

## Impact of Temperature on Ladderane Lipid Distribution in Anammox Bacteria<sup>∇†</sup>

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Anaerobic ammonium-oxidizing (anammox) bacteria have the unique ability to synthesize fatty acids containing linearly concatenated cyclobutane rings, termed “ladderane lipids.” In this study we investigated the effect of temperature on the ladderane lipid composition and distribution in anammox enrichment cultures, marine particulate organic matter, and surface sediments. Under controlled laboratory conditions we observed an increase in the amount of C<sub>20</sub> [5]-ladderane fatty acids compared with the amount of C<sub>18</sub> [5]-ladderane fatty acids with increasing temperature and also an increase in the amount of C<sub>18</sub> [5]-ladderane fatty acids compared with the amount of C<sub>20</sub> [5]-ladderane fatty acids with decreasing temperature. Combining these data with results from the natural environment showed a significant ( $R^2 = 0.85$ ,  $P = <0.0001$ ,  $n = 121$ ) positive sigmoidal relationship between the amounts of C<sub>18</sub> and C<sub>20</sub> [5]-ladderane fatty acids and the *in situ* temperature; i.e., there is an increase in the relative abundance of C<sub>18</sub> [5]-ladderane fatty acids at lower temperatures and *vice versa*, particularly at temperatures between 12°C and 20°C. Novel shorter (C<sub>16</sub>) and longer (C<sub>22</sub> to C<sub>24</sub>) ladderane fatty acids were also identified, but their relative amounts were small and did not change with temperature. The adaptation of ladderane fatty acid chain length to temperature changes is similar to the regulation of common fatty acid composition in other bacteria and may be the result of maintaining constant membrane fluidity under different temperature regimens (homeoviscous adaptation). Our results can potentially be used to discriminate between the origins of ladderane lipids in marine sediments, i.e., to determine if ladderanes are produced *in situ* in relatively cold surface sediments or if they are fossil remnants originating from the warmer upper water column.

Anaerobic ammonium-oxidizing (anammox) bacteria possess the unique ability to oxidize NH<sub>4</sub><sup>+</sup> with NO<sub>2</sub><sup>-</sup> to N<sub>2</sub> under anoxic conditions (42). Since the discovery of the anammox process in a wastewater treatment plant in the Netherlands (21), studies have indicated that anammox bacteria are omnipresent in low-oxygen environments around the world. Anammox therefore forms an important link in both the oceanic (4, 7, 17, 18, 31) and freshwater (14, 33) nitrogen cycles. Unlike other *Planctomycetes*, anammox bacteria contain a unique “organelle” called the anammoxosome (19, 37, 44–46). The membrane of this compartment contains unusual “ladderane” lipids (37). The core ladderane lipids consist of C<sub>18</sub> and C<sub>20</sub> fatty acids containing either 3 or 5 linearly concatenated cyclobu-

tane rings, which are ester bound to a glycerol backbone or ether bound as alkyl chains (35). In addition, the intact polar lipids containing the core lipid structures may have different types of polar head groups, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylglycerol (PG) (1, 22). *In silico* density simulation modeling experiments with a ladderane lipid-containing membrane (glycerol-bound mixed ether-ester containing both ladderane moieties) have indicated that ladderane lipids could provide a denser cell membrane than conventional membrane lipids (37). Since the anammoxosome appears to be impenetrable to fluorophores, the ladderane membrane could function in cell energy conservation (37, 44).

Experimental evidence has shown that anammox bacteria isolated from wastewater treatment reactors grow over a wide range of temperatures (20 to 43°C) and have an optimum temperature of about 35°C (39). In the natural environment the anammox process has been reported to occur at temperatures as low as –2.5°C in sea ice (5, 26) and as high as 70°C in hot springs and hydrothermal vent areas (3, 12). Furthermore, “*Candidatus Scalindua spp.*” has been successfully enriched from marine sediment (Gullmarsfjord, Sweden) in sequencing batch reactors at temperatures of 15 and 20°C (43). In other bacteria containing common fatty acids temperature adaptation can be achieved by (among other things) modifying the

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composition of the membrane bilayers to deal with alterations in membrane viscosity due to changes in temperature. This process has been well documented and is termed "homeoviscous adaptation"; i.e., the fatty acid composition is changed to maintain membrane fluidity (23, 27, 34, 40). Currently, it is not known how anammox bacteria, with their highly unusual ladderane lipids, react to temperature. To investigate this, we analyzed the ladderane lipid composition of anammox bacteria grown at different temperatures in sequencing batch reactors and in samples from different natural environments covering a wide range of temperatures.

