (Whittenbury et al. 1970, Stirling & Dalton 1979) or during non-growth conditions without methane (Leadbetter & Foster 1960). These compounds include ethane, propane, butane and ammonia, which are present in the natural habitat of *Siboglinum poseidoni* in low concentrations and could be used as additional energy sources, especially when methane is limited.

The present study investigates whether low-molecular-weight alkanes or ammonia can be co-oxidized as additional energy sources by symbiotic methanotrophs and examines the influence of these compounds on methane oxidation rates.

MATERIAL AND METHODS

Consumption of light hydrocarbons. The Pogonophora used for these experiments were collected with a beam trawl (Flügel & Langhof 1983) at 2 stations in the central Skagerrak (*Siboglinum poseidoni*: 58°01.3' N, 09°34.6' E, 300 m water depth; *Siboglinum ekmani*: 58°11.5' N, 09°58.0' E, 440 m). After keeping the worms for 3 or 4 d in seawater at 5°C, healthy-looking individuals were sorted out, cleansed of adhering mud and rinsed twice in membrane-filtered (0.2 µm) seawater. Groups of 50 individuals were put into 58 ml crimp vials filled with 0.2 µm filtered seawater. After adjusting the headspace to exactly 5 ml the vials were sealed and the gaseous hydrocarbons were added by injection through the seal. The purity of the hydrocarbons was as follows: methane, 99.995%; ethane and...
propane, 99.95%; butane, 99.5%. In Expt 1 each vial contained the same amount of 1% hydrocarbon (v/v) in air. As solubilities of methane, ethane, propane and butane in water differ considerably, this resulted in different hydrocarbon concentrations in the incubation medium (Table 1). Solubilities of 3 of these hydrocarbons are known only for pure water and, in the case of methane, also for seawater (Wen & Hung 1970). Therefore the solubility of ethane, propane and butane in seawater can only be roughly estimated, assuming the seawater salts cause a similar reduction in solubility as for methane (Table 1). At the incubation temperature of 7°C solubility is highest for ethane, decreasing for propane, butane and methane. To account for the different solubilities of hydrocarbons, roughly equal concentrations of the gases in solution were established in Expt 2, which meant a different concentration of each hydrocarbon in the headspace (Table 1).

During incubation the decrease in hydrocarbon concentration in the headspace was monitored by daily sampling of 0.1 ml gas and subsequent analysis in a gas chromatograph (Packard 438 A) fitted with a TCD and a 3 m x 1/8 in. (3.2 mm) Porapak Q column. Helium was used as carrier gas (14 ml min⁻¹). The analysis was carried out at an initial oven temperature of 100°C, which was raised to 145°C over 3 min by a time program starting after 1 min.

Vials filled with seawater but containing no pogonophora were used as controls for determining loss of hydrocarbons by adsorption or diffusion through the seals. In Expt 2 an additional control was carried out using specimens of a related pogonophoran species (Siboglinum ekmanni), which is known to harbour sulphur-oxidizing symbionts and does not possess enzymes for C₃ oxidation (Southward et al. 1986). The time needed to establish an equilibrium between gas and liquid phase was at least 24 h. This was because earlier experiments had shown the pogonophora to be very sensitive to shaking. The incubation was carried out in darkness at 7°C, while the tubes lay horizontally (maximum fluid depth 2.5 cm) to allow gas exchange between the phases to occur as rapidly as possible.

Hydrocarbon uptake rates were calculated from headspace concentrations during the first 5 days after the beginning of the incubation, taking into account the amount of dissolved gas. As the rates remained stable or showed only a slight decrease during this period, the average rate over the 5 d is reported here. These rates were corrected by subtracting the rates for controls without pogonophora, and related to pogonophoran wet weight. Gas losses in the control tubes were almost entirely due to removal of hydrocarbons by the repeated sampling. Usually no loss of gas by absorption or leaking could be detected.

Some of the rate determinations in Expt 2 were run in duplicate. The measured rates were within the range of +7 to 14% of the average given in the figures. The gas chromatographic measurements were done in duplicate, showing an average divergence of 3.3% between 2 successive analyses.

**Influence of ammonia on methane oxidation rates.** Collection, cleaning and incubation of pogonophora was conducted similarly to the hydrocarbon studies. Different ammonia concentrations were prepared by addition of NH₄Cl (0.05, 0.1, and 0.5% w/v). The amount of ammonia present in the natural seawater (less then 10⁻⁵%) was neglected. After sealing the tubes 1% methane was injected through the septum, and methane consumption was measured daily during the first 5 d of incubation.

