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# Biochimica et Biophysica Acta

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## Biophysical properties of membrane lipids of anammox bacteria: II. Impact of temperature and bacteriohopanoids

Henry A. Boumann<sup>a</sup>, Pieter Stroeve<sup>b</sup>, Marjorie L. Longo<sup>b</sup>, Bert Poolman<sup>c</sup>, Johanna M. Kuiper<sup>c</sup>, Ellen C. Hopmans<sup>a</sup>, Mike S.M. Jetten<sup>d</sup>, Jaap S. Sinninghe Damsté<sup>a</sup>, Stefan Schouten<sup>a,\*</sup>

<sup>a</sup> Dept. of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, PO Box 59, 1790 AB, Den Burg, Texel, The Netherlands

<sup>b</sup> Dept. of Chemical Engineering and Materials Science, University of California Davis, 1 Shields Ave, Davis, CA 95616, USA

<sup>c</sup> Dept. of Membrane Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

<sup>d</sup> Dept. of Microbiology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands

### ARTICLE INFO

#### Article history:

Received 9 November 2008

Received in revised form 31 March 2009

Accepted 8 April 2009

Available online 17 April 2009

#### Keywords:

Anammox bacteria

Ladderane lipid

Lipid packing

Acyl chain ordering

Langmuir monolayer

Fluorescence depolarization

### ABSTRACT

Anammox bacteria possess unique membranes that are mainly comprised of phospholipids with extraordinary “ladderane” hydrocarbon chains containing 3 to 5 linearly concatenated cyclobutane moieties that have been postulated to form relatively impermeable membranes. In a previous study, we demonstrated that purified ladderane phospholipids form fluid-like mono- and bilayers that are tightly packed and relatively rigid. Here we studied the impact of temperature and the presence of bacteriohopanoids on the lipid density and acyl chain ordering in anammox membranes using Langmuir monolayer and fluorescence depolarization experiments on total lipid extracts. We showed that anammox membrane lipids of representatives of *Candidatus* “*Kuenenia stuttgartiensis*”, *Candidatus* “*Brocadia fulgida*” and *Candidatus* “*Scalindua*” were closely packed and formed membranes with a relatively high acyl chain ordering at the temperatures at which the cells were grown. Our findings suggest that bacteriohopanoids might play a role in maintaining the membrane fluidity in anammox cells.

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### 1. Introduction

The discovery of microorganisms responsible for anaerobic ammonium oxidation (anammox) about a decade ago has significantly contributed to our understanding of the global nitrogen cycle [1]. In the anammox reaction, ammonium ( $\text{NH}_4^+$ ) is anaerobically utilized as an electron donor for reduction of nitrite ( $\text{NO}_2^-$ ), yielding dinitrogen gas ( $\text{N}_2$ ) as the final product [2]. The bacteria capable of performing this biochemical reaction were initially identified in wastewater treatment systems, but have now also been detected in various oxygen-limited fresh water and marine ecosystems [1,3–6]. Today, anammox bacteria play a key role in the low cost and environmental-friendly removal of ammonium in wastewater bioreactors and sewage treatment [6–8]. Anammox bacteria belong to the order of the *Planctomycetales* and form a distinct phylogenetic cluster composed of the genera *Candidatus* “*Kuenenia*”, *Candidatus* “*Brocadia*”, *Candidatus* “*Scalindua*”, *Candidatus* “*Jettenia*” and *Candidatus*