#### MATERIALS AND METHODS

**Cultivation of anammox bacteria.** Sequencing batch reactors (SBRs) were used for cultivation of anammox enrichment cultures (16, 38). Before an SBR was started, the percentage of anammox bacteria in the inoculum was determined using fluorescence *in situ* hybridization as previously described (16). "*Candidatus Brocadia fulgida*" was the only anammox species identified in the enrichment culture and accounted for around 75% of the total population. The "*Candidatus Brocadia fulgida*" inoculum biomass was initially cultured at 35°C (16, 38), and then it was split and placed in three 2-liter SBRs set to 16°C, 25°C, and 35°C. Biomass cultured at 35°C remained in the original operational "*Candidatus Brocadia fulgida*" SBR. Enrichment cultures were grown on mineral medium containing  $\text{NO}_2^-$  and  $\text{NH}_4^+$  prepared as described by van de Graaf et al. (41). Anoxic conditions were maintained by continuously flushing the SBRs with 95% Ar-5%  $\text{CO}_2$  at a rate of 10 ml  $\text{min}^{-1}$ , and the SBR contents were mixed using a turbine stirrer (ca. 250 rpm). The temperatures of the three reactors were kept constant using water jackets. The reactors were operated for 85 days, and 20-ml biomass-reactor medium samples were taken weekly. Each biomass suspension was centrifuged, and the biomass pellet was frozen at  $-20^\circ\text{C}$  until it was analyzed. The anammox bacteria in the 16°C and 25°C reactors were not cultured at the optimum temperature (35°C) and as a consequence were less active and thus consumed less  $\text{NH}_4^+$  and  $\text{NO}_2^-$ . As a result, the nutrient load in the SBRs had to be monitored and measured daily to prevent excess  $\text{NO}_2^-$  from poisoning the cultures. When the  $\text{NO}_2^-$  concentration was more than 40 mg/liter, nutrient addition was stopped until the excess  $\text{NO}_2^-$  was consumed; alternatively, the pump system was set to a lower speed to match the consumption rate.

In addition to three SBR cultures, cell material was obtained from full-grown enrichment cultures of the following anammox species grown at a variety of temperatures: "*Candidatus Brocadia anammoxidans*" (35°C) (30), "*Candidatus Brocadia fulgida*" (16, 19, 23, 31, 35, 39, and 40°C) (16), "*Candidatus Anammoxoglobus propionicus*" (33°C for aggregate cells; 23°C for single cells) (15), "*Candidatus Kuenenia stuttgartiensis*" (35°C) (29), and "*Candidatus Scalindua* spp." (15 and 20°C) (43). Enrichment cultures were grown in SBRs as described previously.

**Environmental samples.** Particulate organic matter (POM) and surface sediments were obtained from a variety of sources. POM samples were collected from the Namibian (17), Peruvian (7), and Arabian Sea oxygen minimum zones (11) and the Black Sea (47) as described previously. Surface sediment from the Santa Barbara Basin was collected as described previously (9). Surface sediments were also collected from the Gullmarsfjord (Sweden) in 2005 (8, 31), from the northwest African continental shelf and slope during a cruise of the R/V *Meteor* (leg M65/1) in the summer of 2005 (10a), from the Irish and Celtic Sea during a cruise of the R/V *Pelagia* in the spring of 2006 (10), and from the Black Sea during a cruise of the R/V *Knorr* (KN172/8) in May 2003 (47). Sediment and microbial mat samples were collected from neutral to alkaline hot springs in northern California and western Nevada in February 2007 (12). The environmental conditions found in the mats have been extensively described elsewhere (12, 32).

**Ladderane core lipid analysis.** Approximately 20 mg (dry weight) of enrichment culture biomass was extracted by ultrasonic disruption of cells using methanol three times, methanol-dichloromethane three times, and dichloromethane three times. Combined extracts were dried using rotary evaporation and were methylated using  $\text{BF}_3$  in methanol and heated for 15 min at 60°C. An aliquot of each methylated total-lipid extract was eluted in ethyl acetate over a small silica column containing 5%  $\text{AgNO}_3$  to remove polyunsaturated fatty acids. Fractions were subsequently dissolved in acetone and then filtered through 0.45- $\mu\text{m}$ -pore-size, 4-mm-diameter polytetrafluoroethylene filters and analyzed using high-performance liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry (HPLC-APCI-MS/MS).

The POM total-lipid extracts from Namibian Margin stations M182 and M202 were obtained as previously described by Kuypers et al. (17), and the POM total-lipid extracts from the Peruvian Margin were obtained as previously described by Hamersley et al. (7). The total-lipid extracts were methylated, dissolved in ethyl acetate, and eluted over a small silica column impregnated with 5%  $\text{AgNO}_3$ ; they were subsequently dissolved in acetone and filtered as described above and finally analyzed using HPLC-APCI-MS/MS.

POM from the Black Sea and POM from the Arabian Sea were extracted as described by Wakeham et al. (47) and Jaeschke et al. (11), respectively. Surface sediments from the Santa Barbara Basin were ground as described previously by Huguet et al. (9), and a total-lipid extract was prepared for ladderane lipid analysis (10a). Surface sediments from the Celtic Sea and the Irish Sea were ground and extracted as described by Jaeschke et al. (10).

**HPLC-APCI-MS/MS analysis.** Ladderane lipids in sediments were analyzed for  $\text{C}_{18}$  [5]- and  $\text{C}_{20}$  [5]- and [3]-ladderane fatty acids by HPLC-APCI-MS/MS using the method of Hopmans et al. (8). Ladderane lipids in enrichment culture biomass and POM were analyzed using a method adapted from the method of Hopmans et al. (8), which quantified the  $\text{C}_{18}$  [3]-ladderane fatty acid and the 3 ladderane fatty acids mentioned above (22). Briefly, samples dissolved in acetone were injected onto a Zorbax Eclipse XDB (extra densely bonded)  $\text{C}_8$  column (3.0 by 250 mm; 5  $\mu\text{m}$ ; Agilent) kept at 30°C. Ladderane fatty acids were eluted using methanol at a rate of 0.18 ml  $\text{min}^{-1}$  and were detected with a Quantum TSQ Ultra EM triple quadrupole mass spectrometer (Thermo Inc., San Jose, CA), equipped with an ion max source with an APCI probe in selective reaction monitoring (SRM) mode.

