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On anammox activity at low temperature: Effect of ladderane composition and process conditions

Vojtěch Kouba [a,*], Kamila Hůrková [b], Klára Navrátilová [b], Dana Kok [a], Andrea Benáková [a], Michele Laureni [c,f], Patricie Vodičková [a,e], Tomáš Podzimek [e], Petra Lipovová [e], Laura van Niftrik [d], Jana Hajšlová [b,1], 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] Delft University of Technology, Department of Biotechnology, Building 58, Van der Maasweg 9, 2629 HZ Delft, The Netherlands
[d] Radboud University, Department of Microbiology, Institute for Water and Wetland Research, 1Heyendaalseweg 135, 6525 ED AJ Nijmegen, The Netherlands
[e] University of Chemistry and Technology Prague, Department of Biochemistry and Microbiology, Technická 5, 166 28 Prague, Czechia
[f] TU Delft, Department of Water Management, Building 23, Stevinweg 1, 2628 CN Delft, the Netherlands

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ABSTRACT

The application of partial nitritation-anammox (PN/A) under mainstream conditions can enable substantial cost savings at wastewater treatment plants (WWTPs), but how process conditions and cell physiology affect anammox performance at psychrophilic temperatures below 15 °C remains poorly understood. We tested 14 anammox communities, including 8 from globally-installed PN/A processes, for (i) specific activity at 10–30 °C, (ii) composition of membrane lipids, and (iii) microbial community structure. We observed that membrane composition and cultivation temperature were closely related to the activity of anammox biomasses. The size of ladderane lipids and the content of bacteriohopanoids were key physiological components related to anammox performance at low temperatures. We also indicate that the adaptation of mesophilic cultures to psychrophilic regime necessitates months, but in some cases can take up to 5 years. Interestingly, biomass enriched in the marine genus "*Candidatus* Scalindua" displayed outstanding potential for nitrogen removal from cold streams. Collectively, our comprehensive study provides essential knowledge of cold adaptation mechanism, will enable more accurate modelling and suggests highly promising target anammox genera for inoculation and set-up of anammox reactors, in particular for mainstream WWTPs.

1. Introduction

Anaerobic ammonium oxidation (anammox) is an established microbial process for nitrogen removal from reject water (side streams) from sludge digestion and other nitrogen-rich and warm wastewaters. Compared to nitrification-denitrification, it does not require any exogenous organic carbon consumption and produces by up to 80% less excess sludge due to the autotrophic nature of anammox microorganisms. Because just 57% of the ammonium is oxidized to nitrite only, the combination of anammox with partial nitritation saves more than 50% in aeration energy and aeration system capacity [1–3]. According to Lackner et al. [4], Bowden et al. [5] and our own research, this technology has been implemented at over 150 full-scale anammox installations world-wide for the treatment of concentrated side streams; this makes side-stream anammox an established technology. At these installations, the parameters beneficial for the anammox process are high temperatures 30–37 °C and high concentrations of ammonium nitrogen (outlet concentrations $\geq$100 mgNamon.L^{-1}). A decrease in these process parameters unfavorably impacts process efficiency [4]. At 30–37 °C and an order of magnitude lower ammonium nitrogen, anammox has been reported to spontaneously occur in the more diluted main stream of municipal wastewater treatment plants [6]. This indicated that even a low ammonium concentration is not a bottleneck. In nature, anammox bacteria were detected in both marine and freshwater

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

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mesophilic (25–38 °C) and psychrophilic (10–25 °C) ecosystems [7–9]. This supports the potential to extend the applicability of anammox to the mainstream of wastewater treatment plants (WWTPs).

Currently, the main challenge in anammox research is its implementation in colder main-stream conditions, one of the main bottlenecks being the low activity of anammox bacteria at low temperatures [10–12]. This implementation will reduce operational and capital expenses (*i.e.*, capacity of aeration system) for the removal of nitrogen from colder mainstream WWTPs and enable a more complete utilization of organic carbon in wastewater, e.g., for energy generation [13]. Specifically, the low activity is cumbersome in psychrophilic main-stream reactors inoculated with mesophilic anammox cultures [11]. Per recent evidence, anammox can overcome cold stress and improve activity at low temperatures. This can result from gradual acclimation [14], enrichment of cold-adapted species [15] or cold shocks [16].

Nonetheless, the following questions still waits to be answered. The activity of anammox cultures as a function of temperature has yet to be reported in sufficient detail, such as for anammox genera other than "*Candidatus* Brocadia" and biomasses operated for the long-term in psychrophilic regime. In the few studies available, the effect of temperature on the activity of anammox cultures has been assessed using only a single genus ("*Ca.* Brocadia"), and biomass from either few mesophilic side-stream reactors [17] or marine environments [18]. A proteomic study by Lin et al. [19] has suggested that low temperatures do affect "*Ca.* Kuenenia" and "*Ca.* Jettenia" more strongly than "*Ca.* Brocadia". Some other recent studies have associated anammox cold adaptation with an increased anammox activity and a shift in dominant anammox populations [14,20–25]. Therefore, the currently rare cultures of anammox bacteria operated under a long-term psychrophilic regime may be affected by temperature differently than mesophilic cultures, since they will be dominated by cold-adapted microbial species, or by different species altogether. However, as the psychrophilic cultures have been made available only recently, they have never been characterized to sufficient detail. Specifically, correlations between anammox activities (in terms of absolute activities and activation energies *Ea*) and long-term cultivation temperature and microbial community structure are yet to be addressed in a comprehensive survey.