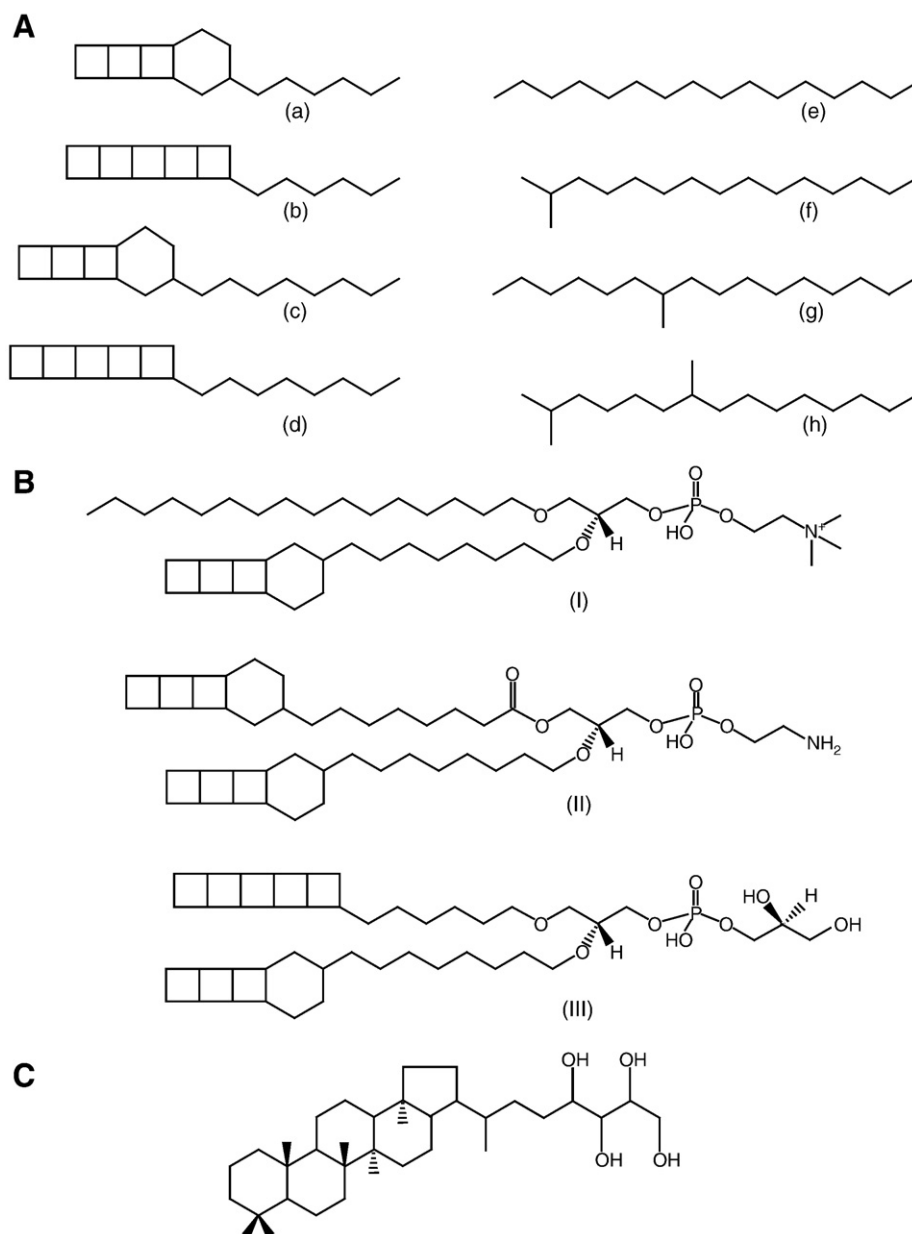
“*Anammoxoglobus propionicus*” [3,9–12]. Although these prokaryotes are widespread, each ecosystem is dominated by a single anammox genus, suggesting that the genera have adapted to distinct niches [10]. At present, pure cultures of anammox bacteria are still lacking mainly because the enrichment of these microbes in reactor batches is challenging and yields only low amounts of biomass as the cells divide only every 10–20 days [8,9,13].

Anammox bacteria contain an intracytoplasmic compartment, the anammoxosome, which accounts for a voluminous fraction of the cell [14]. It has been proposed that the anammox process takes place in this specialized organelle, since the volatile intermediate hydrazine together with hydroxylamine/hydrazine oxidoreductase are exclusively localized in that compartment [14–18]. The latter implies that the anammoxosome necessitates a membrane that facilitates the preservation of an electrochemical proton gradient during the exceptionally slow anammox catabolism and prevents diffusion of the mutagenic anammox intermediates to the exterior of the anammoxosome [17,19,20]. Indeed, studies on the membrane lipid composition of the anammoxosome of *Kuenenia stuttgartiensis* revealed the presence of so-called “ladderane” lipids which contain hydrocarbon chains comprised of three or five linearly condensed cyclobutane rings forming highly strained moieties (Fig. 1) [19]. These lipids are unique in nature and have, thus far, only been reported for anammox bacteria. The majority of the intact membrane lipids have either one or two of these ‘ladderane’ hydrocarbon chains linked to

**Abbreviations:** DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; HPLC-ESI-MS/MS, high performance liquid chromatography electrospray ionization tandem mass spectrometry; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,4-triene

\* Corresponding author. Tel.: +31 222 369300; fax: +31 222 319674.