In order to identify ladderane fatty acid methyl esters with chain lengths less than and/or greater than those previously reported, two enrichment culture biomass extracts, a "*Candidatus Scalindua* spp." extract representing a low growth temperature (15°C) and a "*Candidatus Brocadia fulgida*" extract representing a high growth temperature (35°C), were analyzed using MS/MS in product ion scan mode. The collision energy was 25 V, and the collision gas was 1.5 mtorr argon; the chromatographic and source conditions were the conditions described above, with parent masses set to match the predicted protonated molecules ( $[\text{M}+\text{H}]^+$ ) of all possible  $\text{C}_{16}$  to  $\text{C}_{24}$  ladderane fatty acid methyl esters, i.e.,  $m/z$  261.2, 289.3, 317.3, 345.3, and 373.3 and  $m/z$  263.2, 291.3, 319.3, 347.3, and 375.3 for the even-numbered  $\text{C}_{16}$  to  $\text{C}_{24}$  [5]- and [3]-ladderane fatty acids, respectively. Based on the results of this experiment, the method described by Hopmans et al. (8) was modified to include SRM transitions to quantify the  $\text{C}_{16}$  to  $\text{C}_{24}$  [5]- and [3]-ladderane fatty acids (Fig. 1 and 2) in addition to the  $\text{C}_{18}$  [5]-ladderane fatty acid and  $\text{C}_{20}$  [5]- and [3]-ladderane fatty acids (Fig. 1 and Fig. 2c, e, and f). The SRM transitions used to detect the monoether were removed from the MS method. For the ladderane fatty acids containing five linearly concatenated cyclobutane moieties, transitions from the corresponding protonated molecules ( $[\text{M}+\text{H}]^+$ ) to  $m/z$  91.1, 105.1, 133.1, and 161.1 at collision energies of 37, 35, 24, and 19 V, respectively, were monitored. For the ladderane fatty acid containing three concatenated cyclobutanes, transitions from the corresponding protonated molecules ( $[\text{M}+\text{H}]^+$ ) to  $m/z$  93.1, 121.1, 135.1, and 163.1 at collision energies of 37, 24, 19, and 16 V, respectively, were monitored. Quantitative standards are available only for the  $\text{C}_{20}$  [5]- and [3]-ladderane fatty acids (Fig. 1e and f). Therefore, all ladderane fatty acids containing 5 concatenated cyclobutanes were quantified using an external standard curve for the  $\text{C}_{20}$  [5]-ladderane fatty acid, while the ladderane fatty acids containing 3 cyclobutanes were quantified using an external standard curve for the  $\text{C}_{20}$  [3]-ladderane fatty acid. All samples except the sediment samples were reanalyzed for  $\text{C}_{16}$  to  $\text{C}_{24}$  [5]- and [3]-ladderane fatty acids using this modified method.

#### RESULTS AND DISCUSSION

**Anammox enrichment cultures.** Aliquots of an inoculum of nonaxenic "*Candidatus Brocadia fulgida*" initially cultured at 35°C were grown in three SBRs at 16, 25, and 35°C (Fig. 3). To quantify the change in the relative amount of the  $\text{C}_{20}$  [5]-ladderane fatty acid, its abundance was normalized relative to that of the other ladderane fatty acids. Since fatty acid chain length has been shown to change with temperature for generic fatty acids in other bacteria (23–25, 27, 34, 40), we normalized the fatty acids with equivalent amounts of cyclobutane rings but with different chain lengths. Therefore, we quantified the trends in ladderane chain length using the index of ladderane lipids with 5 cyclobutane rings ( $\text{NL}_5$ ):

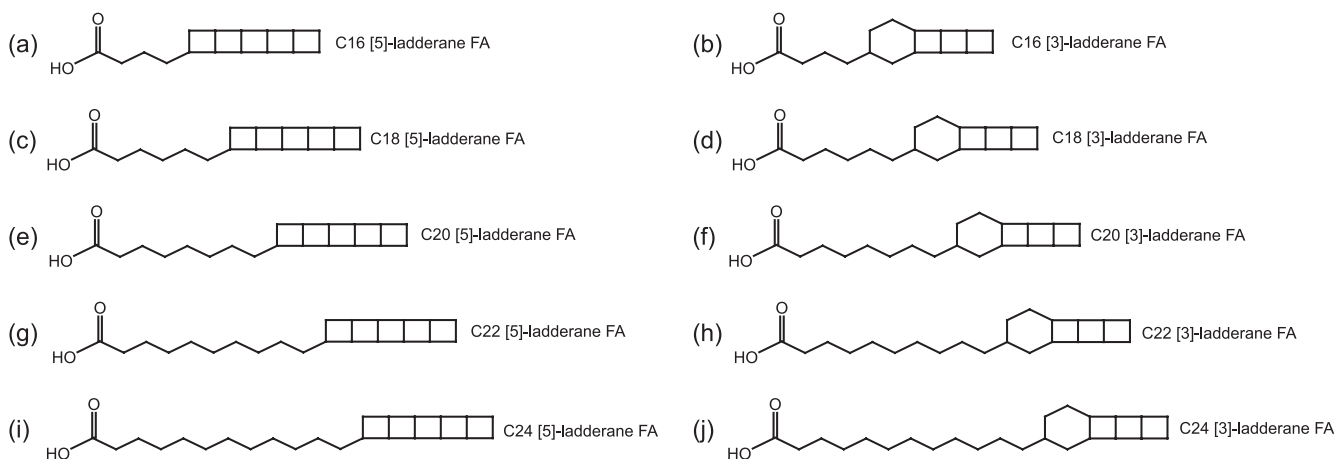


FIG. 1. Homologous series of ladderane fatty acid core lipid structures. FA, fatty acid.