One of the promising mechanisms responsible for anammox adaptation to cold stress is the altered composition of ladderane phospholipids [26]. Ladderanes are unique to anammox, likely reducing the diffusion of protons from anammoxosome organelle, thus enabling the slow anammox reaction [27]. They consist of three or five concatenated cyclobutene rings bound to a polar head group by an ester or ether bond [28]. Generally, cold-adapted bacteria tend to synthesize more branched, unsaturated and shorter fatty acid phospholipids, so that their cytoplasmic membrane remains fluid, thus maintaining function of membrane proteins [29]. Only two studies on ladderanes in cold anammox bacteria have been published, suggesting that anammox cultivated at lower temperatures had contained more C18 than C20 ladderanes [30,31]. The original study also reported the ladderane composition of anammox cultures from multiple environments and WWTPs. However, ladderane composition potentially enabling increased culture activity as a function of temperature has yet to be investigated.

This study assessed the effect of temperature (10, 15, 20, 25, 30 °C) on the activity of 14 anammox biomasses originating from a representative set of full-scale reactors, pilot- and lab-scale models and highly enriched lab-scale cultures. The activities and *Ea* were correlated with ladderane content, dominant anammox populations, cultivation temperature regime and relevant process conditions (*i.e.*, one- or two-stage PN/A, cultivation of anammox in granules/flocs/carriers/free cells). Collectively, our findings identified the most suitable inocula and process conditions applicable for side- and mainstream anammox. They provide essential insights for process acclimation under various temperature regimes. While suggesting mechanisms anammox bacteria use to adapt to low temperature, the *Ea* measured can sustain integration into mathematical models for process anticipation.

2. Materials and methods

2.1. Anammox biomasses

The mesophilic biomasses sampled from full-scale installations (Landshut / DE, Plettenberg / DE, Malmö / SE, Strass / AT, Tilburg / NL, Rotterdam / NL), enrichment of Kuenenia (Delft / NL) and Brocadia (Nijmegen / NL) and psychrophilic cultures from full scale WWTPs (Eisenhüttenstadt / DE, Xi'an / CN), pilot and laboratory reactors (Lemay / FR, Dübendorf1 / CH, Dübendorf2 / CH) and enrichment Scalindua (Nijmegen / NL) are described in Table 1.

2.2. Experimental set-up

The batch experiments were initiated by transferring anammox biomass to two reactors with a working volume of 1 L. The anammox biomass amount was set so that the duration of test at 30 °C was at least 45 min to allow for collection of a minimum of 4–5 samples, and the resulting biomass content was kept consistent during all temperature tests. The biomass in vessels was gently mixed by magnetic stirrers Heidolph MR Hei-Mix L at 250 rpm. To maintain vessel temperature at 5, 10, 15, 20, 25 or 30 °C, the vessels were cooled or heated using thermostats Julabo F12 (Julabo GmbH, Germany). Anoxic conditions were maintained by a gentle flushing of the headspace with dinitrogen gas (grade 4.0) at 50–200 mL.min^{-1}. After the suitable temperature was established, pH was adjusted to 7.40±0.05 by 0.05 mol.L^{-1} HCl and NaOH. Then, $NaNO_2$ and NH_4Cl dissolved in 5–10 mL of tap water was dosed to both reactors, so that each assay started at 40 mg-N.L^{-1} of nitrite and at least 40 mg-N.L^{-1} of ammonium. The tests were done in duplicates, achieving a relative average deviation of 9.3±8.2%. The biomasses we did not sample personally (Lemay, Dübendorf1-2, Malmö, Xi'an) were re-activated in our set-up for at least 12 h with substrate.

Regular sampling of batch reactors was carried out to analyse total ammonium nitrogen (TAN) as the sum of N-NH_3 and N-NH_4^+, N-NO_3^-, N-NO_2^-, and to measure suspended and total solids concentration according to APHA [32]. The anammox activity was determined as a sum of nitrite and ammonium nitrogen removal rate, each calculated as a linear slope of nitrogen concentration development during respective batch tests. To avoid error due to changing affinity to substrate, only concentrations in the linear range were included.

The contribution of denitrification to nitrogen removal was calculated as the removal rate of nitrite higher than according to the anammox stoichiometry given by Strous et al. [33]. Based on our experience with testing the activities of anammox biomasses, heterotrophic denitrification can be judged as negligible with the possible exception of biomasses both: i) under steady and significant input of organic carbon, e.g., biomass combining denitrification with anammox process in the main stream of wastewater treatment plant, and ii) with significant residues of this organic carbon in the biomass. As documented by Lotti et al. [17], the biomass concentration increases during these batch tests are negligible and so the development of biomass concentration during the tests was not monitored.

