Effect of temperature on the compositions of ladderane lipids in globally surveyed anammox populations

Kouba, Vojtěch; Hůrková, Kamila; Navrátilová, Klára; Kok, Dana; Benáková, Andrea; Laureni, Michele; van Niftrik, Laura; van Loosdrecht, Mark C.M.; Weissbrodt, David Gregory; More Authors

DOI
10.1016/j.scitotenv.2022.154715

Publication date
2022

Document Version
Final published version

Published in
Science of the Total Environment

Science of the Total Environment 830 (2022) 154715

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Effect of temperature on the compositions of ladderane lipids in globally surveyed anammox populations

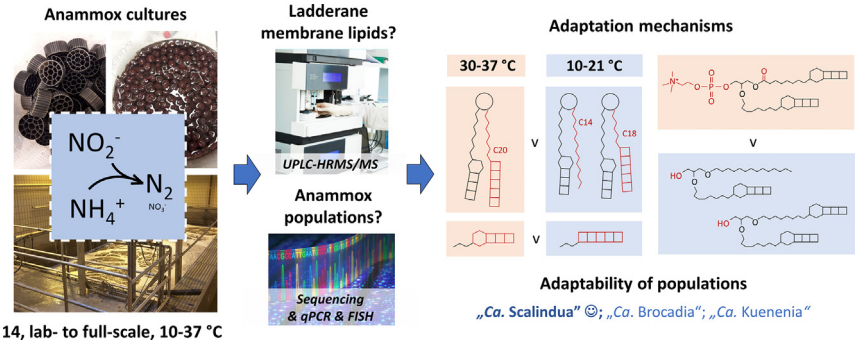
Vojtěch Kouba [a,*], Kamila Hůrková [b], Klára Navrátilová [b], Dana Kok [a], Andrea Benáková [a], Michele Laureni [c,d], Patricie Vodičková [a,f], Tomáš Podzimek [f], Petra Lipovová [f], Laura van Niftrik [e], Jana Hajšlová [b], Mark C.M. van Loosdrecht [c,1], David Gregory Weissbrodt [c,1], Jan Bartáček [a,1]

[a] University of Chemistry and Technology Prague, Department of Water Technology and Environmental Engineering, Technická 5, 166 28 Prague, Czechia
[b] University of Chemistry and Technology Prague, Department of Food Analysis and Nutrition, Technická 5, 166 28 Prague, Czechia
[c] TU Delft, Department of Biotechnology, Building 58, Van der Maasweg 9, 2629 HZ Delft, the Netherlands
[d] TU Delft, Department of Water Management, Building 23, Stevinweg 1, 2628 CN Delft, the Netherlands
[e] Radboud University, Department of Microbiology, Institute for Water and Wetland Research, 1Heyendaalseweg 135, 6525 ED AJ Nijmegen, the Netherlands
[f] University of Chemistry and Technology Prague, Department of Biochemistry and Microbiology, Technická 5, 166 28 Prague, Czechia

HIGHLIGHTS

- The survey involved 14 anammox lab enrichments and biomasses from WWTPs.
- Dominant anammox populations in WWTPs belonged to the genus "Ca. Brocadia".
- Ladderanes correlated with cultivation temperature and anammox populations.
- Promising ladderane adaptation mechanisms were alkyl length, phosphatidyl content.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 January 2022
Received in revised form 15 March 2022
Accepted 17 March 2022
Available online 22 March 2022

Editor: Qilin Wang

Keywords:
Anaerobic ammonium oxidation
Ladderane phospholipids
Candidatus Scalindua
Effect of temperature
Candidatus Brocadia

ABSTRACT

The adaptation of bacteria involved in anaerobic ammonium oxidation (anammox) to low temperatures will enable more efficient removal of nitrogen from sewage across seasons. At lower temperatures, bacteria typically tune the synthesis of their membrane lipids to promote membrane fluidity. However, such adaptation of anammox bacteria lipids, including unique ladderane phospholipids and especially shorter ladderanes with absent phosphatidyl headgroup, is yet to be described in detail. We investigated the membrane lipids composition (UPLC–HRMS/MS) and dominant anammox populations (16S rRNA gene amplicon sequencing, Fluorescence *in situ* hybridization) in 14 anammox enrichments cultivated at 10–37 °C. "*Candidatus* Brocadia" appeared to be the dominant organism in all but two laboratory enrichments of "*Ca.* Scalindua" and "*Ca.* Kuenenia". At lower temperatures, the membranes of all anammox populations were composed of shorter [5]-ladderane ester (reduced chain length demonstrated by decreased fraction of C20/(C18 + C20)). This confirmed the previous preliminary evidence on the prominent role of this ladderane fatty acid in low-temperature adaptation. "*Ca.* Scalindua" and "*Ca.* Kuenenia" had distinct profile of ladderane lipids compared to "*Ca.* Brocadia" biomasses with potential implications for adaptability to low temperatures. "*Ca.* Brocadia" membranes contained a much lower amount of C18 [5]-ladderane esters than reported in the literature for "*Ca.* Scalindua" at similar temperature and measured here, suggesting that this could be one of the reasons for the dominance of "*Ca.* Scalindua" in cold marine environments. Furthermore, we propose additional and yet unreported

* Corresponding author.
E-mail address: vojtech.kouba@vscht.cz (V. Kouba).
[1] These co-authors contributed equally.

http://dx.doi.org/10.1016/j.scitotenv.2022.154715

mechanisms for low-temperature adaptation of anammox bacteria, one of which involves ladderanes with absent phosphatidyl headgroup. In sum, we deepen the understanding of cold anammox physiology by providing for the first time a consistent comparison of anammox-based communities across multiple environments.

1. Introduction

Anaerobic ammonium oxidation (anammox) is globally a significant source of atmospheric dinitrogen from marine, and freshwater environments (Crowe et al., 2017). In the last decade, anammox bacteria became widely used at wastewater treatment plants (WWTPs) in partial nitritation/anammox (PN/A) configurations treating reject water from anaerobic digesters (Lackner et al., 2014). Their autotrophic metabolism enables nitrogen to be removed from wastewater with savings on energy, organic carbon, and lower production of excess sludge, thus reducing WWTPs operational and capital costs (Daigger, 2014). To implement anammox process for the treatment of sewage, one of the issues is the adaptation of anammox populations to low temperatures (Cao et al., 2017). This has stimulated inquiry into the physiology that anammox bacteria use to adapt to the cold such as via cold-shock protein expression or changes in the phospholipid composition in their cell membranes (Kouba et al., 2022a; Kouba et al., 2022b).

Phospholipids in the membranes of anammox bacteria contain a unique ladderane alkyl moiety. The function of ladderane lipids has been hypothesized to limit the diffusion of protons and radicals from the anammoxosome organelle (Moss et al., 2018; Nouri and Tantillo, 2012). Ladderane refers to a moiety composed of five linearly concatenated cyclobutane rings or single cyclohexane and three cyclobutane concatenated rings. Ladderane lipids are typically composed of at least one of these moieties attached to an alkyl chain that connects to glycerol backbone by ether or ester bond (Sinninghe Damsté et al., 2002). At least one of the *sn-1* and *sn-2* of alkyl positions on the glycerol backbone is occupied by a C20-[3]-ladderane, whereas the other contains another ladderane, a common alkyl chain, or no alkyl at all. The *sn-3* position typically contains a polar head group such as phosphatidylcholine, phosphatidylethanolamine, or phosphatidylglycerol (Table 1) (Boumann et al., 2006; Boumann et al., 2009a). The biochemistry of ladderane biosynthesis remains unknown (Javidpour et al., 2016). The same is valid for the factors governing ladderane synthesis, including the cultivation temperature of the cellular environment. Furthermore, we hypothesized that the ladderane lipids in anammox enrichments are structurally much more diverse than reported to date if only due to biosynthesis byproducts accumulation or lipid degradation.

To date, only two studies linked ladderanes to the adaptation of anammox bacteria to temperature. Specifically, the ladderane alkyl chain length has been highlighted as one of the adaptation mechanisms. Increased amounts of C20 compared to C18 [5]-ladderanes have been microbially synthesized at elevated temperatures (Kouba et al., 2022b; Rattray et al., 2010). Other suggested ladderane adaptations include increased content of non-ladderane straight/branched (C14–16) alkyls and PE as polar headgroup (Kouba et al., 2022b). However, the impact of microbial composition and process conditions on this C20:C18 ratio and the other components of anammox membranes such as the number of concatenated cyclobutane rings, length of more common alkyl chains, ether/ester alkyl bonds, and triterpenoids remain to be uncovered. Furthermore, the composition of ladderanes and other lipids varied substantially among anammox enrichments studied by Rattray et al. (2008), while the effect of process conditions was unclear.

We analyzed the composition of phospholipids in 14 different anammox enrichment cultures obtained under various operational conditions (i.e., cultivation temperature, biomass growth mode, one- and two-stage PN/A) and in relation to their microbial compositions. Besides the known ladderane structures, we also detected shorter ladderanes with absent phosphatidyl headgroup (APH ladderanes). These shorter ladderanes can be (i) the products of cell lysis and microbial lipid degradation of phospholipids, (ii) intermediates of ladderane lipid biosynthesis, or (iii) regular membrane constituents (Rattray et al., 2008).