$$NL_5 = \frac{[C_{20} [5]\text{-ladderane fatty acid}]}{([C_{18} [5]\text{-ladderane fatty acid}] + [C_{20} [5]\text{-ladderane fatty acid}])}$$

The  $NL_5$  appeared to function in response to the *in situ* temperature, and the  $NL_5$  for the 35°C cultured biomass remained around 0.9 (Fig. 3). The only exception to this was a change in the fatty acid composition after 14 days, which coincided with the addition of a supplemental amount of “*Candidatus Brocadia fulgida*.” The extra biomass was needed to increase reactor productivity and had been kept at a relatively low (8°C) temperature before addition. In contrast, the  $NL_5$  for anammox biomass cultured at 25°C showed a gradual decrease from 0.89 to 0.84, and the  $NL_5$  for anammox biomass cultured at 16°C displayed a sharp decrease from 0.89 to between 0.65 and 0.75 (Fig. 3). Since the typical doubling time of anammox bacteria is around 14 days, it appears that “*Candidatus Brocadia fulgida*” regulates its membrane lipids to adapt to a new temperature regimen, especially considering that the new ladderane lipid distribution is diluted by the divided cells, which already contain membranes adapted to the *in situ* temperature of the inoculum.

A less defined relationship was also observed between ladderane fatty acids with different numbers of cylobutane rings and different chain lengths in response to temperature (data not shown). An increase in the amount of  $C_{20}$  [5]-ladderane fatty acid compared with the amount of  $C_{20}$  [3]-ladderane fatty acid with increasing temperature was also observed in the laboratory samples. However, changes in cyclization of  $C_{20}$  ladderane fatty acids were not observed in the environmental samples. The reasons for this are unclear and require further investigation.

In order to investigate if other genera of anammox bacteria adjust their ladderane fatty acid chain length with temperature, we analyzed ladderane fatty acids in SBR enrichment cultures of three other genera of anammox bacteria, “*Candidatus Anammoxoglobus propionicus*,” “*Candidatus Scalindua* spp.,” and “*Candidatus Kuenenia stuttgartiensis*” (Table 1; see the supplemental material). The cultures of “*Candidatus Scalindua* spp.” were enriched in SBRs at 15 and 20°C using marine sediment from Gullmarsfjord (Sweden) with an *in situ* temperature of 6°C (43). The results showed that species cultured at lower temperatures contained larger amounts of the  $C_{18}$

[5]-ladderane fatty acid and consequently lower percentages of the  $C_{20}$  [5]-ladderane fatty acid compared to biomass grown at higher temperatures. Therefore, in four anammox genera a lower  $NL_5$  was calculated for biomass cultured at lower temperatures and *vice versa*.

**Particulate organic matter and sediments.** To investigate whether the ladderane fatty acid composition also varied with temperature in natural settings, we analyzed particulate organic matter (POM) and surface sediments where anammox activity had been documented previously (Table 1). Figure 4 shows three base peak chromatograms obtained from POM and surface sediment at *in situ* temperatures at the “extreme temperature ends” of the environmental data set (see the supplemental material). One of these chromatograms clearly shows that the relative abundance of the  $C_{20}$  [5]-ladderane fatty acid was lower in the surface sediment from the northwest African continental slope (at a water depth about of 3,000 m, where the *in situ* temperature was 2°C) than in the Peru POM sample (15.4°C). Since the ladderane fatty acids in POM and sediment showed similar responses to temperature, as observed in the anammox enrichment cultures, we calculated the  $NL_5$  value for the environmental samples and plotted this value together with the microbial mat and enrichment culture data against the *in situ* temperature (see the supplemental material for a detailed list of all data used). This resulted in a significant fourth-order sigmoidal relationship ( $R^2 = 0.85$ ,  $P < 0.0001$ ,  $n = 121$ ) between the  $NL_5$  and temperature (Fig. 5). The greatest change in ladderane fatty acid chain length took place at temperatures between 12°C and 20°C, while there was only a small change in chain length at temperatures less than 12°C and more than 20°C.