To evaluate the effect of temperature on anammox activity, the activities were normalized to 30 °C. The activation energies were calculated according to the Arrhenius' empirical model and its linearized version (equations (1) and (2)), where k is the ratio of anammox activities at the lower (numerator) and higher (denominator) compared temperatures, ln is the natural logarithm, A is a constant pre-exponential factor, *Ea* is the activation energy (J.mol^{-1}), R is the ideal gas constant (J.mol.$^{-1}$.K^{-1}) and T is the thermodynamic temperature (K). The data were linearized using equation (2), yielding either one or two *Ea*. Two *Ea* were chosen when the resulting correlation coefficient R^2 was higher by 0.4. The exception was biomass Tilburg, where individual *Ea* was attributed to each temperature interval of 10–15, 15–20, 20–25, and

Table 1
Description of tested anammox cultures named based on their original location, except for enrichments.

ID	Description of technology	Influent	Sludge character	t (°C)	N-load (kg. $m^{-3}.d^{-1}$)	aeration / DO (mg. L^{-1})
Full-scale installations						
Eisenhüttenstadt / DE	SBR DEMON®, one-stage	reject water	flocs/granules	21	–	intermittent/-
Landshut / DE	TERRAMOX®, two-stage	reject water	flocs	32	0.5	no
Malmö / SE	AnitaMOX™, Anox Kaldnes, two-stage	reject water	biofilm, carriers K5**	30	1.0	no
Plettenberg / DE	SBR DEMON®, one-stage	reject water	flocs/granules	30	0.3	intermittent/-
Strass / AT	continuous DEMON®, one-stage	reject water	flocs/granules	30	0.8	intermittent/0.4
Rotterdam / NL	ANAMMOX®, two-stage	reject water	granules	37	3.4	no
Tilburg / NL	ANAMMOX®, one-stage	reject water, CAMBI	granules	37	1.3	continuous/3.0–3.5
Xian / CN	full-scale WWTP, activated sludge process	municipal sewage	biofilm, carriers	n.a.	n.a.	n.a.
Pilot and lab-scale systems						
Lemay / FR	lab-scale 1.6 m^3, VERI*, one-stage	pre-treated municipal sewage	biofilm, carriers K5**	20	0.2–0.3	continuous/ 0.7
Dübendorf1 / CH	pilot-scale MBBR, EAWAG	municipal sewage pre-treated in A-stage	biofilm, carriers	14	n.a.	n.a.
Dübendorf2 / CH	lab-scale MBBR, EAWAG	synthetic	biofilm, carriers	10	n.a.	n.a.
Enrichments						
Brocadia	enrichment, laboratory of Microbiology, Radboud University, NL	synthetic	granules	30	0.36	no
Kuenenia	enrichment, laboratory of EBT, TU Delft, NL	synthetic	planktonic	30	1	no
Scalindua	enrichment, laboratory of Microbiology, Radboud University, NL	synthetic	flocs	20	0.93	no

25–30 °C.

$$k = Ae^{\frac{-E_a}{RT}} \quad (1)$$

$$lnk = lnA - \frac{E_a}{RT} \quad (2)$$

2.3. Analysis of bacterial community compositions by amplicon sequencing

All biomasses were analysed for their bacterial community compositions by 16S rRNA gene amplicon sequencing of distinct regions (16S V4 / 16S V3 / 16S V3-V4 / 16S V4-V5, 18S V4 / 18S V9, ITS1 / ITS2, Arc V4). Genomic DNA was extracted using the DNeasy® PowerBiofilm® Kit (MO BIO GmBH, Germany) following the manufacturer's protocol and submitted to Novogene (Hong Kong, PRC) for amplicon sequencing using the MiSeq workflow (Illumina, US). Details on the method are described in the section Amplicon Sequencing Methodology of the Supporting Information.

2.4. Ladderane analysis

We used U-HPLC–HRMS/MS which is exceptionally sensitive and provided insight into the number of carbon atoms of these lipids. Per Rattray et al. [34], the polar headgroup ionization differs substantially. Also, to the best of our knowledge, ladderane lipid standards are not on the market. Thus, the detection results can be characterized qualitatively and in relative quantification between lipids with the same polar headgroup.

2.4.1. Reagents and chemicals

Deionized water was obtained from a Milli-Q® Integral 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).

2.4.2. Sample preparation

To extract ladderane phospholipids, a mixture of MeOH:DCM:10 mM ammonium acetate (2:1:0.8, v/v/v) was chosen according to Lanekoff & Karlsson (2010). Lyophilized anammox cultures 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, centrifuged (5 min, 10,000 rpm, 5 °C). Finally, 1 mL of supernatant was transferred into the vial before further analysis by ultra-high performance liquid chromatography coupled to high-resolution tandem mass spectrometry (U-HPLC–HRMS/MS).