E-mail address: [schouten@nioz.nl](mailto:schouten@nioz.nl) (S. Schouten).



**Fig. 1.** (A) Abundant lipid hydrocarbon chains in anammox bacteria: (a)  $C_{18}$ -[3]-ladderane, (b)  $C_{18}$ -[5]-ladderane, (c)  $C_{20}$ -[3]-ladderane, (d)  $C_{20}$ -[5]-ladderane, (e) hexadecane, (f) 14-methylpentadecane, (g) 10-methylhexadecane, (h) 9,14-dimethylpentadecane. (B) Examples of the ladderane lipid classes PC, PE and PG: (I) 1-hexadecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine, (II) 1-(8-[3]-ladderane-octanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine and (III) 1-(6-[5]-ladderane-hexanyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophospho-3-phospho-(1'-sn-glycerol), respectively. (C) Molecular structure of bacteriohopanetetrol.

the glycerol backbone with a phosphocholine, phosphoethanolamine or phosphoglycerol headgroup [21,22]. Whereas the sn-1 position of the ladderane phospholipids is usually linked to a  $C_{20}$ -hydrocarbon chain with three cyclobutane rings, the sn-2 position has a more diverse repertoire of hydrocarbon chains that includes etherified or esterified ladderane, straight or methyl branched hydrocarbon chains (Fig. 1). In addition to the ladderane phospholipids, the cholesterol-like  $C_{35}$  bacteriohopanetetrol has been identified in the different anammox genera [20,21].

At present, relatively little is known about membranes composed of these extraordinary ladderane lipids. Since the permeability of fluorophores is significantly lower across anammoxosomal membranes relative to conventional membranes, it has been proposed that the ladderane barrier may preserve concentration gradients of other relative small molecules like hydrazine or protons [17,19]. Molecular modeling experiments, in which a

bilayer composed of an ether-ester lipid with one  $C_{20}$ -[3]- and one  $C_{20}$ -[5]-ladderane unit was modeled, suggested that such a membrane was exceptionally dense [19]. Recently, we have demonstrated that both a PC and a mixed PE/PG ladderane lipid fractions form tightly packed but still fluid mono- and bilayer systems [23]. In another study, we showed that anammox species cultured at different temperatures change their lipid composition: with increasing temperature the relative amount of  $C_{18}$ -ladderane hydrocarbon chains was decreasing, while the longer  $C_{20}$ -ladderane hydrocarbon chains were increasing [24]. A similar phenomenon was noted for ladderane lipids in environmental samples from a range of temperatures and has been associated with maintaining the optimal membrane viscosity for cell viability [24,25]. Furthermore, the membrane lipid composition of anammox bacteria also contains bacteriohopanetetrol [20,21]. These bacteriohopanoids have been observed to play a role as a rigidifier in membrane

environments [26,27]. However, the significance of bacteriohopanoids in membranes composed of ladderane lipids is currently unknown.

To investigate the adaptation of anammox membranes to temperature, we examined the total lipid extracts from representatives of three different anammox genera, using Langmuir monolayers experiments. In addition, large unilamellar vesicles (LUVs) have been constructed to gain insight into the acyl chain ordering of lipid bilayers of the different anammox lipid extracts in fluorescence depolarization experiments. The results were compared to those of conventional phospholipid membranes of *Escherichia coli* and revealed unusual acyl chain ordering and highly packed lipid membranes for different anammox genera at physiological temperatures.