Most of the  $NL_5$  data obtained for temperatures less than 12°C are data for environmental samples in which the marine anammox bacteria belonged exclusively to the genus “*Candidatus Scalindua*” (7, 17, 31, 43, 47). In contrast, most of the  $NL_5$  data obtained for temperatures more than 20°C are data for cultures (this study) and environments (12) in which organisms phylogenetically related to “*Candidatus Brocadia fulgida*,” “*Candidatus Brocadia anammoxidans*,” and “*Candidatus Kuenenia stuttgartiensis*” were present. Therefore, the differences in  $NL_5$  values could be attributed to differences in

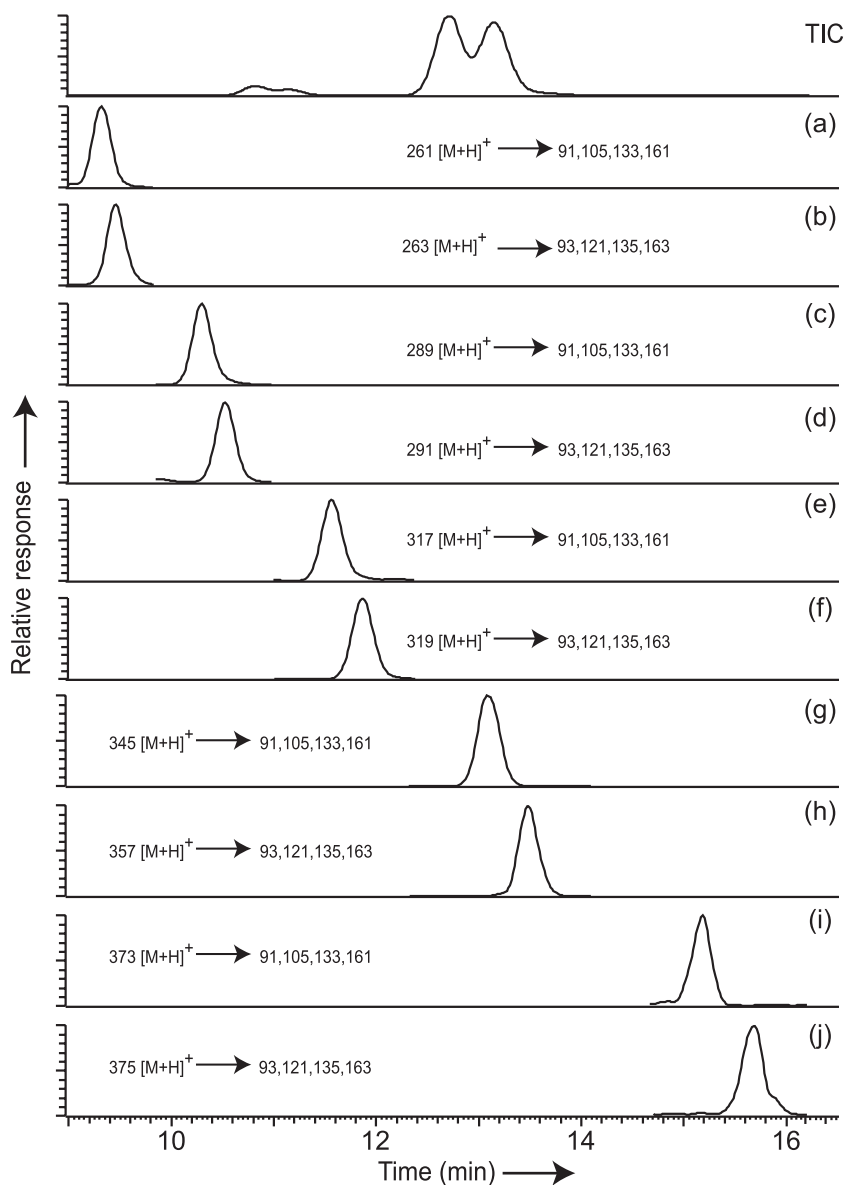


FIG. 2. HPLC-APCI-MS/MS chromatogram illustrating previously unreported protonated molecules and selected product ions for shorter  $C_{16}$  and longer  $C_{24}$  ladderane fatty acids and previously reported  $C_{18}$  to  $C_{22}$  ladderane fatty acids. Panels a to j correspond to structures in Fig. 1a to j, respectively. Ladderane fatty acids were analyzed as methyl esters.

anammox phylogeny rather than temperature. However, the data set shown in Fig. 5 also includes data for a marine surface sediment from Gullmarsfjord (*in situ* temperature, 6°C) and anammox cultures enriched from this sediment (15°C and 20°C) (43). The fact that the  $NL_5$  values of the sediment and biomass fit well in the curve in Fig. 5 indicates that the observed trend observed was probably not only the result of genetic differences between the different anammox species but was mainly due to acclimation of individual anammox cells. The trend shown in Fig. 5 therefore suggests that the species of anammox bacteria analyzed in this study likely change their ladderane fatty acid chain length mainly as an adaptation to the *in situ* temperature over the range from 12 to 20°C. An exception to the trend was the data for two of the hot spring microbial mats, and the reasons for this could include diurnal

temperature variations or a response to other variables in the mat environment.

**Longer and shorter ladderane fatty acids.** The apparently constant ladderane fatty acid chain lengths at temperatures less than 12°C and more than 20°C raise the question of whether anammox bacteria adapt to temperature regimens by synthesizing fatty acids with chain lengths other than  $C_{18}$  and  $C_{20}$ . Therefore, we investigated whether ladderane fatty acids with acyl chains containing less than 7 and more than 9 carbon atoms are present in anammox bacteria, in addition to the previously identified  $C_{18}$ ,  $C_{20}$ , and  $C_{22}$  ladderane fatty acids (22, 35).