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

The 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, Ontario, Canada) was used to analyze ladderane phospholipids. Chromatographic separation of extracts was carried out using U-HPLC system, which was equipped with Acquity UPLC BEH C18 column, 100 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 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 set at 5 °C. A quadrupole-time-of-flight TripleTOF® 6600 mass spectrometer (SCIEX, Concord, Ontario, Canada) was used. 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 to: 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 methodology were consistent with Hurkova et al. (2019), except of confirmation of compound identification, which used accurate mass, isotopic pattern and MS/MS characteristic fragments.

3. Results

3.1. All biomasses were dominated by "Ca. Brocadia", except enrichments

3.1.1. Bacterial community compositions by 16S rRNA gene amplicon sequencing

The main anammox genera detected across the lab-scale, pilot, and full-scale biomasses by amplicon sequencing were "*Ca.* Brocadia" (1–50%), "*Ca.* Scalindua" (0–11%), and "*Ca.* Kuenenia" (0–76%) (Fig. 1). Small relative abundances of "*Ca.* Anammoxomicrobium" were detected in several samples, less than 0.08% of relative abundance normalized to all bacteria OTU. Aside from the two enrichments ("*Ca.* Scalindua", "*Ca.* Kuenenia"), "*Ca.* Brocadia" was the dominant anammox genus in all biomasses. Total anammox sequencing read counts relative to total bacteria varied from 1 to 78%. Detailed results of amplicon sequencing are described in supporting materials (Table S1).

3.1.2. qPCR

qPCR provided a proxy for anammox abundance in the biomass, expressed as the ratio of anammox and the bacterial 16S rRNA genes. The efficiencies of the qPCR reaction for anammox and the bacterial 16S assays varied between 0.853 and 1.08 and 0.792–1.18, respectively. The ratios between the gene abundances varied from 0.02 to 0.41 (Fig. 2).

3.2. Effect of temperature on anammox performance: activity and activation energy (Ea)

The activity of various anammox cultures was expressed as the mass of ammonium and nitrite nitrogen metabolized per biomass weight (as volatile suspended solids, VSS) and time at 10–30 °C. As shown in Fig. 3, in the whole temperature range, the most active biomass of all was the marine enrichment of "*Ca.* Scalindua". At 25–30 °C, similar activity was achieved by the enrichment of "*Ca.* Kuenenia". Further, at 30 °C, the most active biomasses were Rotterdam (ANAMMOX®), Strass (DEMON®) and Malmö (AnitaMOX®). Following "*Ca.* Scalindua" and "*Ca.* Kuenenia" enrichments, these three mesophilic cultures (Rotterdam, Strass, Malmö) were also most active at 10 °C. Among psychrophilic cultures, the most active at 10 °C were the enrichment of "*Ca.* Scalindua", followed by Lemay (0.031 kg-N.kg-VSS^{-1}.d^{-1}), Dübendorf1 (0.024 kg-N.kg-VSS^{-1}.d^{-1}) and Dübendorf2 (0.019 kg-N.kg-VSS^{-1}.d^{-1}).

To describe the effect of temperature on anammox biomasses, the activities were normalized at 30 °C and Ea was determined as a temperature coefficient for each culture (Table 2). At 15–30 °C, all anammox cultures were characterized by a similar Ea of 79±19 kJ.mol^{-1}. All but one psychrophilic culture could be described by a single Ea over the range from 10 to 30 °C, similar to some mesophilic ones (enrichments "*Ca.* Kuenenia" and "*Ca.* Brocadia", and biomass Plettenberg). Other mesophilic cultures were affected by temperature at 10–15 °C more severely (Table 2). Tilburg could only be described by a separate Ea for each of the four temperature intervals. Alternately, Tilburg and also some other cultures (e.g., Strass, Malmö) could be more accurately described by a quadratic function (Fig. S1).

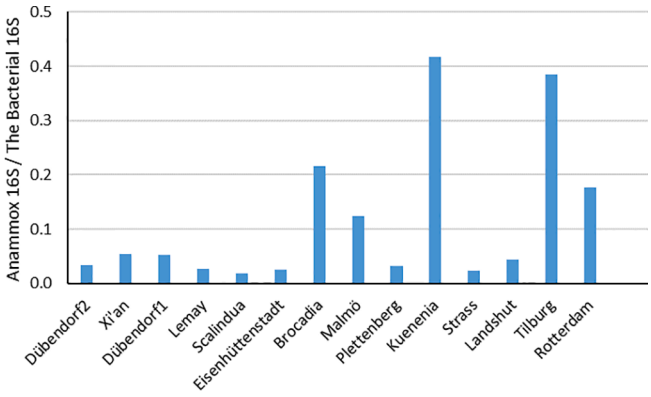

Fig. 2. The ratio of abundance change of anammox-specific 16S rRNA genes and the total amount of bacterial 16S rRNA genes in anammox biomasses.