2. Materials and methods

2.1. Anammox enrichments

Anammox biomasses were sampled from the mesophilic (full-scale: Landshut, Plettenberg, Malmö, Strass, Tilburg, Rotterdam; lab-scale enrichments: Kuenenia, Brocadia) and psychrophilic (full-scale: Xi'an, Eisenhüttenstadt, pilot-scale: Lemay, Dübendorf1; lab-scale: Dübendorf2; lab-scale enrichment: Scalindua) environments described in Table 2. The table includes the process configuration of the original reactor, influent, sludge character, cultivation temperature, and dominant anammox species. Brocadia enrichment was cultivated using 45 mM $NaNO_2$ and 22.5 mM ammonium sulfate; the other components were described in Van De Graaf et al. (1996). Scalindua cultivation used a medium 100 mM $NaNO_2$ and 50 mM ammonium sulfate, along with 30 g L^{-1} of red sea salt, 12.5 mL of $FeSO_4$ (5 g L^{-1}), and 5 mL of KH_2PO_4 (100 g L^{-1}). Cultivation of planktonic Kuenenia was described elsewhere (Lotti et al., 2014).

2.2. Ladderane lipid analysis

We used ultra-high performance liquid chromatography coupled to high-resolution tandem mass spectrometry (U-HPLC–HRMS/MS) to analyze ladderane lipids (in addition to other selected lipids such as bacteriohopanoids) from the anammox biomasses at exceptionally high detection sensitivity and to identify the number of carbon atoms of these lipids. According to Rattray et al. (2008), the polar headgroup ionization of ladderanes differs substantially, and, to the best of our knowledge, ladderane lipid standards are not available. Therefore, the analytical results can be characterized qualitatively and in relative quantification between lipids with the same polar headgroup.

2.2.1. Reagents and chemicals

Deionized water was obtained from a Milli-Q® Integral membrane filtration system supplied by Merck (Darmstadt, Germany). HPLC-grade methanol, isopropyl alcohol, formic acid and ammonium formate (purity ≥ 99%) were purchased from Sigma-Aldrich (St. Luis, MO, USA).

2.2.2. Sample preparation

Samples of anammox biomasses were obtained from the sources described in Table 2 and promptly lyophilized. The biomass on plastic carriers (Malmö, Xi'an, Lemay, Dübendorf2) was manually scratched off. To extract ladderane phospholipids, a mixture of MeOH:DCM: 10 mmol L^{-1} ammonium acetate (2,1:0.8, *v*/v/v) was chosen as solvent according to Lanekoff and Karlsson (2010). Lyophilized anammox biomasses were weighted (0.2 g) into a plastic cuvette and automatically shaken for 2 min with 2 mL of extraction solvent. The suspensions were sonicated for 10 min and centrifuged (5 min, 10,000 rpm, 5 °C). Finally, 1 mL of supernatant was transferred into the vial pending analyses by ultra-high performance liquid chromatography coupled to high-resolution tandem mass spectrometry (U-HPLC–HRMS/MS).

2.2.3. Ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (U-HPLC-HRMS)

A Dionex UltiMate 3000 RS U-HPLC system (Thermo Fisher Scientific, Waltham, USA) coupled to quadrupole-time-of-flight SCIEX TripleTOF® 6600 mass spectrometer (SCIEX, Concord, ON, Canada) was used to analyze the ladderane phospholipids.

Chromatographic separation of ladderane extracts was carried out using the U-HPLC system, equipped with an Acquity UPLC BEH C18 column, 100

Table 1
Structure of anammox lipids: ladderane phospholipids (composed of glycerol backbone, one C20 [5]-ladderane alkyl moiety, in some cases one other ladderane or non-ladderane alkyl moiety, in some cases phosphate-bound polar headgroup) and terpenoids, adapted from Rattray et al. (2008) and Boumann et al. (2009a).

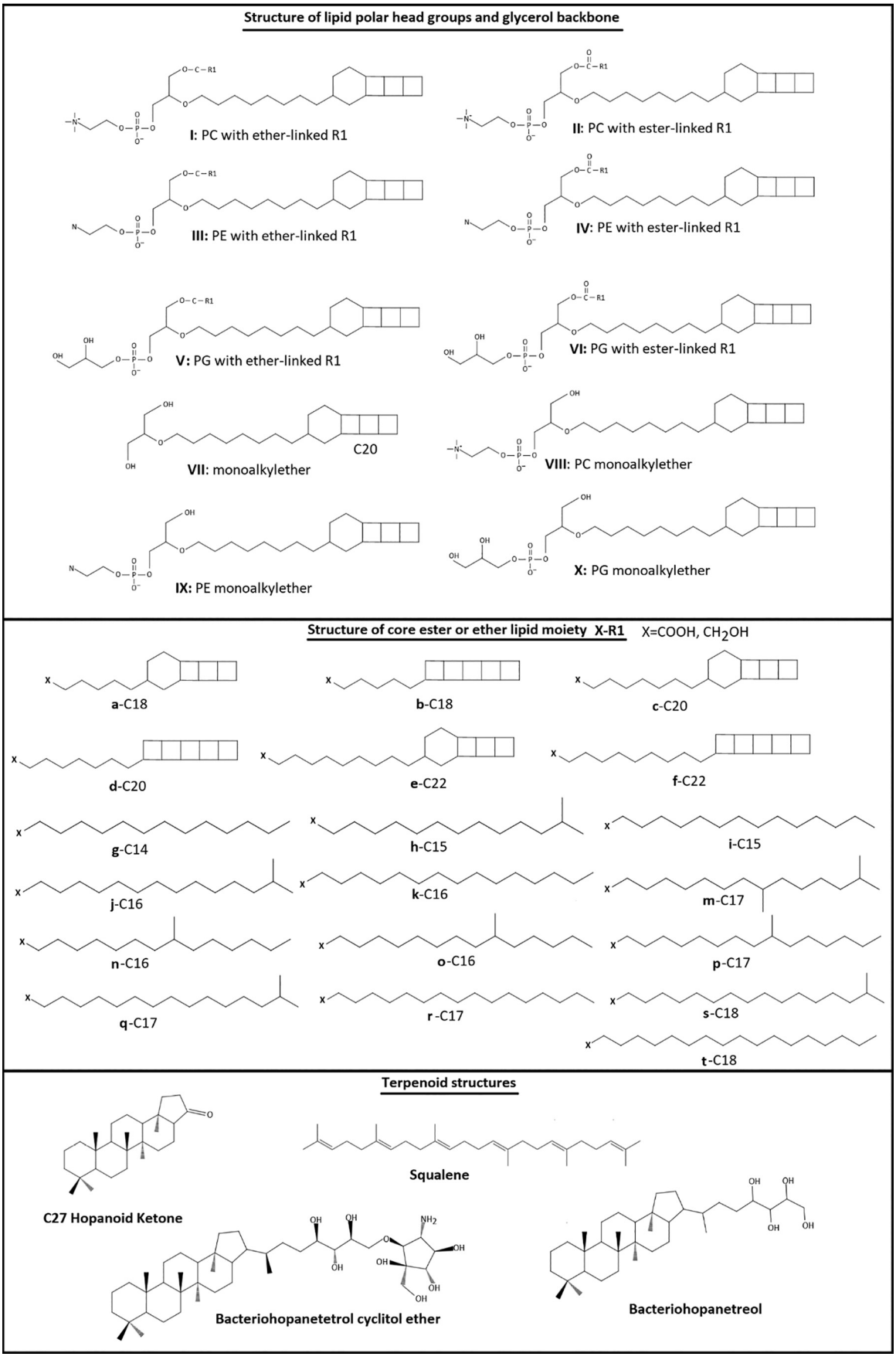


Table 2
Description of the examined anammox biomasses and their dominant anammox species. *VERI = Veolia Research and Innovation; ** specific surface 800 $m^2.m^{-3}$. Dominant anammox genera was determined by 16S rRNA gene amplicon sequencing, and dominant species was determined by Fluorescence in situ hybridization. More detailed information is contained in Kouba et al. (2019).

ID	Description of technology	Influent	Sludge character	t (°C)	Dominant anammox species
Full-scale installations					
Eisenhüttenstadt / DE	SBR DEMON®, one-stage	reject water	flocs/granules	21	"*Ca.* Brocadia anammoxidans", "*Ca.* Brocadia fulgida"
Landshut / DE	TERRAMOX®, two-stage	reject water	flocs	32	"*Ca.* Brocadia fulgida"
Malmö / SE	AnitaMOX™, Anox Kaldnes, two-stage	reject water	biofilm, carriers K5**	30	"*Ca.* Brocadia anammoxidans", "*Ca.* Brocadia fulgida"
Plettenberg / DE	SBR DEMON®, one-stage	reject water	flocs/granules	30	"*Ca.* Brocadia anammoxidans", "*Ca.* Brocadia fulgida"
Strass / AT	continuous DEMON®, one-stage	reject water	flocs/granules	30	"*Ca.* Brocadia fulgida"
Rotterdam / NL	ANAMMOX®, two-stage	reject water	granules	37	"*Ca.* Brocadia anammoxidans"
Tilburg / NL	ANAMMOX®, one-stage	reject water, CAMBI	granules	37	"*Ca.* Brocadia anammoxidans"
Xian / CN	full-scale WWTP, activated sludge process	municipal sewage	biofilm, carriers	–	"*Ca.* Brocadia anammoxidans"
Pilot and lab-scale systems					
Lemay / FR	lab-scale 1.6 m^3, VERI*, one-stage	pre-treated municipal sewage	biofilm, carriers K5**	20	"*Ca.* Brocadia anammoxidans"
Dübendorf1 / CH	pilot-scale MBBR, EAWAG	municipal sewage pre-treated in A-stage	biofilm, carriers	14	"*Ca.* Brocadia anammoxidans", "*Ca.* Brocadia fulgida"
Dübendorf2 / CH	lab-scale MBBR, EAWAG	synthetic	biofilm, carriers	10	"*Ca.* Brocadia anammoxidans", "*Ca.* Brocadia fulgida"
Enrichments					
Brocadia	enrichment, laboratory of Microbiology, Radboud University, NL	synthetic	granules	30	"*Ca.* Brocadia anammoxidans"
Kuenenia	enrichment, laboratory of EBT, TU Delft, NL	synthetic	planktonic	30	"*Ca.* Kuenenia stuttgartiensis"
Scalindua	enrichment, laboratory of Microbiology, Radboud University, NL	synthetic	flocs	20	"*Ca.* Scalindua spp."