Ammonia concentration in sediment of the habitat of Siboglinum poseidoni was measured by suspending 10 ml of sediment in 100 ml of 3.5% NaCl solution and subsequently determining ammonia by the method of Grasshoff (1964).

**Assays for nitrifying enzymes.** Freshly sampled Siboglinum poseidoni were pulled out of their tubes and separated into anterior parts (without endosymbionts) and posterior parts containing the trophosome with the symbiotic bacteria. Groups of 30 to 50 specimens were put into 0.5 ml of 0.2 μm filtered seawater and frozen in liquid nitrogen. After thawing, the pogonophoran tissue was ruptured by gentle treatment with a glass homogenizer and centrifuged for 10 min at 12,000 x g. Bacteria in the pellet were afterwards re-
homogenized with 0.5 ml ice-cold distilled water to rupture the bacteria, and this second homogenate was used for the assays.

Assays for nitrifying enzymes were performed according to Dando et al. (1986). Homogenate (50 μl) was incubated for 24 h with 1 ml of 100 mM hydroxylamine or 2.5 mM ammonium sulphate in 50 mM diethanolamine, at pH 7.5 and 20°C. Controls incubated without substrate were used to correct for internal nitrite formation. After incubation, aliquots of the reaction mixture were centrifuged and analyzed for nitrite according to Strickland & Parsons (1972). Protein contents of the homogenate were determined with the method of Bradford (1976).

**RESULTS**

Consumption of hydrocarbons

In Expt 1 either methane, ethane, propane and butane were added singly at a 1% concentration to the headspace of the incubation vials, or methane was combined with one of the higher homologues (at an equal concentration in the gas phase). Results are given in Fig. 1. Methane alone was taken up most rapidly by *Siboglinum poseidoni*, followed by ethane and only minor amounts of propane, while butane was not consumed. If methane was added simultaneously, the amount of ethane and propane taken up was larger than that without methane. Ethane was even preferred to methane in this case, probably because of its higher solubility. Butane, however, could not be taken up even in the presence of methane. Methane consumption in combination with higher alkanes was highest with butane, decreasing for propane and ethane. The difference in uptake rates with methane and butane compared to those with methane alone was not significant.

In Expt 2 equal concentrations of hydrocarbons in solution were applied, and the results for *Siboglinum poseidoni* (with methanotrophic symbionts) were compared with *S. ekmami* (with sulphur-oxidizing symbionts). As in Expt 1, uptake of methane alone by *S. poseidoni* was higher than that of the other hydrocarbons, and the simultaneous presence of methane enhanced uptake of ethane and propane (Fig. 2). The methane consumption rate was higher than in Expt 1, probably due to higher methane concentrations. *S. ekmami* produced only a very low decrease in methane, ethane or propane concentrations, apparently due to adsorption of hydrocarbons to the pogonophore tube or to uptake by bacterial contaminants adhering to it. As these low rates are comparable to propane uptake by *S. poseidoni* without methane, propane uptake by this species is also probably not due to oxidation by the symbionts.

Influence of ammonia on methane oxidation rates

*Siboglinum poseidoni* individuals incubated without additional ammonia consumed methane at rates comparable to those in Expt 1 (which had an identical methane concentration in the medium) (Table 2). Addition of 0.05 or 0.1% NH₄Cl resulted in a decrease in methane uptake rate to 22%, while 0.5% NH₄Cl in the medium led to a nearly total breakdown of methane oxidation.

The ammonia concentrations used in this experiment were selected to compare the degree of inhibition with data from Whittenbury et al. (1970), who employed identical concentrations for free-living methanotrophs. Compared to naturally occurring ammonia concentrations in the sediment, which range between 0.1 and 0.7 mM kg⁻¹ wet wt (Table 3) (corresponding to ca 0.002 to 0.015% w/v in pore water), these concentrations are very high.

![Fig. 1 Siboglinum poseidoni. Consumption of gaseous alkanes in Expt 1 (incubation with initial concentration of 1% hydrocarbon in the gas phase). M: methane; E: ethane; P: propane; B: butane](image-url)
Oxidation of ammonia and hydroxylamine to nitrite

Homogenates of *Siboglinum poseidoni* were able to oxidize ammonia as well as hydroxylamine to nitrite (Table 4). This activity was confined to the posterior parts of the Pogonophora, containing the trophosome with methanotrophic endosymbionts. The low activity in the worms' anterior parts was probably due to contamination with trophosome material during preparation.