Two enrichment cultures, a culture of “*Candidatus Scalindua* spp.” representing low-temperature growth conditions (15°C) and a culture of “*Candidatus Brocadia fulgida*” repre-

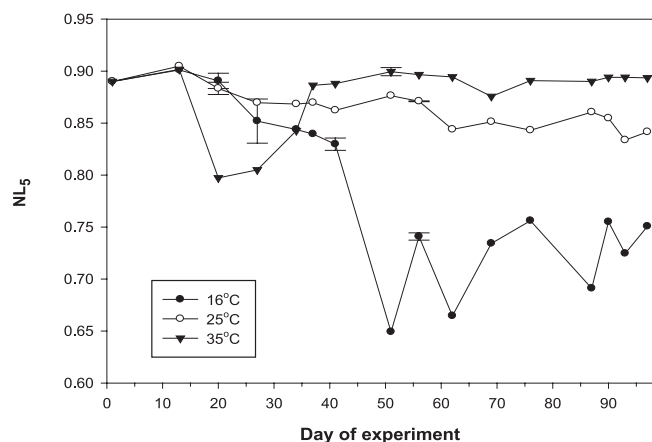


FIG. 3.  $NL_5$  plotted over time for anammox biomass of “*Candidatus Brocadia fulgida*” cultured in 16, 25, and 35°C reactors. The control reactor was kept at 35°C, whereas the biomass in the 16°C and 25°C reactors was originally cultured at 35°C. The error bars indicate the standard deviations for the  $NL_5$  values calculated using duplicate or triplicate samples prepared and analyzed separately.

senting high-temperature growth conditions (35°C), were analyzed by MS/MS in product ion scan mode to search for shorter or longer ladderane fatty acids. In both cases, the resulting traces showed a series of peaks eluting in pairs (data not shown). Each pair of peaks appeared to consist of one ladderane fatty acid containing 5 cyclobutane rings and one ladderane fatty acid containing 3 cyclobutane rings, based on the parent mass and the MS/MS spectra (data not shown). Besides the previously reported  $C_{18}$  [5]-ladderane fatty acid and  $C_{20}$  [5]- and [3]-ladderane fatty acids (37),  $C_{18}$  [3]-ladderane fatty acid (18), and  $C_{22}$  [5]- and [3]-ladderane fatty acids (22),  $C_{16}$

and  $C_{24}$  ladderane fatty acids with 5 and 3 cyclobutane moieties were also tentatively identified based on their elution behavior, parent mass, and product ion mass spectra.

Based on these results, the SRM method (8) was modified to include SRM transitions specific for  $C_{16}$ ,  $C_{22}$ , and  $C_{24}$  [5]- and [3]-ladderane fatty acids. This analysis showed that the  $C_{18}$  and  $C_{20}$  [5]- and [3]-ladderane fatty acids were always dominant and that the  $C_{16}$ ,  $C_{22}$ , and  $C_{24}$  [5]- and [3]-ladderane fatty acids constituted only a minor fraction of the ladderane fatty acids. In fact, the latter fatty acids never comprised more than ~2% of the total ladderane fatty acids extracted from the “*Candidatus Brocadia fulgida*” biomass grown at 16°C, 25°C, and 35°C. These lipids were also observed to form a minor fraction of the total ladderane lipids of the POM and sediment samples. Since the  $C_{16}$ ,  $C_{22}$ , and  $C_{24}$  [5]- and [3]-ladderane fatty acid components were a minor part of the total ladderane fatty acids, we assume here that they do not play a major role in the biochemical functioning of membranes containing ladderane lipids.

**Implications.** Our results suggest that temperature may have an effect on the membrane composition of anammox bacteria, i.e., that there is an increase in the amount of  $C_{20}$  [5]-ladderane fatty acids compared with the amount of  $C_{18}$  [5]-ladderane fatty acids with increasing temperature and *vice versa*. Adjusting the production of fatty acids with different chain lengths in response to temperature is termed “addition synthesis” (23) and has been reported for a number of bacteria. For example, when *Micrococcus cryophilus* was grown over a temperature range from  $-4$  to 25°C, a 4-fold change in the ratio of the  $C_{18:1}$  fatty acid to the  $C_{16}$  fatty acid (and no change in the type of unsaturation) was observed (27). In the gammaproteobacterium *Escherichia coli* the membrane lipid properties are also dependent on the temperature at which the cells are grown (6).

TABLE 1. Sources of samples investigated for ladderane lipids in this study

Origin of samples	<i>n</i>	Environment	Anammox species	Temp range (°C)	Reference(s)
Namibian Margin	15	Water column	“ <i>Candidatus Scalindua sorokinii</i> ” and “ <i>Candidatus Scalindua brodae</i> ”	13–15	17
Peruvian Margin	20	Water column	“ <i>Candidatus Scalindua sorokinii</i> ”	10–17	7
Gullmarsfjord (Sweden)	3	Surface sediment	“ <i>Candidatus Scalindua</i> ”	6–7	43
Northwest African Margin	13	Surface sediment	“ <i>Candidatus Scalindua brodae</i> ”	2–12	10a
Black Sea	13	Surface sediment and water column	“ <i>Candidatus Scalindua</i> sp.”	8–9	47
Santa Barbara Basin (CA)	2	Surface sediment	ND <sup>a</sup>	6.6	– <sup>b</sup>
Irish Sea	3	Surface sediment	“ <i>Candidatus Scalindua sorokinii</i> ” and “ <i>Candidatus Scalindua wagneri</i> ”	7–9	10
Celtic Sea	3	Surface sediment	“ <i>Candidatus Scalindua sorokinii</i> ” and “ <i>Candidatus Scalindua wagneri</i> ”	5–10	10
Arabian Sea	5	Water column	ND	12–13	– <sup>b</sup>
California and Nevada hot springs	5	Microbial mats and surface sediment	“ <i>Candidatus Brocadia anammoxidans</i> ,” “ <i>Candidatus Brocadia fulgida</i> ,” and “ <i>Candidatus Kuenenia stuttgartiensis</i> ”	36–65	12
Enrichment cultures	39	Wastewater treatment plants and marine sediments	“ <i>Candidatus Brocadia anammoxidans</i> ,” “ <i>Candidatus Brocadia fulgida</i> ,” “ <i>Candidatus Kuenenia stuttgartiensis</i> ,” “ <i>Candidatus Anammoxoglobus propionicus</i> ,” and “ <i>Candidatus Scalindua</i> spp.”	15–40	15, 16, 43