Å, 100 mm × 2.1 mm; 1.7 μm particles (Waters, Milford, MA, USA). The mobile phase consisted of (A) 5 mM ammonium formate in Milli-Q water: methanol with 0.1% formic acid (95:5 *v*/v) and (B) 5 mM ammonium formate in isopropyl alcohol:methanol: Milli-Q water with 0.1% formic acid (65:30:5, v/v/v). The following elution gradient was used only in positive ionization mode: 0.0 min (90% A; 0.40 mL min^{-1}), 2.0 min (50% A; 0.40 mL min^{-1}), 7.0 min (20% A; 0.40 mL min^{-1}), 13.0 min (0% A; 0.40 mL min^{-1}), 20.0 min (0% A; 0.40 mL min^{-1}), 20.1 min (95% A; 0.40 mL min^{-1}), 22.0 min (90% A; 0.40 mL min^{-1}).

The sample injection volume was set at 2 μL, the column temperature was kept constant at 60 °C and the autosampler temperature was permanently refrigerated at 5 °C.

A quadrupole-time-of-flight TripleTOF® 6600 mass spectrometer (SCIEX, Concord, ON, Canada) was used for detection. The ion source Duo Spray™ with separated ESI ion source and atmospheric-pressure chemical ionization (APCI) was employed. In the positive ESI mode, the source parameters were set for: nebulizing gas pressure: 55 psi; drying gas pressure: 55 psi; curtain gas 35 psi; capillary voltage: +4500 V; temperature: 500 °C; and declustering potential: 80 V. The other aspects of the analytical method were consistent with Hurkova et al. (2019) except for confirmation of compound identification, for which we used accurate mass, isotopic pattern, and MS/MS characteristic fragments.

2.3. Analysis of microbial community composition by rRNA gene amplicon sequencing

For detecting the dominant anammox genera and quantifying their relative abundance, the bacterial community compositions of all biomasses were analyzed by rRNA gene amplicon sequencing targeting different hypervariable regions (bacterial 16SV4/16SV3/16SV3-V4/16SV4-V5, eukaryal 18S V4/18S V9, ribosomal DNA internal transcribed spacers ITS1/ITS2, archaeal Arc 16S V4). Genomic DNA was extracted from the anammox biomasses using the DNeasy® PowerSoil® Kit (Quiagen GmBH, Germany) following the manufacturer's protocol. The purified DNA extracts were submitted to Novogene (Hong Kong, PRC) for amplicon sequencing analysis using the MiSeq workflow (Illumina, US). More details on sequencing are described in the Supplementary Materials.

2.4. Fluorescence in situ hybridization (FISH)

Samples aliquots of all anammox biomasses were fixed and hybridized according to Nielsen et al. (2009). Since the anammox bioaggregates were very compact, the samples were gently homogenized with plastic tissue grinder prior to hybridization. The oligonucleotide FISH probes listed in Table S 1 were used to detect the anammox populations. Visualization was done under an epifluorescence microscope Olympus BX51 (Olympus Corporation of the Americas, USA). Microphotographs were taken with a LeicaDFC320 camera (Leica Microsystems Imaging Solutions Ltd., UK). The Cy3 fluorochrome (red emission filter) was used for all specific probes and DAPI (blue emission filter) was used to detect all biomass. Vectashield mounting medium was applied for the prevention of fluorescence bleaching.

3. Results and discussion

3.1. Analysis of dominant microbial populations in anammox biomasses

The combination of molecular ecology methods of rRNA-gene amplicon sequencing and fluorescence in situ hybridization (Table 2, Table S 2) showed that the "*Ca.* Brocadia anammoxidans" and "*Ca.* Brocadia fulgida" were the dominant anammox populations in the mixed cultures from WWTPs, pilots and most laboratory biomasses. Only the marine enrichment was dominated by "*Ca.* Scalindua". The planktonic anammox enrichment was dominated by "*Ca.* Kuenenia stuttgartiensis" and another laboratory enrichment by "*Ca.* Brocadia anammoxidans". More detailed information on these anammox biomasses has been given in Kouba et al. (2019).

3.2. Composition of ladderane alkyl moiety in phospholipids

All anammox biomasses contained C20 and C18 ladderane alkyl moieties with three and five concatenated cyclobutane rings, [3]- and [5]-ladderanes. The Table S 3 describes evidence on the content of these ladderane moieties among anammox genera. The "*Ca.* Scalindua" enrichment had an exceptionally high content of [3]-ladderanes of 56%, when compared to "*Ca.* Brocadia" cultures (only 9–23%) and "*Ca.* Kuenenia" (39%). In the "*Ca.* Kuenenia" enrichment, C22 [5]-ladderanes were also detected in trace amounts, but not in the others.

In biomasses dominated by "*Ca.* Brocadia", the fraction of C20/(C18 + C20) [5]-ladderanes correlated with temperature: the biomasses sampled from environments at higher temperatures harbored an elevated content of C20 [5]-ladderanes compared to C18 ones and vice versa (Fig. 1 A). In comparison, the enrichment cultures of "*Ca.* Kuenenia" and biomasses of "*Ca.* Brocadia" shared a similar fraction (81%), while the enrichment culture of "*Ca.* Scalindua" was composed of a much lower fraction of C20 [5]-ladderanes (37%).

In addition, the total MS signal of all ladderane phospholipids also varied widely in all biomasses, ranging from 350,000 to 34,000,000 counts per second (standard qualitative measurement unit in MS, Fig. 2).

3.3. Composition of non-ladderane alkyl moiety

All anammox cultures contained straight-chain C14 alkyls, and straight or branched C15–16 (Table 3), attached to the *sn-1* position on the glycerol backbone. Also, the fraction of C15/(C14 + C15) correlated to the cultivation temperature: anammox biomasses at lower temperatures presented shorter alkyl chains in their membranes.

3.4. Composition of phospholipids

The ladderane lipids of the investigated anammox enrichments contained phosphatidylcholine (PC), phosphatidylglycerol (PG) and/or phosphatidylethanolamine (PE) as polar headgroups.

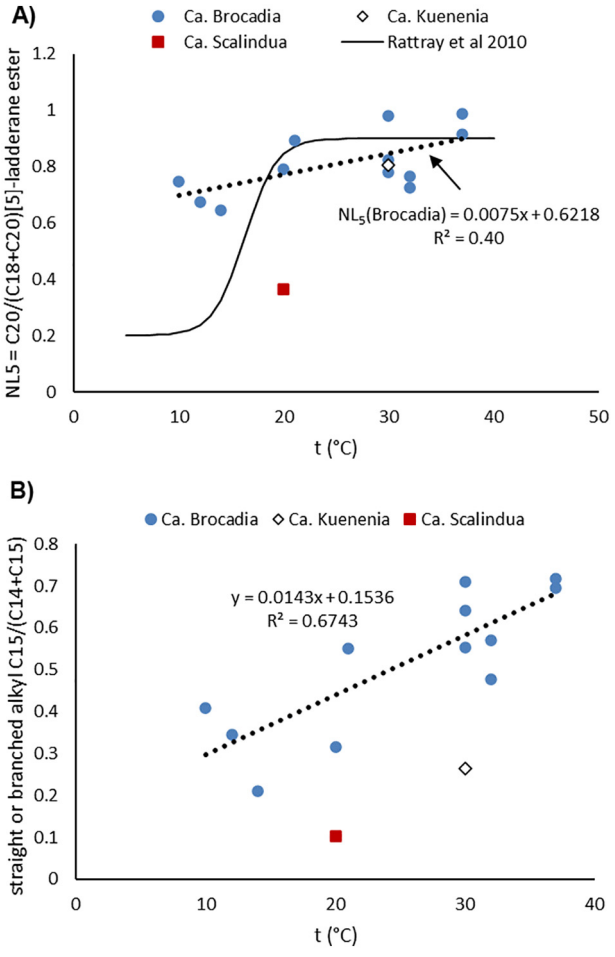


Fig. 1. Relationship between cultivation temperature and ladderane phospholipid composition in presently analyzed various anammox cultures dominated by "*Ca.* Brocadia" (circle), "*Ca.* Scalindua" (square) and "*Ca.* Kuenenia" (diamond), and the relationship proposed by (Rattray et al., 2010) where samples under 20 °C were marine "*Ca.* Scalindua".

3.4.1. Phospholipids in "*Ca.* Scalindua"

The "*Ca.* Scalindua" culture contained almost exclusively PG ladderane lipids, most abundant was **Vc** ("V" describes the combination of backbone, polar headgroup, and alkyl ether at *sn-2* position; "c" describes alkyl ether or ester on *sn-1* position; exact % described in Table 3)**, Vg**, **VIa** and **VIg**. Only trace amounts of a few PC and PE, as well as monoalkylethers and C20[3] monoether, were detected. Alkyl moieties were mostly bound to glycerol backbone by an ether bond.