## 2. Material and methods

### 2.1. Lipid material derived from anammox bacteria and *E. coli*

Three different anammox genera were incubated in individual sequencing batch reactors for 5 months under anoxic conditions [7,9,12,13]. The microbial population of the enrichment cultures was monitored by fluorescence *in situ* hybridization [21,28]. The reactor for *Candidatus* “*B. fulgida*” was maintained at  $\pm 35$  °C and  $\sim 75\%$  the obtained cell material was composed of this anammox bacterium [10]. For *Candidatus* “*K. stuttgartiensis*”, the reactor was set at 35 °C and  $\sim 74\%$  of the biomass was composed of this anammox bacterium, whereas the *Scalindua* spp. biomass was maintained at 20 °C and yielded cell material composed for 90% of this anammox bacterium [12,29]. Furthermore, *Candidatus* “*K. stuttgartiensis*” biomass was obtained from a suspension of anammox biomass in an oxygen-limited wastewater treatment plant at 25 °C from Paques B.V. (Balk, The Netherlands). The cell material of this wastewater bioreactor was composed of  $\sim 80\%$  of *Candidatus* “*K. stuttgartiensis*” cells.

All anammox biomasses were lyophilized and the total lipids extract were obtained according to a slightly modified Bligh and Dyer method [30,31]. The composition of the total lipid extracts were analyzed by high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) as described previously and was found to be dominated by phospholipids with  $C_{18}$ -[3],  $C_{18}$ -[5],  $C_{20}$ -[3] and  $C_{20}$ -[5]-ladderane hydrocarbon chains (see Fig. 1 and [21,22]). For control experiments, *E. coli* total lipid extract was purchased from Avanti Polar Lipids (Alabaster, AL) and washed with acetone followed by diethylether to remove residual proteins.

### 2.2. Langmuir monolayers

Surface pressure–mean area per molecule isotherms were obtained using a Teflon® Langmuir–Blodgett trough with dimensions 20 cm by 30 cm (Type 611, Nima, Coventry, United Kingdom). Lipids were dissolved in chloroform at  $1.0 \text{ mg mL}^{-1}$ . About  $75 \mu\text{L}$  of a given lipid solution was gently deposited at the air–water interface using a  $100 \mu\text{L}$  Hamilton microsyringe (Hamilton, Reno, NV) at the indicated temperature. The lipid films were compressed at a constant speed of  $20 \text{ cm}^2 \text{ min}^{-1}$ , while the surface pressure was measured using a Wilhelmy plate made of filter paper. Each surface pressure–area curve was recorded at least three times. Langmuir data was analyzed by determining the isothermal compressibility,  $k_s$ , as shown in Eq. (1), where  $A$  and  $\pi$  represent the molecular occupied area and film pressure, respectively:

$$k_s = - \frac{1}{A} \frac{dA}{d\pi} \quad (1)$$

The lipids 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Avanti Polar

Lipids, Alabaster, AL) were analyzed as references at room temperature. Based on the total lipid composition of anammox cells as determined by HPLC/MS, we estimated the mean molecular weight of the anammox total lipid extracts to be  $\sim 725 \text{ g mol}^{-1}$ .