Table 2. *Siboglinum poseidoni*. Inhibition of methane oxidation rates by different concentrations of NH₄Cl. Rates are given in nmol g⁻¹ wet wt h⁻¹ (1 individual corresponds to between 3 and 4 mg). Groups 1 and 2 were duplicates

<table>
<thead>
<tr>
<th>Conc. of NH₄Cl</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.00005 %</td>
<td>60.4</td>
<td>69.7</td>
<td>65.05</td>
</tr>
<tr>
<td>0.05 %</td>
<td>13.7</td>
<td>14.2</td>
<td>14.15</td>
</tr>
<tr>
<td>0.10 %</td>
<td>14.2</td>
<td>14.1</td>
<td>14.15</td>
</tr>
<tr>
<td>0.50 %</td>
<td>0</td>
<td>4.7</td>
<td>2.35</td>
</tr>
</tbody>
</table>

Table 3. Concentrations of NH₄⁺ in Skagerrak sediments containing *Siboglinum poseidoni*

<table>
<thead>
<tr>
<th>Sediment 1</th>
<th>Sediment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (cm)</td>
<td>NH₄⁺ (μmol kg⁻¹ wet wt)</td>
</tr>
<tr>
<td>0-1</td>
<td>190</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
</tr>
<tr>
<td>6</td>
<td>531</td>
</tr>
<tr>
<td>10</td>
<td>724</td>
</tr>
<tr>
<td>15</td>
<td>290</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The ability of *Siboglinum poseidoni*, or rather its methanotrophic endosymbionts, to consume ethane and propane was demonstrated by gas chromatographic analysis. It has been shown for several free-living obligate methanotrophic bacteria that the enzyme responsible for the first step in methane oxidation, a methane mono-xygenase, can also oxidize its gaseous homologues ethane, propane and butane, in addition to a number of other compounds (Anthony 1982). These compounds cannot be used as the sole carbon source by obligate methanotrophs, but they can be oxidized and used as additional energy sources. It has been shown by Davidova et al. (1988) that during growth of *Methylococcus capsulatus* in a gaseous mixture containing methane and ethane the latter is primarily oxidized to water-soluble products which are released to the medium. According to Leadbetter & Foster (1960) these are mainly ethanol, acetaldehyde and acetic acid. Especially in the case of an endosymbiosis these exudates could be directly used by the host. A similar excretion of metabolites also occurs during methane oxidation (Higgins et al. 1982).

An inhibitory effect of ethane and propane on methane consumption has been reported by several authors (Leadbetter & Foster 1960, Davidova et al. 1988). This inhibition was not effective if the released metabolites were removed by an associate culture,
indicating that not only the substrate competition is important but also an inhibition by the released metabolites. In this respect as well, an endosymbiosis could favour the co-oxidation of gaseous alkanes.

The hydrocarbon uptake rates found in the present experiments cannot be extrapolated to in situ conditions. Natural concentrations of ethane, propane and butane in the habitat of *Siboglinum poseidoni* are much lower than that of methane. Free gases extracted from pore water contained no measurable C$_2$+ hydrocarbons, whereas gas adsorbed to sediment amounted to 86.7 ppb methane, 7.1 ppb ethane, 2.8 ppb propane and 1.9 ppb butane (w/w) (Schmaljohann et al. 1990). In areas where thermogenic gas seeps from the sediment the gaseous alkanes are much more important. Anderson et al. (1983) report C$_1$/C$_2$+C$_3$ ratios between 0.52 and 56.5 for the Louisiana (USA) slope seeps. It would be interesting to investigate the impact of these compounds on the symbiosis between mytilid mussels and methanotrophs occurring in this area (Childress et al. 1986).

The finding that methane oxidation by *Siboglinum poseidoni* is inhibited by ammonia is consistent with reports from free-living methanotrophs. Whittenbury et al. (1970), who applied the same NH$_4$Cl concentrations to cultures of *Methylosinus trichosporium* strain OB 3B, noted that methane oxidation rates with 0.05% NH$_4$Cl in the medium decreased to ca 73% and with 0.5% NH$_4$Cl to 7%, of rates without addition of ammonia. These authors observed similar ratios for other methanotroph strains. This comparison shows that the symbionts of *S. poseidoni* behave like true methanotrophs and that the host obviously does not hinder high ammonia concentrations from entering through its tissue.