3.4.2. Phospholipids in "*Ca.* Kuenenia suttgartiensis"

The enrichment of "*Ca.* Kuenenia suttgartiensis" contained a number of phospholipids with all three PC, PG and PE headgroups. A majority of those were diethers. PC phospholipids were dominating the spectrum: the most abundant were **Ic**, **IIc**, and **IIg**, while a similarly high content of **VIII** PC monoalkylether was also detected. Second most abundant were PG: **Vc**, **Vg** and **Vb** were the most numerous. The most abundant PE phospholipids were **IVd**, **IVc** and **IIIc**.

3.4.3. Phospholipids in "*Ca.* Brocadia anammoxidans"

The two mixed psychrophilic cultures dominated by only "*Ca.* Brocadia anammoxidans" were Lemay (20 °C) and Xi'an. Both biomasses displayed similar phospholipid spectra that were dominated by PC phospholipids: the highest contents were detected for **IIj / IIk / IIm**, **IIg**, **Ig** and **IIh / IIi**. PE were dominated by **IVj / IVk / IVm** and **IVg**. PG contained mostly **VIj / VIk / VIm**, **Vg** and **VIh / VIi**. Both PG and PE headgroups were less abundant than PC ones. Also, the Lemay culture contained a notable amount of **VIII**, ladderane C20[3] monoether.

The mesophilic biomasses of WWTPs Rotterdam and Tilburg as well as the "*Ca.* Brocadia" enrichment were dominated by "*Ca.* Brocadia anammoxidans". Overall, their spectra were also relatively consistent, dominated by PC phospholipids (**IIh / IIi**, **IIj / IIk / IIm**, **IIc** and **IIg**). Smaller amounts of PG (**VIh / VIi**, **VIj / VIk / VIm** and **Vc**) and PE (**IVj / IVk / IVm, IVh / IVi** and **IVc**) were also detected. The Rotterdam biomass also contained an especially high content of **VIII**, a ladderane C20[3] monoether.

3.4.4. Phospholipids in "*Ca.* Brocadia fulgida"

Two of the mesophilic biomasses were dominated by "*Ca.* Brocadia fulgida" (Landshut, Strass). Again, there were many similarities, such as dominant PC phospholipids **IId** and **IIc**, and the content of **VIII**, ladderane C20[3] monoether. Further, lesser amounts of PG (**VIh / VIi**) and PE (**IVd**) phospholipids were detected.

3.4.5. Phospholipids in other "*Ca.* Brocadia"

In five other mixed cultures (Eisenhüttenstadt, Malmö, Plettenberg and Dübendorf1–2), both "*Ca.* Brocadia fulgida" and "*Ca.* Brocadia anammoxidans" were predominant. In all these cultures, PC phospholipids were dominating the spectra.

Psychrophilic biomasses (Dübendorf) contained mostly PC (**IIj / Iik / Iim**, **Iig** and **Iic**), followed by PG (**VIj / VIk / VIm**, **VIg**, and **VIh / VIi**). PE (**IVd** and **IVg**) were least abundant.

Mesophilic cultures were dominated again by PC (**IId**, **IIc** and **IIh / IIi**). The most abundant PG were **VIh / VIi**, **VIj / VIk / VIm** and **VIg**, while PE were dominated by **IVd**.

3.5. Composition of ladderanes with absent phosphatidyl headgroup

In anammox communities, we detected not only ladderanes typically reported in the literature (Boumann et al., 2006; Boumann et al., 2009a) but also shorter ladderanes with absent phosphatidyl headgroups ('APH ladderanes') depicted in Fig. 3 and Table 4, respectively. All shorter lipids we searched for were shown at Fig. S 1. Two cultures (Plettenberg, Eisenhüttenstadt) were dominated by C15-[3]-ladderane fatty acid (**3p** – "3" means that this ladderane is comprised of only single alkyl with 3 concatenated cyclobutane rings and "p" specifies its structure according to the Table 4), another culture by C18-[3]-ladderane alcohol (**3c**,

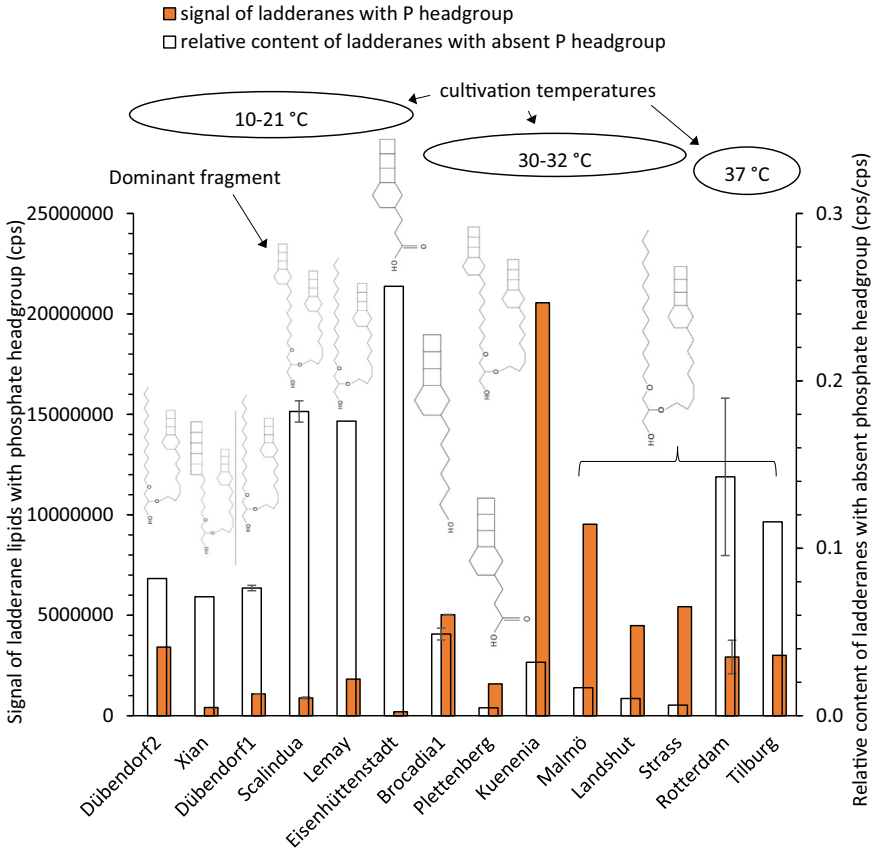


Fig. 2. Total signal for ladderane phospholipids and relative content of ladderanes with absent phosphate headgroup (APH ladderanes) in all the anammox biomasses examined in this study. The cultures were arranged from the lowest to highest cultivation temperature. For each culture, the most abundant APH ladderane is depicted. The error bars are minimum and maximum values.

Brocadia). Two enrichments (Kuenenia and Scalindua) were dominated by APH ladderane with two C20-[3]-ladderane ethers (**3.3e** – "3.3" means that the lipid is composed of C20 [3]-ladderane ether and another [3]-ladderane whose structure is given by "e" according to the Table 4, both bound to a glycerol backbone). One culture (Xi'an) contained an equally high content of APH ladderanes with i) C18-[5]- and C20-[3]-ladderane ethers (**3.5f** "5" refers to [5]-ladderane with structure given by "f" in the Table 4) and ii) C14-alkyl and C20-[3]-ladderane ethers (**3.14** – "14" refers to non-ladderane C14 alkyl bound to the glycerol backbone in addition to C20 [3]-ladderane ether). The majority of cultures (8) were dominated by APH ladderane with C14-alkyl and C20-[3]-ladderane ethers (**3.14**).

3.6. Triterpenoids content in anammox biomasses

The anammox communities were also evaluated for the content of triterpenoids. The most abundant triterpenoids were bacteriohopanetetrol cyclitol ether, followed by bacteriohopanetetrol and squalene. The C27 hopanoid ketone was not detected (signal specified in Table S 4). "*Ca.* Scalindua" contained exceptionally high amounts of all detected triterpenoids. The second most triterpenoid-rich organism was "*Ca.* Brocadia anammoxidans". The least triterpenoid-rich organism was "*Ca.* Kuenenia stuttgartiensis", containing an order of magnitude lower signal of bacteriohopanetetrol cyclitol ether.

3.7. Effect of cultivation temperature on ladderane and other alkyl moieties

Anammox biomasses dominated by "*Ca.* Brocadia" were composed of shorter length of [5]-ladderane ester alkyl chain at low cultivation temperature, from C20 to C18 (Fig. 1 A). Previously, Rattray et al. (2010) have proposed a relationship between anammox biomass cultivation temperature and the length of alkyl chain in [5]-ladderane ester moiety, showing that anammox populations cultivated at higher temperatures synthesized increased amounts of C20 [5]-ladderane esters and at lower temperatures synthesized increased amounts of C18 [5]-ladderane esters. This was based on monitoring ladderanes in "*Ca.* Brocadia" in cultivations under 16, 25 and 35 °C as well as on ladderane lipids composition from anammox biomasses collected from various environments at distinct temperatures. This was initially corroborated by our previous study cultivating anammox enrichment at reduced temperatures and analyzing ladderanes after adaptation in comparison to control (Kouba et al., 2022b). The present study was able to further corroborate this by analyzing ladderanes in anammox biomasses collected from reactors under various cultivation temperatures. Furthermore, the combined datasets from this study and Rattray et al. (2010) provide further evidence (Fig. 1 A) that "*Ca.* Brocadia" are able to modulate fractional content of C20 and C18 [5]-ladderane esters in a distinct, much narrower, range (0.6–1.0) than "*Ca.* Scalindua" (0.1–0.7) which could predispose "*Ca.* Scalindua" to being more efficiently adaptable to low temperatures.