### 2.3. Fluorescence depolarization

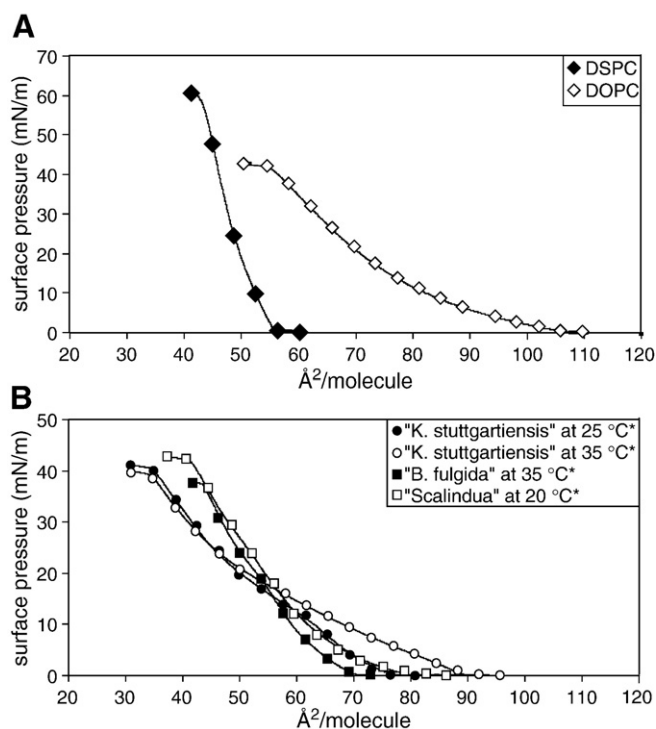
Fluorescence depolarization experiments were conducted for the anammox and *E. coli* total lipid extracts using a Fluorolog®-3 Instrument (Horiba Jobin Yvon, Edison, NJ), according to Shinitzky and Barenholz [32]. For experiments using 1,6-diphenyl-1,3,5-hexatriene (DPH) (Invitrogen, Eugene, OR), lipids were dried by rotary evaporation and hydrated in 50 mM  $\text{KP}_i$  buffer, pH 7.0. Five consecutive freezing in liquid nitrogen and thawing at room temperature steps, followed by extrusion through 200 nm polycarbonate filter yielded LUVs with an average diameter of  $\sim 170 \text{ nm}$  [33].  $5.0 \cdot 10^{-5} \text{ M}$  LUVs in 50 mM  $\text{KP}_i$  buffer, pH 7.0 were incubated in the presence of  $5.0 \cdot 10^{-8} \text{ M}$  DPH. Excitation and emission wavelength were set at 360 nm and 428 nm, respectively. For the 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,4-triene (TMA-DPH) (Invitrogen) labeling of liposomes,  $5 \mu\text{L}$  TMA-DPH stock solution (4 mM in dimethylsulfoxide) was diluted in  $245 \mu\text{L}$  50 mM  $\text{KP}_i$  buffer, pH 7.0. Subsequently, the liposomes were mixed with TMA-DPH solution in a 1:1 volume ratio resulting in a TMA-DPH/lipid ratio of 1:125 (mol/mol). After incubation for 1 h at room temperature, the steady-state fluorescence depolarization was measured upon 40-fold dilution of the liposomes. The excitation and emission wavelength were recorded at 360 nm and 430 nm, respectively. All experiments were performed using a Lauda Model RE 106 temperature controlled water bath (Lauda-Königshofen, Germany).

## 3. Results and discussion

### 3.1. Langmuir trough experiments

To obtain insight into the biophysical properties of the ladderane-containing anammox membranes, we first carried out Langmuir monolayer experiments. For comparison, the well-studied DSPC and DOPC lipids were analyzed in parallel at a temperature of 25 °C. Fig. 2A depicts the surface pressure *versus* area per molecule curve for DSPC lipid with an onset pressure increase at about  $57 \text{ \AA}^2$  per molecule and a collapse pressure at 61 mN/m (closed diamonds). By extrapolating the slope of the solid phase isotherm to zero pressure, we observed a minimal packing density of  $\sim 54 \text{ \AA}^2$  per DSPC molecule. For the DOPC lipid, an initial pressure increase was observed at  $108 \text{ \AA}^2$  per molecule and the minimal lipid packing was established at  $\sim 85 \text{ \AA}^2$ /molecule (Fig. 2A, open diamonds). Ongoing compression of this liquid-expanded phase monolayer resulted in instability at about 43 mN/m. These findings are in agreement with previous studies and clearly demonstrate that minimal lipid packing in monolayer films is already profoundly affected by a double bond (‘kink’) in the hydrocarbon chains of the lipids [34,35].

Fig. 2B illustrates representative curves of surface pressure–area per mean molecule of the total lipid extracts of the different anammox genera. The optimal lipid packing was determined at the temperature corresponding to that of cell growth and at the surface pressure of 30 mN/m at which the lipids in a monolayer are thought to occupy a molecular area close to that for lipids in a biological membrane [36]. The monolayer of the lipid extract from *Candidatus* “*K. stuttgartiensis*” grown at 25 °C exhibited a first pressure increase at approximately  $77 \text{ \AA}^2$ /molecule and remained stable in the liquid-expanded phase until it collapsed at  $\sim 41 \text{ mN/m}$  (Fig. 2B, closed circles). Interestingly, the mean area per molecule for this monolayer at 30 mN/m was about  $62 \text{ \AA}^2$  per molecule, suggesting a relatively high lipid density close to that of the DSPC lipid. Since the HPLC-ESI-MS/MS analysis revealed that this lipid extract was dominated by ladderane