<sup>a</sup> ND, not determined.

<sup>b</sup> –, ladderane lipids determined in this study.

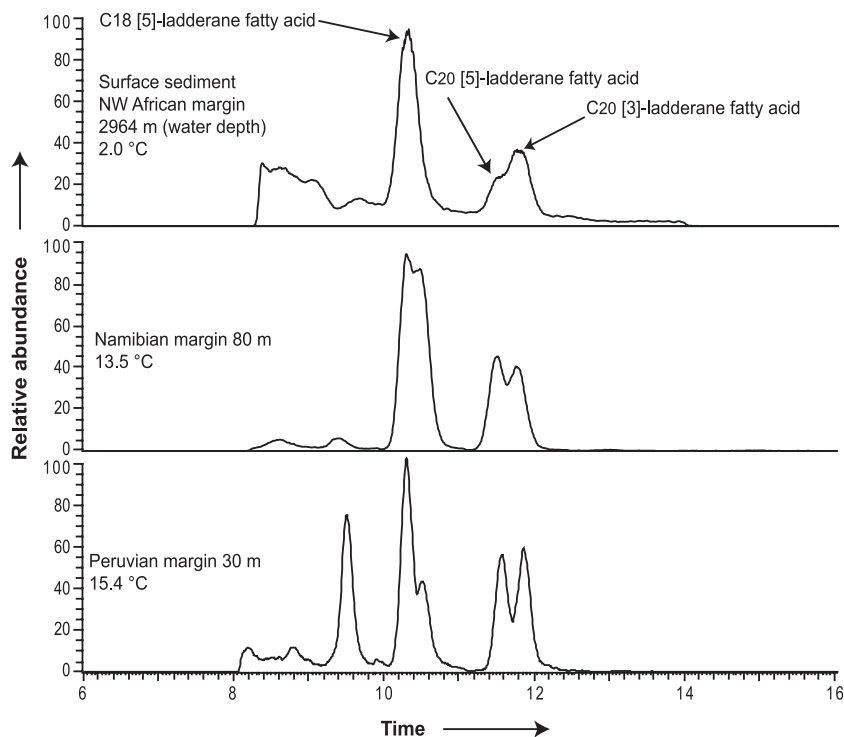


FIG. 4. HPLC base peak chromatograms showing the differences in the distribution of ladderane fatty acids produced at the *in situ* temperatures in surface sediment from the northwest African continental slope and POM from the Namibian and Peruvian margins. Ladderane fatty acids were analyzed as methyl esters.

In this case, adaptation was achieved by increasing the relative abundance of saturated long-chain fatty acids in response to an increase in the growth temperature (34).

The response of membrane lipids in *M. cryophilus* and *E. coli* to temperature is similar to the response observed for ladderane fatty acid chain length in anammox bacteria. This suggests that the homeoviscous adaptation process might occur with at least two types of ladderane fatty acids. Shortening the acyl chain length has the effect of disrupting acyl chain packing and thereby lowering the liquid-crystalline transition gel phase transition temperature compared to packing with only longer-chain lipids (23). In turn, the alteration of membrane lipids allows an organism to modulate the activity of intrinsic proteins and maintain the rates of reactions for functions like electron transport, ion pumping, and nutrient uptake (20, 24, 28). It is therefore possible that the same mechanisms occur in the ladderane lipid-containing membranes of anammox bacteria. This hypothesis is supported by a recent study of Boumann et al. (2) on the biophysical properties of artificial ladderane lipid membranes. These authors showed that the lipid-packing densities for ladderane membranes at different temperatures were similar despite differences in lipid composition. However, it should be noted that the effect of temperature on the lipid composition of microorganisms can be highly complex (40). It is therefore probable that other types of lipids, besides ladderane fatty acids, also moderate the fluidity of the membrane; e.g., anammox bacteria are known to contain bacteriohopanepolyols that influence the rigidity of the membrane (22, 36). Furthermore, our data do not exclude the possibility that the ladderane lipid composition is affected to some extent by other

environmental factors, such as nutrient or dissolved organic carbon concentrations. However, the strong empirical relationship between chain length and temperature and the results of Boumann et al. (2) suggest that temperature has a major impact on ladderane lipid distribution and thereby on the biophysical properties of the membranes of anammox bacteria.