In "*Ca.* Brocadia" genus, lower temperatures trigger the synthesis of shorter alkyl chains, C15 alkyls giving ground to C14 ones (Fig. 1 B). Compared to mesophilic biomasses dominated by "*Ca.* Brocadia anammoxidans", the psychrophilic conditions induced the production of more (i) [5]-ladderane alkyl moieties instead of [3]-ladderanes, (ii) fatty acids compared to ethers and (iii) more C14 and C16 non-ladderane alkyls. This highlights physiological mechanisms at a cell membrane level for the adaptation of "*Ca.* Brocadia" to cold stress. Increased content of [5]-ladderanes would make the membrane more rigid which goes against the typical adaptive measures of bacteria at low temperatures and suggests that the function of these unique structures, such as the maintenance of proton motive force or possibly retention of crucial anammox metabolites (Moss et al., 2018; Nouri and Tantillo, 2012), needs to be enhanced at low temperature. The latter two phenomena are in line with the theory of homeoviscous adaptation of bacterial

Table 3
Composition of ladderane phospholipids (% of the whole ladderane signal = counts per second (standard measurement unit in MS), per 0.2 g of dry weight biomass) in the anammox biomasses, as analyzed by U-HPLC-HR-MS/MS.

	Scalindua enrichment	Kuenenia enrichment	Tilburg	Rotterdam	Brocadia enrichment	Dübendorf1	Dübendorf2	Xi'an	Plettenberg	Landshut	Eisenhüttenstadt	Strass	Malmö	Lemay
PC headgroup														
Ia	-	5.1	0.3	0.9	1.1	0.6	0.9	1.0	0.0	1.0	0.1	0.7	1.4	1.0
Ib	-	4.3	0.4	0.7	0.3	3.1	4.2	3.8	0.7	5.1	0.9	4.2	8.4	3.1
Ic	-	11.0	3.7	2.5	8.2	2.2	2.8	2.7	0.4	2.2	0.5	2.6	5.3	2.0
Id	-	0.5	0.3	0.0	0.0	0.2	0.7	0.4	0.1	0.6	0.2	0.9	2.4	0.4
Ig	-	3.5	2.6	2.2	4.5	3.4	3.6	5.3	0.04	2.3	0.5	1.4	2.4	10.2
Ih / Ii	-	0.9	2.0	1.4	4.8	0.1	0.4	0.1	-	0.4	0.1	0.2	0.5	1.1
Ij / Ik / In / Io	-	0.1	0.7	0.3	1.7	0.3	0.5	0.3	-	0.1	0.1	0.0	0.2	1.6
IIa	-	2.3	0.05	0.3	0.04	1.8	1.0	0.5	1.0	1.4	0.4	1.3	0.9	0.4
IIb	-	1.2	0.03	0.3	-	4.0	3.5	2.5	7.8	7.2	2.7	5.3	4.1	1.4
IIc	-	7.0	8.3	7.2	7.9	11.0	11.0	5.1	14.5	14.8	27.2	14.8	14.5	5.2
IId	-	3.7	2.9	2.5	0.4	6.6	9.8	5.0	32.7	17.5	24.1	15.4	14.7	5.0
IIf	-	0.04	-	-	-	-	-	-	-	-	-	-	-	-
IIg	-	5.5	7.1	7.3	6.3	15.1	9.5	12.8	4.5	3.0	9.4	8.9	7.1	10.5
IIh / IIi	0.1	2.7	18.9	18.6	18.7	5.2	8.2	9.4	5.6	4.6	10.4	8.7	10.2	8.3
IIj / IIk / IIn / Iio	0.1	4.9	18.1	12.5	13.6	20.7	16.6	34.0	6.2	3.8	13.2	7.1	8.6	23.0
PE headgroup														
IIIa	-	0.8	-	0.01	0.02	-	-	-	-	-	-	-	-	0.010
IIIb	-	1.0	0.02	0.02	0.02	0.04	0.2	0.1	-	0.02	-	0.03	0.03	0.1
IIIc	-	2.0	0.1	0.1	0.3	-	0.01	-	-	-	-	0.005	0.005	0.006
IIId	-	0.8	0.015	-	-	-	-	-	-	-	-	0.005	0.005	0.004
IIIg	-	0.3	0.024	0.014	0.039	0.027	0.042	0.016	-	-	-	0.006	0.005	0.2
IIIh / IIIi	-	0.01	0.014	0.005	0.1	-	0.03	-	-	-	-	-	-	-
IVa	0.4	1.0	-	0.024	0.02	-	0.1	-	-	0.03	-	0.03	0.05	0.02
IVb	0.9	0.3	0.024	0.1	0.02	0.6	1.2	0.2	0.1	0.6	0.1	0.7	0.7	0.1
IVc	-	2.4	1.8	1.6	2.3	0.7	1.2	0.1	0.3	0.7	0.3	1.0	0.6	0.2
IVd	-	2.6	2.1	1.1	0.6	1.5	3.7	0.5	2.9	3.1	-	3.6	2.5	0.7
IVg	0.7	1.8	1.4	1.2	1.5	1.8	1.8	0.9	0.02	0.1	0.1	0.6	0.5	1.7
IVh / IVi	0.1	0.4	3.0	1.6	2.6	0.4	1.1	0.1	0.04	0.1	0.1	0.4	0.2	0.2
IVj / IVk / IVn / IVo	0.2	0.4	5.9	3.1	3.9	0.8	1.7	1.8	0.01	0.2	0.1	0.1	0.3	2.4
PG headgroup														
Va	4.0	2.0	0.1	0.3	0.2	-	0.1	-	-	0.4	-	0.1	0.2	0.1
Vb	-	3.2	0.1	0.2	0.1	0.2	0.6	0.3	0.1	2.0	-	0.6	1.0	0.5
Vc	33.2	4.6	2.3	1.5	3.1	0.1	0.6	0.2	-	0.8	-	0.5	0.6	0.3
Vd	1.4	0.7	0.4	-	0.1	-	0.2	0.1	0.1	0.2	-	0.2	0.3	0.1
Vg	18.3	3.6	0.6	0.5	1.1	2.2	2.0	1.5	0.1	1.1	0.1	1.0	0.9	3.2
Vh / Vi	3.1	0.7	1.6	0.9	3.1	0.2	0.6	0.2	0.1	0.5	0.1	0.3	0.6	0.8
VIa	15.2	0.2	-	0.02	-	-	-	-	-	0.1	-	0.03	0.02	-
VIb	4.1	0.4	-	0.02	-	-	-	-	0.4	1.0	0.1	0.2	0.03	-
VIc	3.3	1.2	0.6	0.6	0.3	0.2	0.3	0.1	1.9	1.9	0.2	1.1	-	0.03
VId	2.9	1.7	0.2	0.1	0.04	0.2	0.6	0.2	3.2	2.6	0.2	1.4	-	0.1
VIg	10.5	2.7	1.7	2.2	1.6	6.4	2.2	2.2	3.1	1.6	0.2	3.2	2.2	1.7
VIh / VIi	0.2	1.5	8.5	8.0	7.9	1.7	3.1	2.2	8.0	5.3	1.8	4.3	4.8	2.2
VIj / VIk / VIn / VIo	0.6	2.6	3.5	1.4	2.3	8.1	5.3	6.4	6.0	1.3	0.9	3.5	1.9	3.4
VII	0.8	0.3	0.02	0.03	0.002	0.04	0.04	0.03	-	0.2	0.2	0.3	0.01	0.05
VIII	0.1	6.0	0.7	14.6	1.3	0.4	0.7	0.1	0.2	10.7	5.3	4.2	1.8	7.8
X	0.1	2.0	0.1	4.3	0.05	0.03	0.05	0.01	0.1	1.6	0.6	1.0	0.2	0.8