**Fig. 2.** Surface pressure-area per molecule isotherms of (A) DSPC (closed diamonds) and DOPC (open diamonds) determined at room temperature; and (B) the total lipid extracts derived from *Candidatus* "K. stuttgartiensis" grown at 25 °C (closed circles), *Candidatus* "K. stuttgartiensis" cultured at 35 °C (open circles), *Candidatus* "B. fulgida" grown at 35 °C (closed squares) and *Candidatus* "Scalindua" cultured at 20 °C (open squares) determined at the temperature corresponding to that of cell growth. Data points are representative for three independent measurements. Asterisk-marks indicated growth temperature.

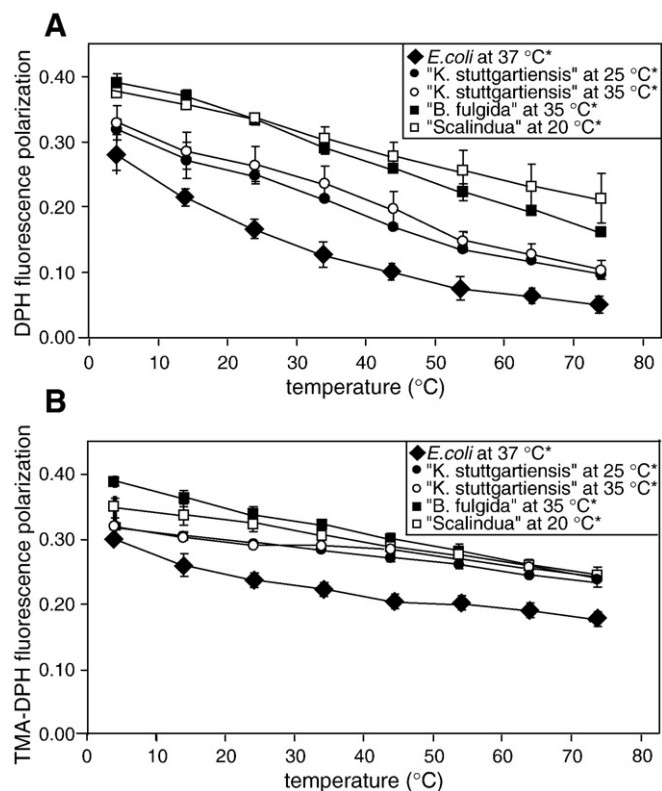
phospholipids, this finding suggests that the cyclobutane ring structures of the ladderane hydrocarbon chains result in relatively tight lipid packing. Indeed, ladderane phospholipids are capable of forming densely packed monolayers, as we have previously observed averaged minimal area of 65 and 59 Å<sup>2</sup>/molecule, respectively, for purified PC- and the PE/PG ladderane fractions [23]. These results are also in agreement with the molecular modeling experiments in which a membrane composed of ladderane lipids showed a high acyl chain density [19]. Similar as for DOPC, the isothermal surface compressibility analysis of this ladderane lipid extract yielded  $k_s$  values higher than 0.010 m/mN between the lift-off and collapse of the monolayer. This points to the absence of a liquid-expanded to liquid-condensed phase transition with increasing pressure and that the lipids formed a fluid membrane at the physiological temperature of the anammox cells (Fig. 2B). Hence, despite the high lipid density, the membrane remained relatively fluid.

It is interesting that the isotherm for *Candidatus* "K. stuttgartiensis" cultured at 35 °C also revealed a mean minimum area of ~62 Å<sup>2</sup> per molecule at the above mentioned conditions (Fig. 2B). Similarly, the isotherms for *Candidatus* "B. fulgida" grown at 35 °C and *Candidatus* "Scalindua" cultured at 20 °C exhibited mean minimum areas of near 62 Å<sup>2</sup> per molecule at 30 mN/m (Fig. 2). None of these isotherms showed a transition from a liquid-expanded to a liquid-condensed phase with increasing pressure (all  $k_s$  values  $\geq 0.011$  m/mN), suggesting that they were all fluid under these conditions (Fig. 2B). Near a surface pressure of 15 mN/m, we observed a slight plateau in the isotherms of the two *K. stuttgartiensis* lipid extracts. This type of behavior is indicative of the presence of two coexisting mixtures, where each will collapse independently. The finding that all these anammox bacteria have equivalent lipid packing under these biological relevant conditions implies that the optimal lipid packing