3.3. Ladderane composition

Membranes in original samples of anammox bacteria were investigated for the length of ladderane core lipids on *sn-1* and *sn-2* positions and polar head composition by U-HPLC-MS/MS. We detected one or two ether-bound C20-[5]-ladderanes; one of these positions were occupied by C20-[3]-, C18-[5]- and C18-[3]-ladderane ether or ester, in one case also a C22-[5]-ladderane ester, or a straight or branched alkyl chain with 14–16 carbon atoms. The polar head groups detected were either choline, ethanolamine or glycerol, mostly choline except for "*Ca.* Scalindua" enrichment (Fig. 4). Finally, the following triterpenoids were identified in anammox enrichments (ordered from lowest to highest abundance): squalene, bacteriohopanetetrol, and bacteriohopanetetrol cyclitol ether (for more details refer to Kouba et al. [49]).

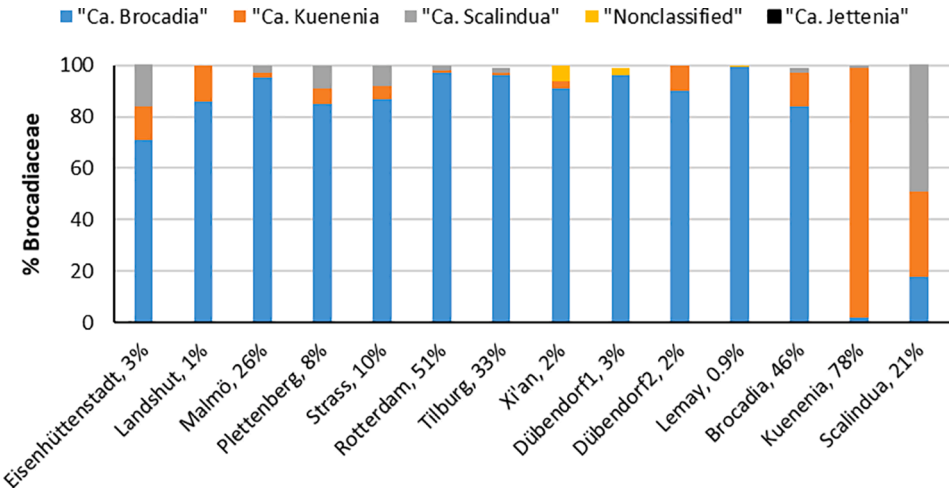

Fig. 1. Relative abundances of all operational taxonomic units (OTUs) detected for each anammox genus within the anammox bacterial family of *Brocadiaceae*. The percentage of *Brocadiaceae* within the kingdom of Bacteria, as estimated by 16S rRNA gene-based amplicon sequencing analysis, is shown next to the name of the source of anammox culture. Aside from two enrichments, all biomasses were dominated by "*Ca.* Brocadia".