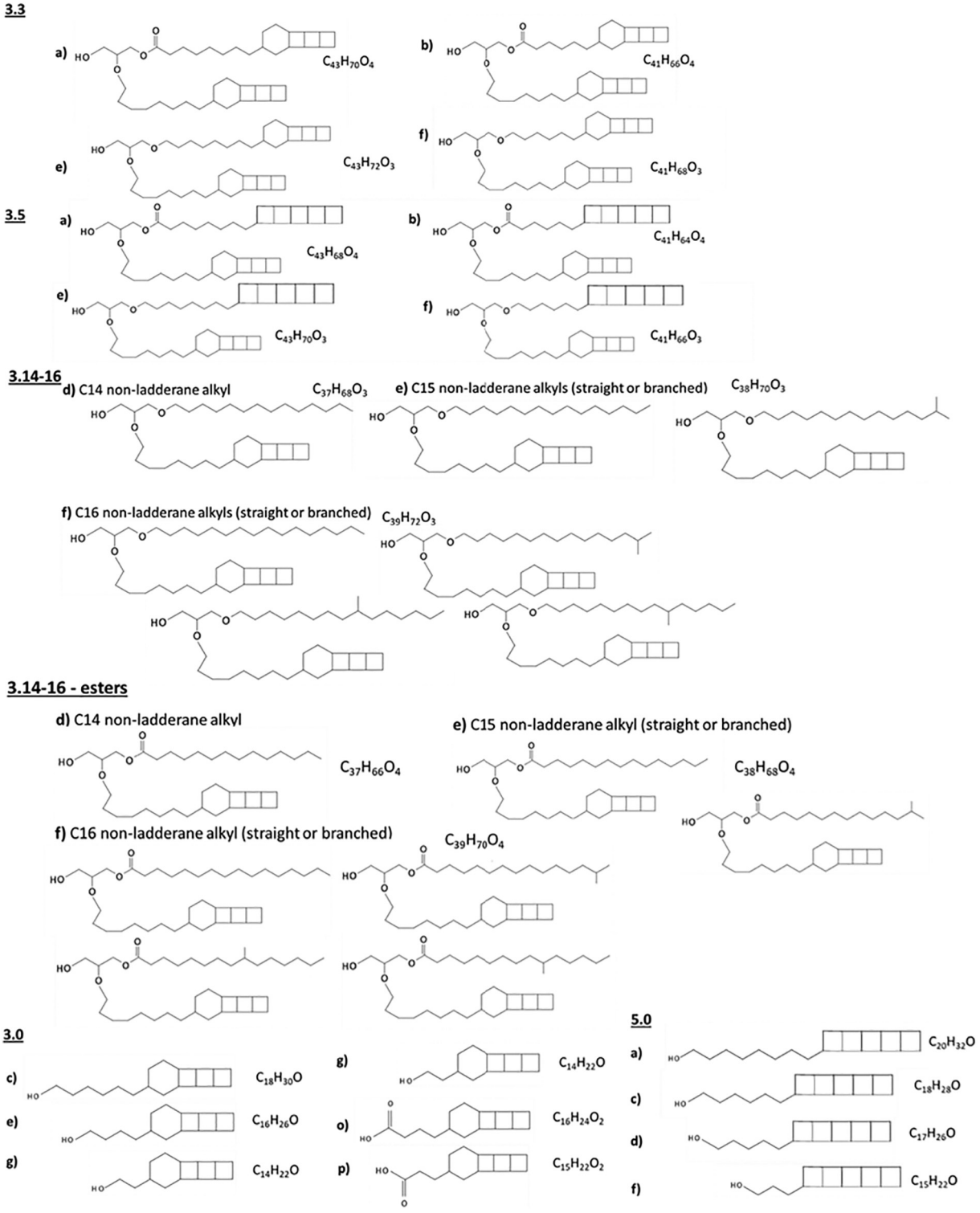


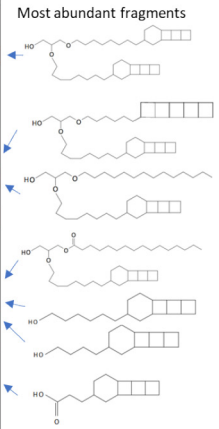
Fig. 3. Ladderanes with absent phosphate headgroup detected in some of the present anammox cultures besides those already listed in Table 1. Relative abundance depicted in Table 4.

membranes, suggesting that bacteria maintain their membrane viscosity at lower temperatures by synthesizing shorter, more branched and unsaturated alkyls to reduce the membrane phase transition temperature. This maintenance of membrane viscosity is crucial for the function and mobility of membrane proteins, diffusion of nutrients and proper separation during cell division (Siliakus et al., 2017).

Regarding the shorter ladderanes with absent phosphatidyl headgroup (APH ladderanes), the highest relative content was detected in the biomass of the full-scale WWTP Eisenhüttenstadt, adapted to 21 °C (29%, signal of APH ladderanes compared to the sum of phospholipids and APH ladderanes). Other cold-adapted (10–20 °C) enrichments also contained elevated amounts of APH ladderanes compared to the cultures adapted to mesophilic conditions at 30–32 °C (Fig. 2). The elevated amounts of APH ladderanes in psychrophilic cultures are also illustrated in Fig. 4 B that shows the relation between cultivation temperature (psychrophilic vs mesophilic), the accumulation of APH ladderanes in anammox enrichments

Table 4
Composition of ladderane lipids with absent phosphate headgroup (APH ladderanes) (% of signal of all APH ladderanes = counts per second (standard measurement unit in MS), per 0.2 g of dry weight biomass) in the anammox biomasses, as analyzed by U-HPLC-HR-MS/MS. Structure of detected APH ladderanes depicted at Fig. 3.

Name	Formula	Düben..2	Xian	Düben..1	Scalindua	Lemay	Eisenh..	Brocadia	Pletten..	Kuenenia	Malmö	Landshut	Strass	Rotterdam	Tilburg
3.3_a	C43H70O4	3.6	0.9	3.0	1.9	0.3	0.0	0.3	1.9	7.8	0.9	1.3	2.1	0.5	1.0
3.3_b	C41H66O4	0.2	0.0	0.4	15.4	0.1	0.0	0.0	0.0	0.1	0.3	0.0	0.8	0.0	0.1
3.3_e	C43H72O3	11.3	10.8	2.2	40.1	4.6	0.0	12.0	0.0	42.6	10.7	3.1	7.2	18.9	30.2
3.3_f	C41H68O3	1.2	2.3	0.5	4.3	0.9	0.0	0.4	0.0	10.1	1.6	1.7	0.0	5.8	1.8
3.5_a	C43H68O4	1.7	0.7	0.9	0.2	0.1	0.0	0.0	3.0	5.5	0.8	1.1	0.9	0.0	0.2
3.5_b	C41H64O4	0.5	0.5	0.5	0.8	0.1	0.0	0.0	0.0	0.3	0.3	0.6	0.6	0.0	0.1
3.5_e	C43H70O3	2.7	3.0	0.5	0.3	0.6	0.0	0.1	0.0	3.6	2.7	0.7	1.8	0.2	1.8
3.5_f	C41H66O3	16.4	25.5	6.4	0.3	9.6	0.2	0.2	2.2	16.2	27.6	10.0	10.7	6.2	2.7
3.14	C37H68O3	42.1	24.7	49.8	29.9	69.8	0.5	14.9	2.1	5.1	40.3	45.7	28.4	43.6	33.0
3.15	C38H70O3	5.3	2.0	2.9	0.5	4.0	0.0	0.7	0.0	0.3	1.3	6.5	1.3	6.1	0.9
3.16	C39H72O3	8.6	1.9	2.1	2.6	5.2	0.2	11.7	2.1	4.4	8.6	5.7	2.7	17.7	20.7
3.14 ester	C37H66O4	0.2	3.1	2.7	0.2	0.3	7.4	0.0	0.0	0.5	0.7	1.5	1.9	0.4	0.1
3.15 ester	C38H68O4	0.2	5.2	0.9	0.1	0.3	3.8	0.0	0.0	0.4	0.9	2.1	1.8	0.6	0.2
3.16 ester	C39H70O4	3.1	7.1	0.7	0.2	3.2	4.5	0.0	0.0	1.4	1.1	14.2	24.8	3.0	0.5
3_c	C18H30O1	0.0	0.0	0.0	0.0	0.0	0.0	56.6	0.0	0.0	0.2	0.0	0.0	0.0	0.0
3_e	C16H26O1	0.2	1.5	27.0	0.4	0.5	6.9	0.0	5.5	0.0	0.4	1.5	2.4	0.2	3.4
3_g	C14H22O1	0.0	0.0	0.2	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.5	1.7	0.0	1.8
3_o	C16H24O2	0.0	0.0	0.0	0.2	0.0	0.6	0.2	3.7	3.6	0.0	0.0	0.7	0.0	0.0
3_p	C15H22O2	2.7	10.1	2.5	2.0	0.4	68.4	0.7	73.6	0.3	1.5	2.7	8.8	0.4	0.3
5_a	C20H32O1	0.0	0.0	0.0	0.0	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5_c	C18H28O1	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5_d	C17H26O1	0.0	0.6	1.1	0.5	0.1	6.0	0.1	5.9	0.0	0.3	1.2	1.3	0.1	1.0
5_f	C15H22O1	0.0	0.0	0.0	0.5	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.2
!		10	12	14	20	20	21	30	30	30	30	32	32	37	37



and the degree of enrichment of anammox bacteria (given as percentage of operational taxonomical unit within the bacterial family *Brocadiaceae*).

The APH ladderanes can be (i) the products of cell lysis and microbial lipid degradation of phospholipids, (ii) intermediates of ladderane lipid biosynthesis, or (iii) regular membrane constituents (Rattray et al., 2008). In the present biomasses, the dominant APH ladderanes did not match the dominant phospholipids. Specifically, *sn-1* positions of APH ladderanes contained mostly ethers, whereas the phospholipids contained mainly esters. Moreover, most dominant APH ladderanes were shorter than C20 ladderanes in phospholipids: a) individual C18-C15s ladderane fatty acids and alcohols, b) more complex APH ladderanes with either the C14 straight alkyl or C18-[5]-ladderane alkyl. Thus, we hypothesize that these APH ladderanes are regular membrane constituents (hypothesis iii) linked to low-temperature adaptation: These shorter APH ladderanes could be introduced to anammox membranes at low temperatures similarly to phospholipids with shorter ladderane alkyls. On the other hand, the increased relative content of APH ladderanes was also detected in cultures adapted to 37 °C (up to 16%); these matched the dominant phospholipids which suggests that these could be the products of more efficient lysis of dead anammox cells and subsequent degradation of anammox membrane lipids by heterotrophs.