under different growth (*i.e.* temperature) conditions for anammox bacteria is around 62 Å<sup>2</sup> per molecule. Rattray and coworkers have shown that the lipid composition varies strongly with temperatures, *i.e.* in the cultures grown at 20 and 25 °C the relative amount of C<sub>18</sub>-ladderanes versus C<sub>20</sub>-ladderanes was larger than for those grown at 35 °C [24]. Our results suggest that this chain length adaptation may have contributed to reaching similar packing densities at different physiological temperatures. Indeed, when we used the lipid extract of *Candidatus* "K. stuttgartiensis" grown at 35 °C to obtain monolayers at room temperature, thus well below the growth temperature, we observed a packing density that was substantially different, *i.e.* at about 53 compared to ~62 Å<sup>2</sup> per molecule at a pressure of 30 mN/m. Presumably, this lipid membrane packing was "too dense" in order for the membrane to function optimally within the anammox bacteria. It is worth mentioning that the surface pressures of the four anammox lipid extracts were in equilibrium near 20 mN/m, corresponding with a lipid packing of ~53 Å<sup>2</sup>/molecule (Fig. 2B). This minimal area per molecule resembled that of DSPC near 20 mN/m and thus supports the notion that the monolayers of the anammox lipid extracts show a comparable biophysical behavior to that of the tightly packed DSPC equivalent.

### 3.2. Fluorescence depolarization experiments

The proposed dense anammox membrane architecture was further investigated as part of a bilayer structure, *i.e.* the membrane of a large unilamellar vesicle (LUV). First, we determined the acyl chain



**Fig. 3.** (A) Fluorescence depolarization of DPH in LUVs prepared from the total lipid extracts of *E. coli* (closed diamonds), *Candidatus* "K. stuttgartiensis" grown at 25 °C (closed circles), *Candidatus* "K. stuttgartiensis" cultured at 35 °C (open circles), *Candidatus* "B. fulgida" grown at 35 °C (closed squares) and *Candidatus* "Scalindua" cultured at 20 °C (open squares). (B) TMA-DPH fluorescence depolarization in LUVs prepared from total lipid extracts of *E. coli* (closed diamonds), *Candidatus* "K. stuttgartiensis" grown at 25 °C (closed circles), *Candidatus* "K. stuttgartiensis" cultured at 35 °C (open circles), *Candidatus* "B. fulgida" grown at 35 °C (closed squares) and *Candidatus* "Scalindua" cultured at 20 °C (open squares). The data points shown are the mean values  $\pm$  the standard deviation of three independent experiments. Asterisk-marks indicated growth temperature.

ordering in the hydrophobic core region of the membranes of LUVs by employing the hydrophobic probe DPH; DPH is thought to reside in the hydrophobic core of the membrane [32,37–39]. As a reference for the anammox lipid extracts, we have used the total lipid extract derived from *E. coli*. The latter is mainly composed of PE, PG and cardiolipin phospholipids with saturated and mono-unsaturated hydrocarbon chains (reviewed by [40]). As shown in Fig. 3A (closed diamonds), the DPH steady-state fluorescence depolarization intensity for the *E. coli* membranes gradually declined from  $0.27 \pm 0.03$  to  $0.07 \pm 0.01$  as the temperature increased. This can be explained by an increase in the rotational freedom of the hydrocarbon chains with no apparent phase changes, which is in good agreement with earlier studies [41–43].