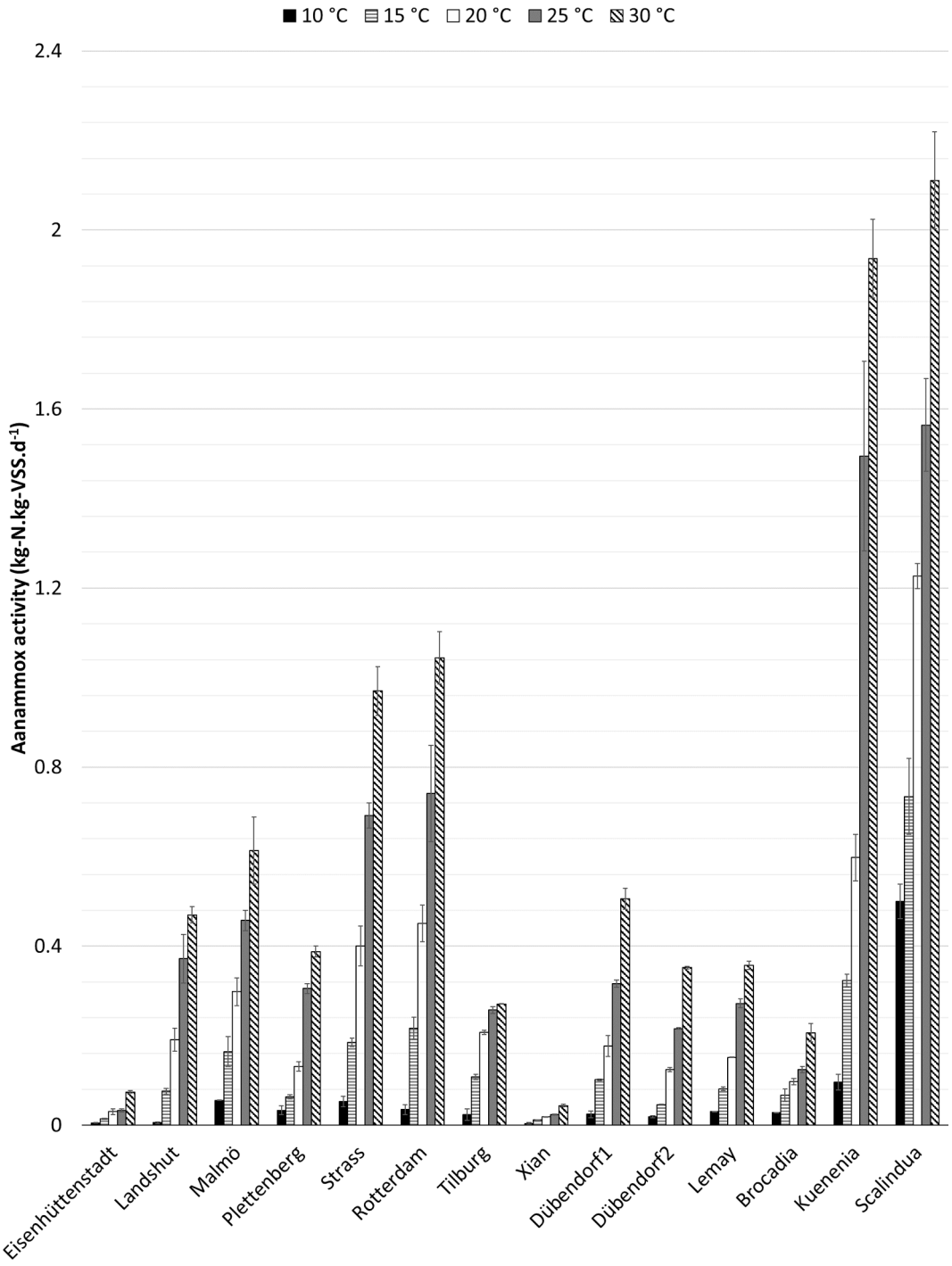

Fig. 3. Effect of temperature on the specific anammox activity of multiple anammox biomasses from pilot and full scale installations and from laboratory enrichments ("*Ca.* Brocadia", "*Ca.* Kuenenia", "*Ca.* Scalindua"). "*Ca.* Scalindua" had the highest activity at 10–20 °C of all cultures. Snow-flake – psychrophilic cultures.

4. Discussion

4.1. Cultivation temperature impacts Ea10-15

To date, literature provided only limited evidence on the long-term effect of low cultivation temperatures on the performance of originally mesophilic anammox cultures [10,23]. Our results in combination with literature data show that long-term cultivation at low temperature has a positive impact on the Ea of anammox conversion in the range of 10 to 15 °C, as cultures operated at lower temperatures typically had lower Ea values (Fig. 5). Psychrophilic cultures in this study as well as the literature had been operated under this regime for 8 months to 5 years. Across this timeframe, the operation length seemed neither to affect the anammox *Ea* from 10 to 30 °C nor the activity from 10 to 20 °C. Thus, within these ranges, exposure to psychrophilic conditions for tens of months seems to be sufficient for inducing such adaptive response.

In terms of absolute activity at 10–15 °C, mixed cultures operated in the psychrophilic regime (not "*Ca.* Scalindua" enrichment) were not the most active (Fig. 3). Thus, our data do not confirm the hypothesis emitted by Lotti et al. [17] that operation under psychrophilic temperatures improves the maximum anammox activities beyond values achieved by mesophilic cultures.

Table 2
Activation energies (Ea) of anammox cultures reported in the literature and determined in this study. The cultures are ranked from lowest to highest Ea under 10–15 °C, *i.e.*, anammox cultures most adapted to low temperatures are on top.