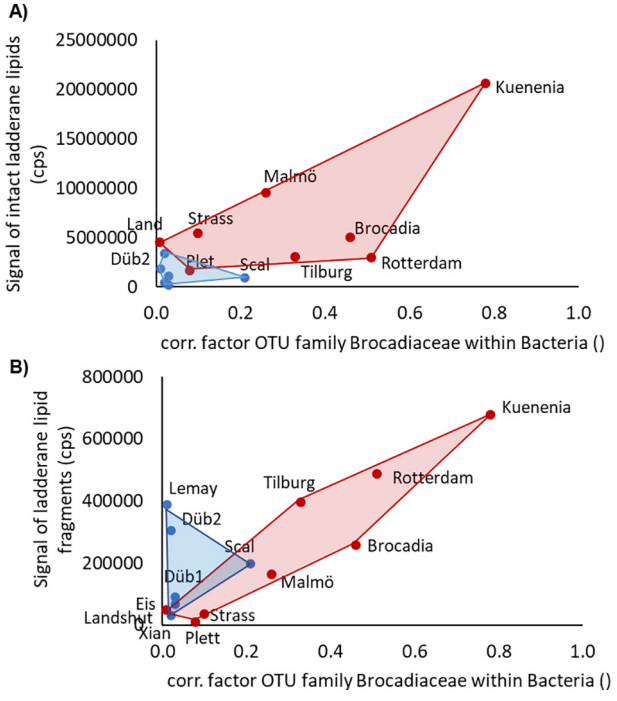


Fig. 4. Relation between the accumulation of ladderane phospholipids (A) and ladderanes with absent phosphate headgroup (B) in anammox enrichments, the degree of enrichment of anammox populations (given by the percentage of operational taxonomic units OTU within the bacterial family *Brocadiaceae*) and the cultivation temperature. Red – mesophilic; Blue – psychrophilic.

3.8. Distinct ladderane lipid composition between anammox genera and species

Several notable differences in the composition of i) lipid alkyl moiety, ii) polar headgroup, iii) alkyl moiety bond to glycerol backbone, and iv) terpenoids were highlighted among the anammox populations (Table 3, Table S 3, Table S 4). Overall, the present study found some similarities with Rattray et al. (2008) as well as some distinctions and characterized extra anammox species to a previously unreported level of detail.

"*Ca.* Scalindua" contained an exceptionally high amount of [3]- compared to [5]-ladderanes, as well as terpenoids. The high terpenoid abundance matches with Rattray et al. (2008). In our previous study (Kouba et al., 2019), we showed that the enrichment of the marine "*Ca.* Scalindua" was one of the biomasses that were the most adapted to low temperatures. Specifically, it had the highest activity of all 14 anammox cultures at 10–20 °C. The most abundant terpenoids were bacteriohopanoids, which have been suggested to facilitate an optimal equilibrium between membrane fluidity and rigidity (Boumann et al., 2009b). While the evidence provided on the link between ladderane lipids composition and the dominant anammox genera are preliminary, the specificities in membrane physiology appear to make "*Ca.* Scalindua" exceptionally suited for adaptation at low temperatures. "*Ca.* Scalindua" was also the only biomass that contained almost exclusively PG as the headgroup. In contrast, "*Ca.* Scalindua" in Rattray et al. (2008) contained also PC and PE, and the PG lipids had different compositions. Hypothetically, this could be explained by these two enrichments of "*Ca.* Scalindua" containing different species.

The "*Ca.* Kuenenia stuttgartiensis" biomasses investigated in this study and by Rattray et al. (2008) and Neumann et al. (2014) shared many similarities in their phospholipid profile: they all contained all the three polar headgroups. Furthermore, alkyl moieties were equally diethers and fatty acids and some dominant phospholipids were Ic, IIc, IVc, and VIII. In addition, we detected the IIa, IIb, IIIc, IIIg, Va-Vc, VIa, VIb, and X phospholipids

in "*Ca*. Kuenenia stuttgartiensis" enrichment, as well as some non-ladderane phospholipids, mainly those with PC headgroup.

The mesophilic cultures of "*Ca.* Brocadia fulgida" (Landshut, Strass) were remarkably similar to those of Rattray et al. (2008) in the content of (i) all three polar headgroups, (ii) the dominant PC IIc and IId, (iii) the alkyl moieties bound mostly by fatty acids, and (iv) the abundant content of VIII. In contrast, we detected a higher abundance of IIb and IVd, and a small amount of Ia, Id, IIIc, IIIg, IVa, IVg, Vb-Vd, VII and X ladderanes.

Further, compared to all other biomasses examined, "*Ca*. Brocadia anammoxidans" had twice as many non-ladderane alkyl moieties and correspondingly less ladderane moieties than biomasses dominated by other populations. The only available report on "*Ca.* Brocadia anammoxidans" ladderane lipids was provided by Sinninghe Damsté et al. (2005) using GC with much lower resolution than the present study.

The comparison of the terpenoid signals revealed a higher content in the "*Ca.* Brocadia anammoxidans" than the "*Ca.* Kuenenia stuttgartiensis" enrichments. The signal of the bacteriohopanetetrol cyclitol ether was higher by an order of magnitude. Interestingly, the "*Ca.* Brocadia anammoxidans" enrichment had lower activation energy of the anammox reaction between 10 and 15 °C, thus being less susceptible to low temperature (Kouba et al., 2019). Thus, our data reinforce the hypothesis initially suggested by Boumann et al. (2009b) that bacteriohopanoids may play a role in maintaining membrane fluidity in anammox cells.

3.9. Impact of other process conditions on ladderanes

The only planktonic enrichment of "*Ca.* Kuenenia stuttgartiensis" was characterized by an exceptionally high signal of total ladderane lipids (Fig. 2). This corresponded to the high degree of enrichment of this population (78%) within the family Brocadiaceae (Table S 2; (Kouba et al., 2019) and likely to low content of extracellular polymeric substances (EPS) diluting ladderane-rich bacteria (Lotti et al., 2019). EPS are much more abundant in biofilms and bioaggregates than in cells in suspension. In theory, the absence of EPS could also affect lipid ionization; however, we do not know whether EPS were extracted by our solvent and if and when they were eluted. If they were eluted in the same retention time as ladderanes, they could theoretically compete for ionization charge and affect our analysis. At this point, the effect of EPS on ladderane analysis is pure speculation. Out of the PN/A process parameters, salinity is an intriguing parameter to investigate further, as moderate halophiles and halotolerant were shown to adapt to saline environment by increasing the proportion of anionic lipids, e.g., phosphoglycerols (Russell, 1989). The most important outlier was indeed formed by the enrichment of the marine "*Ca*. Scalindua" which was cultivated with the most saline medium when compared to other biomasses and, hypothetically, the salt content could also induce changes in the lipid content. Also, "*Ca.* Scalindua" is phylogenetically more distant which also may affect the lipid content.

3.10. Implications for the production of ladderanes

We suggest that anammox biomasses rich in ladderanes can be obtained in a planktonic mixed culture enriched in "*Ca.* Kuenenia". Such planktonic cultivation mode of anammox microorganisms has first been reported by Van Der Star et al. (2008) using a membrane bioreactor operated with a biomass retention time of 12 days. At the time of this writing, the solids retention time of our planktonic enrichments had been reduced to 10 days. The cultivation also involved low levels of calcium and magnesium to prevent biomass aggregation, and that has resulted in a biomass concentration of 0.5 g-VSS/L and enrichments of up to 98%.

We have shown that in addition to ladderane phospholipids, anammox enrichments can also contain significant amounts of ladderanes with absent phosphate headgroup (APH ladderanes) besides the ones already identified by Rattray et al. (2008). Yet their origin and putative physiological function need to be determined. Fig. 4 shows the relation between the cultivation temperature, the accumulation of ladderanes in anammox enrichments, and the degree of enrichment of anammox lineages within the family Brocadiaceae. The results provide evidence that cultivation temperature plays a crucial role in the accumulation of either ladderane phospholipids (30–32 °C) or APH ladderanes (<21 °C). Specifically, psychrophilic enrichments seemed to accumulate fewer phospholipids and more APH ladderanes compared to mesophilic enrichments, potentially facilitating homeoviscous membrane adaptation.

Overall, the specific composition of ladderane phospholipids seems to depend on anammox populations that dominate the biomass, the cultivation regime and conditions, and temperature. These factors impact the alkyl chain length of ladderanes, the number of cyclobutane rings on ladderane moiety, presence of non-ladderane alkyls and/or the phospholipid polar headgroup composition, as well as the presence of APH ladderanes, which in turn crucially affect the ladderane utilization potential in biotechnologies. For example, biomass containing more [5]- or at least [3]-ladderanes on the *sn-1* position as compared to straight or branched alkyls has potential for yielding more energy-dense extracts.

4. Conclusions

We aimed to comprehensively analyze the ladderane lipid composition of various anammox biomasses and to identify the major factors influencing the lipid composition, including ladderanes with absent phosphatidyl headgroup that could play an important role in anammox membrane physiology but remained to be characterized.

- Compared to the mesophilic biomasses, the psychrophilic populations of "*Ca.* Brocadia" had substantially lower fraction of C20/(C18 + C20) [5]-ladderane esters which is an important confirmation of previous preliminary evidence on the involvement of these fatty acids in low-temperature adaptation of anammox bacteria. We provide further evidence that "*Ca.* Brocadia" can only modulate this fraction in a narrower range (0.6–1.0) than the salt-adapted marine "*Ca.* Scalindua" (0.1–0.7).
- Ladderanes with absent phosphatidyl headgroup were detected in high amounts particularly in anammox enrichments adapted to low temperatures, possibly linking to homeoviscous membrane adaptation. In turn, relatively lower levels of ladderane phospholipids were detected in biomasses adapted to low temperatures, most likely due to the lower level of anammox biomass enrichment.
- The composition of ladderane lipids (e.g., Nr of cyclobutane rings, ether/ester bonds, chain length, polar headgroup), along with hopanoids and squalene abundances can vary across anammox populations. "*Ca.* Scalindua" contained an exceptionally high content of [3]-ladderanes (56% vs 9–39% for other genera). "*Ca.* Brocadia anammoxidans" from psychrophilic biomasses contained more [5]-ladderane alkyl moieties, more fatty acids and more C14 and C16 non-ladderane alkyls compared to the mesophilic ones.