Our results suggest that different species of anammox bacteria inhabiting different environments, modify ladderane lipid fatty acid chain length in similar ways in response to temperature. Therefore, a possible application of our results could be to discriminate between the origins of ladderane lipids in marine sediments, i.e., to determine if ladderane lipids are produced *in situ* in cold surface sediments or if they originate from the warmer upper water column. The origin of these lipids can be especially complex in cases where there is a suboxic water column overlying an anoxic sediment, as anammox activity can be expected in the water column and/or the sediment environment. This can be illustrated by analysis of surface sediment from the Santa Barbara Basin, which has an overlying anoxic water column. Analysis of the ladderane fatty acid composition gave relatively high  $NL_5$  values (0.69 and 0.72) for the surface sediment, corresponding (using our sigmoidal correlation curve) to temperatures of about 18°C. This temperature is substantially higher than the *in situ* temperature of the Santa Barbara Basin surface sediment (6.6°C). Thus, the high  $NL_5$  value for the surface sediment appears to indicate that the ladderane fatty acids are fossil remnants predominantly originating from the warmer upper water column rather than from the colder surface sediments. Evidence for the

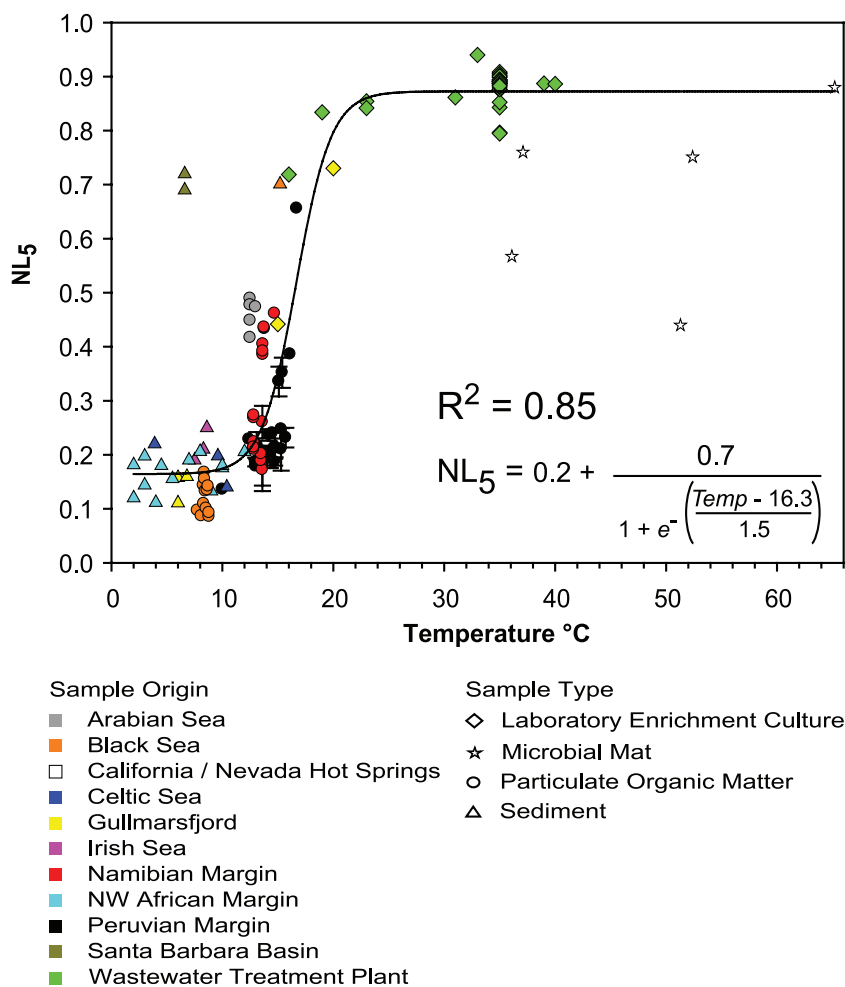


FIG. 5. The  $NL_5$  calculated using ladderane fatty acid concentrations from sequencing batch reactor enrichment cultures, POM, marine sediments, and microbial mats plotted against temperature. The equation relates to an empirical fourth-order sigmoidal regression. The error bars indicate the standard deviations for samples prepared and analyzed independently, which ranged from 0.01 to 0.04. A full data set is shown in the supplemental material. All data points at 35°C represent laboratory SBR enrichment cultures with a wastewater treatment plant origin.

production of anammox lipids in surface waters and their subsequent transport to the sediment has also been reported for the Arabian Sea (11), and fossil ladderane fatty acids in sediments up to 140,000 years old have been used to reveal past anammox activity in the Arabian Sea (13). This approach could therefore be especially helpful in studies in which fossil ladderane lipids are used to reconstruct past anammox activity.

**Conclusions.** Our study provides preliminary evidence that anammox bacteria produce increasing amounts of  $C_{20}$  [5]-ladderane fatty acids at increasing temperatures and increasing amounts of  $C_{18}$  [5]-ladderane fatty acids as the temperature decreases. The variation in ladderane fatty acid chain length could be a form of homeoviscous adaptation by anammox bacteria. Changes in the amounts of ladderane fatty acids have been quantified using the  $NL_5$ , which strongly correlates with the *in situ* temperature. Using the  $NL_5$ , it may be possible to determine the origin of ladderane lipids in sediments, i.e., whether they were produced *in situ* in the relatively cold sediment or whether they originated from transport and preservation of lipids derived from the warmer upper water column.

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