Reference	Character/culture origin, operational temperature/dominant anammox genera/species	t (°C) range for Ea	Ea (kJ. mol^{-1})
This study	**"*Ca.* Scalindua" enrichment, 20 °C**	**10–30**	**51 ± 16**
[35]	sediments, Greenland	−2–13	51
[36]	Expanded bed reactor combining anammox (granules) and hydroxyapatite crystallization, "*Ca.* Kuenenia", 15 °C	10–35	56
[37]	sediments, Skagerrak, North Sea	6.5–37	61
[38]	biofilm, granules, "*Ca.* Kuenenia Stuttgartiensis", 30 °C, two-stage	10–40	63
[39]	granules, "*Ca.* Brocadia fulgida", 30 °C, two-stage	10–30	64
[10]	free cells, MBR, 20–30 °C, "*Ca.* Brocadia fulgida", two-stage	15–30	64 ± 28
[15]	flocculent sludge, "*Ca.* Brocadia fulgida", 10 °C, two-stage	5–17	66
This study	**enrichment, "*Ca.* Brocadia anammoxidans"**	**10–30**	**71 ± 36**
[40]	granules, biofilm, "*Ca.* Brocadia fulgida", 20 °C, two-stage	10–20	72
[41]	biofilm, non-identified anammox or planktomycete bacteria, 20 °C, two-stage	10–30	73
[36]	Expanded bed reactor combining anammox (granules) and hydroxyapatite crystallization, "*Ca.* Kuenenia", 35 °C	10–35	76
[42]	Expanded bed reactor combining anammox (granules) with hydroxyapatite granules, 7 °C, "*Ca.* Kuenenia"	4–35	78
This study	**Eisenhüttenstadt – suspension/granules, full scale, DEMON®, 21 °C, "*Ca.* Brocadia"**	**10–30**	**79 ± 13**
This study	**Xi'an, main stream of WWTP, full scale, "*Ca.* Brocadia"**	**10–30**	**83 ± 41**
This study	**enrichment, "*Ca.* Kuenenia stuttgartiensis", 30 °C**	**10–30**	**83 ± 42**
This study	**Lemay – moving bed biofilm reactor, K5 carriers, Veolia Research and Innovation, 1.6 m^3 reactor, 20 °C, "*Ca.* Brocadia"**	**10–30**	**86 ± 3**
[41]	granules, "*Ca.* Kuenenia Stuttgartiensis", 35 °C, two-stage	10–35	89
[43]	encapsulated biomass, "*Ca.* Kuenenia stuttgartiensis", "*Ca.* Brocadia anammoxidans", Planctomycetes KSU-1 (AB057453), two-stage	6–28 28–37	93 33
This study	**Plettenberg – granules, full scale, DEMON®, 30 °C, one-stage, "*Ca.* Brocadia"**	**10–30**	**93 ± 14**
This study	**Dübendorf2 – moving bed biofilm reactor (K5, Anox Kaldnes), 10 °C, "*Ca.* Brocadia"**	**10–30**	**104 ± 32**
[41]	biofilm, "*Ca.* Kuenenia stuttgartiensis", "*Ca.* Brocadia caroliniensis", "*Ca.* Brocadia fulgida" and other planctomycete bacteria, 20 °C, two-stage	10–25	108
[17]	granules, 10 °C, "*Ca.* Brocadia sinica", one-stage	10–30	110
[17]	IC, full-scale, granules, "*Ca.* Brocadia fulgida", 30–35 °C	10–30	117
[39]	flocs, "*Ca.* Brocadia caroliniensis", 30 °C, two-stage	10–30	124
[44]	Immobilized filter, "*Ca.* Brocadia" a "*Ca.* Kuenenia", 8.5—32 °C	10–15 15–30	139 59
[17]	SBR (granules, 20 °C, "*Ca.* Brocadia fulgida"), one-stage	10–15 15–30	140 52.3
This study	**Malmö – moving bed biofilm reactor, K5 carriers, full scale, Anox Kaldnes, 30 °C, two-stage, "*Ca.* Brocadia"**	**10–15** **15–30**	**150±10** **63±12**
[45]	granules, "*Ca.* Kuenenia Stuttgartiensis", two-stage	10–20 20–33	153 9.4
This study	**Strass – granules/flocs, full scale, DEMON®, 30 °C, one-stage, "*Ca.* Brocadia"**	**10–15** **15–30**	**170±50** **80±7**
This study	**Dübendorf1 – biofilm on MBBR, carriers Wabag Fluopur®, 14 °C, pilot-scale, one-stage, "*Ca.* Brocadia"**	**10–15** **15–30**	**193** **78±7**
[17]	airlift, granules, "*Ca.* Brocadia sinica", 30–35 °C	10–15 15–30	204 60
[46]	Fluidized bed membrane reactor, "*Ca.* Brocadia fulgida", "*Ca.* Anammoxoglobulus propionicus", "*Ca.* Kuenenia", "*Ca.* Brocadia sinica", a – 25 °C; b – 35 °C	a:15–25 b:20–35	72 5.8
This study	**Tilburg – granules, full scale, ANAMMOX®, 37 °C, one-stage "*Ca.* Brocadia"**	**10–15** **15–20** **20–25** **25–30**	**207** **92** **31** **8**
[47]	aggregate biomass, 32–33 °C,	20–43	70
This study	**Rotterdam – granules, full-scale, two-stage, ANAMMOX®, 37 °C, "*Ca.* Brocadia"**	**10–15** **15–30**	**248** **76±26**
[25]	SBR, granules, "*Ca.* Kuenenia", 25 °C	15–21	93–94
[17]	enrichment in MBR, free cells, "*Ca.* Brocadia fulgida", 30 °C	10–15 15–30	325 94
This study	**Landshut – flocs, full scale, TERRAMOX®, 32 °C, two-stage, "*Ca.* Brocadia"**	**10–15** **15–30**	**360** **±140** **89±20**
[48]	SBR, granules, "*Ca.* Brocadia", 30 °C	10–15 15–40	437 50.5

*unclear detemination of Ea, arguable linear fit for biofilm (Fig. 1 in [38]; +calculated by the authors of this study based on data published in [17].