The nitrification rates obtained from assays with homogenates of *Siboglinum poseidoni* are qualitative rather than quantitative in nature. Oxidation rates cannot be compared with those found e.g. by Dalton (1977) for crude extracts of *Methyllococcus capsulatus*, because of the long incubation time applied and different assay parameters in the present study. But the data clearly indicate that nitrifying activity associated with the symbionts in the posterior parts of Pogonophora occurs, which is lacking in the bacteria-free anterior parts. These data show for the first time that endosymbiotic bacteria in marine invertebrates can also oxidize potential energy sources other than methane or reduced sulphur compounds. Dando et al. (1986), applying the same assay to several symbiotic associations including Pogonophora and bivalves in a Norwegian fjord, did not find any nitrifying activity in these organisms.

The higher activity observed for hydroxylamine oxidation compared to ammonia oxidation might be explained by the observation of Dalton (1977) that there was always a lag phase before nitrite was formed from ammonia, and that a certain concentration of hydroxylamine (ca 0.25 mM) had to be formed from ammonia before nitrite could be formed.

Ammonia oxidation by methanotrophs has also been reported by other authors (Dalton 1977, O’Neill & Wilkinson 1977, Romanovskaya et al. 1977). The data suggest that nitrification is a normal element of the metabolism of methanotrophs, supplying these organisms with an additional source of energy. Though some obligate methanotroph strains possess ribulosebisphosphate-carboxylase (*Methylococcus capsulatus*), they are not capable of autotrophic growth in the absence of methane and with NH$_4^+$ as their energy source, as are ammonia-oxidizing bacteria (Stanley & Dalton 1982). There are also ammonia-oxidizing bacteria such as *Nitrosococcus oceanus* that are able to obtain energy from the oxidation of methane to CO$_2$ and to incorporate this into cell carbon. These bacteria have a high affinity to methane and can metabolize it at rates approximately equal to ammonia during ammonia oxidation (Ward 1987). As these organisms also have internal membrane systems quite similar to those of the endosymbionts of *Siboglinum poseidoni*, it could be suggested that the symbionts are in reality ammonia-oxidizing bacteria. It has, however, been shown that the bulk of cell carbon in *S. poseidoni* originates from biogenic methane, which is evident from the extremely $^{13}$C-depleted state of its cell carbon (Schmaljohann et al. 1990). Nitrifying bacteria cannot grow on methane as the sole source of carbon and energy, and especially strains isolated from marine water samples seem to incorporate only negligible amounts of methane carbon during methane oxidation (Jones & Morita 1983).

The rates for methane oxidation by whole pgonophores (Table 2) cannot be directly compared with the measured nitrification rates by homogenates of *S. poseidoni* (Table 4). However, if the rates for homogenates of posterior parts are expressed in mmol g$^{-1}$ wet wt h$^{-1}$ (0.54 nmol g$^{-1}$ wet wt h$^{-1}$ for 2.5 mM ammonium sulphate and 1.43 nmol g$^{-1}$ wet wt h$^{-1}$ for 100 $\mu$mol hydroxylamine) these rates can be compared to methane oxidation rates (65.05 nmol g$^{-1}$ wet wt h$^{-1}$ at a concentration of 11.9 $\mu$M methane).

### Table 4. Siboglinum poseidoni. Formation of nitrite (nmol mg$^{-1}$ protein d$^{-1}$) from ammonia or hydroxylamine by homogenates of pgonophores. Values are results of duplicate measurements

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NH$_4$SO$_4$</th>
<th>NH$_2$OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior parts</td>
<td>11.8; 13.2</td>
<td>32.4; 34.6</td>
</tr>
<tr>
<td>Anterior parts</td>
<td>3.0; 3.1</td>
<td>2.2; 1.8</td>
</tr>
</tbody>
</table>
To evaluate the possibility of ammonia oxidation by *Siboglinum poseidoni* symbionts it is necessary to consider the concentrations of methane and ammonia in the natural habitat of this pogonophore. Concentrations of methane in the sediment varied between 1 and 44 μmol kg⁻¹ wet wt (Schmaljohann & Flügel 1987, Schmaljohann et al. 1990), whereas ammonia concentrations were between 123 and 724 μmol kg⁻¹ (Table 3). This means that ammonia concentrations were 1 to 2 orders of magnitude higher than methane concentrations. Dalton (1977) gives $K_M$ (Michaelis constant) values for the methane monooxygenase of *Methylococcus capsulatus* of 160 μM for methane and 96 μM for ammonia. Ammonia oxidation in this methanotroph would therefore only be significant if there were either very much ammonia or very little methane in the sediment. In the sediment from the S. *poseidoni* site, with its low and varying methane concentrations, the possibility of switching from methane to ammonia oxidation might be advantageous to a sedentary organism.

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