For the anammox lipid extracts, the DPH fluorescence intensities were markedly higher than those of the *E. coli* membranes. These membranes showed more acyl chain ordering, as the DPH depolarization intensities of both lipid extracts from *Candidatus* “*K. stuttgartiensis*” ranged from about  $0.33 \pm 0.02$  to  $0.12 \pm 0.01$  (Fig. 3, closed and open circles). Similar as for *E. coli*, no abrupt changes in the fluorescence depolarization intensities were observed, indicating the absence of a phase transition. For *Candidatus* “*B. fulgida*” and *Candidatus* “*Scalindua*”, the DPH fluorescence depolarization intensities showed a similar trend and gradually decreased from about 0.39 to 0.24 (Fig. 3, closed and open squares). The later values are higher than those of *Candidatus* “*K. stuttgartiensis*” lipid extracts and hence consistent with the Langmuir monolayer results that show a higher slope, i.e. a higher elastic modulus and thus more acyl chain ordering and less viscosity, for *Candidatus* “*B. fulgida*” and *Candidatus* “*Scalindua*” (cf. Fig. 2B). As the DPH probe monitors the acyl chain ordering in the interior of the lipid bilayers, this may be caused by variation in the abundance of the membrane-rigidifying bacteriohopanetetrol.

At the biological relevant temperature, the ladderane containing LUVs have DPH fluorescence depolarization intensities ranging from about 0.24 to 0.36, whereas *E. coli* showed a considerably lower DPH fluorescence depolarization intensity of  $\sim 0.12$  at 37 °C, corresponding to the growth temperature of *E. coli* (Fig. 3). This result indicates that the bilayers composed of the anammox lipids are less fluid than those composed of conventional lipids. The higher ordering of acyl chains in ladderane-containing membranes is likely reflect the dense lipid packing of the anammox membrane lipids (see Fig. 2B). Interestingly, the acyl chain ordering of the total lipid extract of *Candidatus* “*K. stuttgartiensis*” is slightly more pronounced than that of the corresponding PC- and PE/PG ladderane fractions [23]. We speculate that bacteriohopanoids might moderately reduce the anammox membrane fluidity, as this rigidifying role for bacteriohopanoids has also been observed in other membrane environments [26,27].

The use of the fluorescent probe TMA-DPH allowed us to monitor the acyl chain ordering near to the periphery of the membrane as this probe anchors with its TMA moiety in the headgroup region of the lipids [39]. As expected, the fluorescence depolarization intensities of TMA-DPH for *E. coli* membrane lipids decreased at higher temperatures (Fig. 3B, closed diamonds) [42]. However, both *Candidatus* “*K. stuttgartiensis*” lipid extracts had relatively higher TMA-DPH depolarization intensities. The temperature dependence of TMA-DPH depolarization intensity for the *Candidatus* “*K. stuttgartiensis*”, *Candidatus* “*B. fulgida*” and *Candidatus* “*Scalindua*” lipid extracts all showed a comparable decline (Fig. 3B open squares, closed and open circles). Whereas the TMA-DPH fluorescence intensity of *E. coli* was near 0.26 at 37 °C, the anammox lipid extracts had slightly higher TMA-DPH values ranging from about 0.32 to  $\sim 0.34$  at their biological relevant temperatures (Fig. 3). Hence, the findings above demonstrate that the relatively high acyl chain ordering in the anammox membranes can be assigned primarily to the nature of the cyclobutane rings of the ladderane hydrocarbon chains in agreement with previous modeling results which showed the highest densities in the hydrophobic region around the cyclobutane moieties [19].

## 4. Conclusions

The current study provides experimental evidence for the high ordering of acyl chains in membranes composed of anammox lipids. The ladderane hydrocarbon chains are likely to play a major role in accomplishing these biophysical features, since we have observed similar properties for mono- and bilayers systems composed of pure ladderane phospholipids [23]. However, despite the high lipid density and high acyl chain ordering, both monolayer and fluorescence depolarization experiments showed that the ladderane-containing membranes are in the fluid phase at biological relevant conditions. Likely, bacteriohopanoids will facilitate in obtaining the optimal equilibrium between membrane fluidity and rigidity necessary for the insertion and function of membrane proteins in these unusual membranes [27,44–46].

## Acknowledgements

The authors would like to thank Paques B.V. (Balk, the Netherlands) and Drs W. Geerts, J. van de Vossenberg and J.E. Rattray for kindly providing anammox lipid material. This work was supported by the ‘From Molecule to Cell Program’ of the Netherlands Organization for Scientific Research (NWO) and granted to S.S. and J.S.S.D (805.47.097).

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