Overall, this study provides important description of the membrane physiology of anammox populations in relation to temperature conditions. Importantly, the generated scientific knowledge can be utilized in the future to tailor the production of ladderanes from anammox cultures using microbial community engineering principles, besides a better management of anammox-based wastewater treatment performance in cold climates.

CRediT authorship contribution statement

Vojtěch Kouba: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, review & editing. **Kamila Hůrková**: Methodology, Data curation, Writing – review. **Klára Navrátilová**: Methodology, Data curation, Writing – review. **Dana Kok**: Methodology, Data curation, Writing – review & editing. **Andrea Benáková**: Methodology, Data curation, Writing – review. **Michele Laureni**: Methodology, Writing – review & editing. **Patricie Vodičková**: Writing – review & editing. **Tomáš Podzimek**: Writing – review & editing. **Petra Lipovová**: Writing – review & editing. **Laura van Niftrik**: Methodology, Writing – review & editing. **Jana Hajšlová**: supervision, Writing – review & editing. **Mark C.M. van Loosdrecht**: supervision, Writing – review & editing. **David Gregory**

Weissbrodt: supervision, Writing – review & editing. **Jan Bartáček**: supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors acknowledge the financial support of the Czech Ministry of Education Youth and Sports through project GACR 17–25781S. Michele Laureni was supported by a Marie Skłodowska-Curie Individual Fellowship (MixAmox; 752,992). The authors also thank the researchers, process engineers and operators for providing and maintaining the anammox biomass, namely Katinka van de Pas-Schoonen and Guylaine Nuijten (Radboud University, The Netherlands), Albert Regiert (Stadtwerke Landshut, Germany), Martin Hell (AIZ, Strass im Zillertal, Austria), Jürgen Koepke (TAZV Oderaue, Germany), Jon Albizuri (Anox Kaldnes, Sweden), Romain Lemaire (Veolia Research and Innovation, France), Job Robben and Gijs Lavrijsen (Waterschap De Dommel, Netherlands), Jeroen van Waveren (Waterschap Hollandse Delta, Netherlands), Hans-Joachim Hölter (Ruhrverband, Germany), Xiaochang Wang and Jin Pengkang (Xi'a University of Architecture and Technology, China) and Adriano Joss and Damian Hausherr (EAWAG, Switzerland). Vojtěch acknowledges helpful feedback on the manuscript from Craig Alfred Riddell.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154715.

References

Boumann, H.A., Hopmans, E.C., Van De Leemput, I., Op den Camp, H.J., Van De Vossenberg, J., Strous, M., Jetten, M.S., Sinninghe Damsté, J.S., Schouten, S., 2006. Ladderane phospholipids in anammox bacteria comprise phosphocholine and phosphoethanolamine headgroups. FEMS Microbiol. Lett. 258 (2), 297–304.

Boumann, H.A., Longo, M.L., Stroeve, P., Poolman, B., Hopmans, E.C., Stuart, M.C., Damsté, J.S.S., Schouten, S., 2009a. Biophysical properties of membrane lipids of anammox bacteria: I. Ladderane phospholipids form highly organized fluid membranes. Biochim. Biophys. Acta Biomembr. 1788 (7), 1444–1451.

Boumann, H.A., Stroeve, P., Longo, M.L., Poolman, B., Kuiper, J.M., Hopmans, E.C., Jetten, M.S., Damsté, J.S.S., Schouten, S., 2009b. Biophysical properties of membrane lipids of anammox bacteria: II. Impact of temperature and bacteriohopanoids. Biochim. Biophys. Acta Biomembr. 1788 (7), 1452–1457.

Cao, Y., van Loosdrecht, M.C., Daigger, G.T., 2017. Mainstream partial nitritation-anammox in municipal wastewater treatment: status, bottlenecks, and further studies. Appl. Microbiol. Biotechnol. 101 (4), 1365–1383.

Crowe, S.A., Treusch, A.H., Forth, M., Li, J., Magen, C., Canfield, D.E., Thamdrup, B., Katsev, S., 2017. Novel anammox bacteria and nitrogen loss from Lake Superior. Sci. Rep. 7 (1), 13757.

Daigger, G.T., 2014. Oxygen and carbon requirements for biological nitrogen removal processes accomplishing nitrification, nitritation, and anammox. Water Environ Res 86 (3), 204–209.

Hurkova, K., Uttl, L., Rubert, J., Navratilova, K., Kocourek, V., Stranska-Zachariasova, M., Paprstein, F., Hajslova, J., 2019. Cranberries versus lingonberries: a challenging authentication of similar vaccinium fruit. Food Chem. 284, 162–170.

Javidpour, P., Deutsch, S., Mutalik, V.K., Hillson, N.J., Petzold, C.J., Keasling, J.D., Beller, H.R., 2016. Investigation of proposed ladderane biosynthetic genes from anammox bacteria by heterologous expression in E. coli. PLoS ONE 11 (3).

Kouba, V., Hurkova, K., Navratilova, K., Vejmelkova, D., Benakova, A., Laureni, M., Vodickova, P., Podzimek, T., Lipovova, P., van Niftrik, L., Hajslova, J., van Loosdrecht, M., Weissbrodt, D., Bartacek, J., 2019. On anammox activity at low temperature: effect of ladderane composition, process conditions and dominant anammox population. bioRxiv 2019.12.15.873869.

Kouba, V., Bachmannova, C., Podzimek, T., Lipovova, P., van Loosdrecht, M.C., 2022. Physiology of anammox adaptation to low temperatures and promising biomarkers: a review. Bioresour. Technol. 349, 126847.

Kouba, V., Vejmelkova, D., Zwolsman, E., Hurkova, K., Navratilova, K., Laureni, M., Vodickova, P., Podzimek, T., Hajslova, J., Pabst, M., 2022b. Adaptation of anammox bacteria to low temperature via gradual acclimation and cold shocks: distinctions in protein expression, membrane composition and activities. Water Res. 209, 117822.

Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C.M., 2014. Full-scale partial nitritation/anammox experiences - an application survey. Water Res. 55, 292–303.

Lotti, T., Kleerebezem, R., Lubello, C., Van Loosdrecht, M., 2014. Physiological and kinetic characterization of a suspended cell anammox culture. Water Res. 60, 1–14.

Lanekoff, I., Karlsson, R., 2010. Analysis of intact ladderane phospholipids, originating from viable anammox bacteria, using RP-LC-ESI-MS. Anal. Bioanal. Chem. 397, 3543–3551.

Lotti, T., Carretti, E., Berti, D., Martina, M., Lubello, C., Malpei, F., 2019. Extraction, recovery and characterization of structural extracellular polymeric substances from anammox granular sludge. J. Environ. Manag. 236, 649–656.

Moss, F.R., Shuken, S.R., Mercer, J.A.M., Cohen, C.M., Weiss, T.M., Boxer, S.G., Burns, N.Z., 2018. Ladderane phospholipids form a densely packed membrane with normal hydrazine and anomalously low proton/hydroxide permeability. Proc. Natl. Acad. Sci. 115 (37), 9098–9103.

Neumann, S., Wessels, H.J., Rijpstra, W.I.C., Sinninghe Damsté, J.S., Kartal, B., Jetten, M.S., van Niftrik, L., 2014. Isolation and characterization of a prokaryotic cell organelle from the anammox bacterium K uenenia stuttgartiensis. Mol. Microbiol. 94 (4), 794–802.

Nielsen, P.H., Daims, H., Lemmer, H., 2009. FISH Handbook for Biological Wastewater Treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH. IWA Publishing, London, UK.

Nouri, D.H., Tantillo, D.J., 2012. Attack of radicals and protons on ladderane lipids: quantum chemical calculations and biological implications. Organic & biomolecular chemistry 10 (29), 5514–5517.

Rattray, J.E., Van De Vossenberg, J., Hopmans, E.C., Kartal, B., Van Niftrik, L., Rijpstra, W.I.C., Strous, M., Jetten, M.S.M., Schouten, S., Damsté, J.S.S., 2008. Ladderane lipid distribution in four genera of anammox bacteria. Arch. Microbiol. 190 (1), 51–66.

Rattray, J.E., Van Vossenberg, J.D., Jaeschke, A., Hopmans, E.C., Wakeham, S.G., Lavik, G., Kuypers, M.M.M., Strous, M., Jetten, M.S.M., Schouten, S., Sinninghe Damsté, J.S., 2010. Impact of temperature on ladderane lipid distribution in anammox bacteria. Appl. Environ. Microbiol. 76 (5), 1596–1603.

Russell, N.J., 1989. Adaptive modifications in membranes of halotolerant and halophilic microorganisms. J. Bioenerg. Biomembr. 21 (1), 93–113.

Siliakus, M.F., van der Oost, J., Kengen, S.W., 2017. Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. Extremophiles 21 (4), 651–670.

Sinninghe Damsté, J.S., Strous, M., Rijpstra, W.I.C., Hopmans, E.C., Geenevasen, J.A.J., Van Duin, A.C.T., Van Niftrik, L.A., Jetten, M.S.M., 2002. Linearly concatenated cyclobutane lipids form a dense bacterial membrane. Nature 419 (6908), 708–712.

Sinninghe Damsté, J.S., Rijpstra, W.I.C., Geenevasen, J.A., Strous, M., Jetten, M.S.M., 2005. Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox). FEBS J. 272 (16), 4270–4283.

Van De Graaf, A.A., De Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen, J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142 (8), 2187–2196.

Van Der Star, W.R.L., Miclea, A.I., Van Dongen, U.G.J.M., Muyzer, G., Picioreanu, C., Van Loosdrecht, M.C.M., 2008. The membrane bioreactor: a novel tool to grow anammox bacteria as free cells. Biotechnol. Bioeng. 101 (2), 286–294.