4.2. Implications for mathematical modelling of anammox activation energies (Ea)

Most authors view 15 °C as a breaking point under which anammox bacteria may be more negatively affected by temperature [11]. This can be interpreted as if the effect of temperature on anammox activity as expressed by *Ea* is consistent throughout two ranges: 10–15 °C (hereafter referred to as Ea10-15) and 15–30 °C (Ea15-30). We show that this was true only for some mesophilic cultures. Therefore, to assume a single Ea10-15 for all mesophilic cultures is short-sighted. For modelling purposes, Ea10-15 specific to the anammox biomass used should be obtained experimentally. However, almost all psychrophilic anammox cultures in this study could be described by a single *Ea* for the whole range from 10 to 30 °C (Table 2), showing that there is no intrinsic

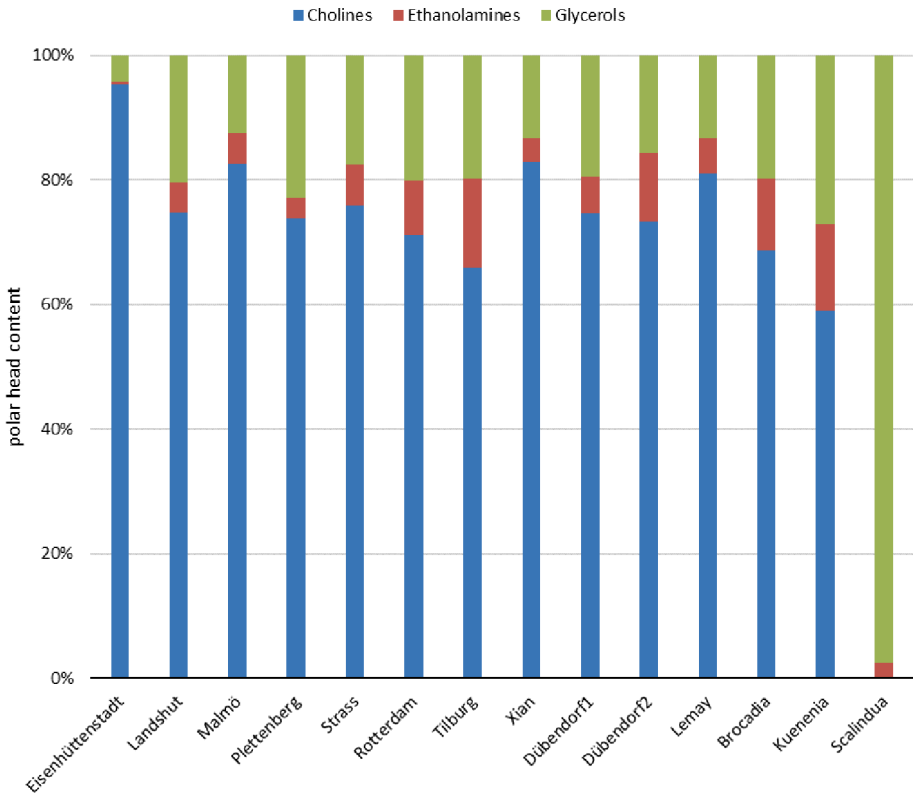

Fig. 4. Polar head content of ladderane phospholipids in various anammox cultures. The ladderane phospholipid headgroup was mostly phosphatidylcholine in all cultures except of "*Ca.* Scalindua" enrichment.

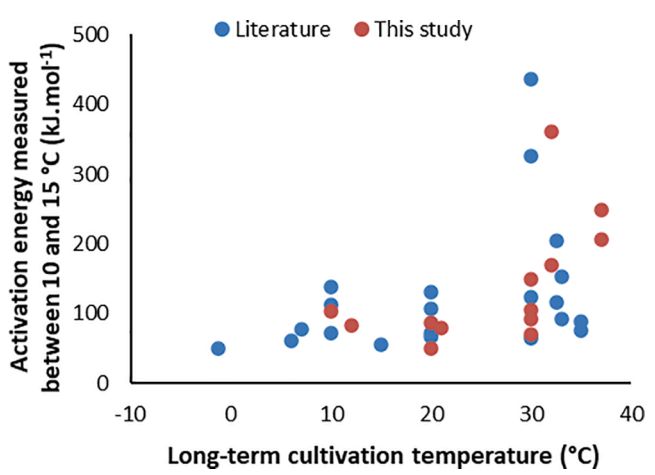

Fig. 5. Long-term cultivation temperature of anammox biomasses and their activation energy between 10 and 15 °C, summarizing our and literature data. Activation energy at 10–15 °C of anammox biomasses cultivated in the mesophilic temperatures (30–37 °C) was in the range of 64–360 kJ.mol.$^{-1}$, whereas the cultures operated at low temperatures up to 21 °C had substantially lower Ea (51–131 kJ.mol^{-1}).

difference in temperature response below 15 °C.

In our experiments at 15–30 °C, the anammox cultures had an *Ea* of 79±18 kJ.mol^{-1} (average ± standard deviation), which was identical to the average value 79±31 kJ.mol^{-1} calculated from the literature data (Table 2). In comparison, the standard *Ea* for nitrification is similar 70 kJ.mol^{-1} [50]. The *Ea* range of mesophilic cultures at 10–15 °C was 71–360 kJ.mol^{-1}.

4.3. Anammox performance of various genera: potential of marine "Ca. Scalindua"

The marine enrichment of "*Ca.* Scalindua" displayed the highest specific anammox activity at 10–20 °C. This was shown only in activities expressed per g-VSS (Fig. 3). In the literature, "*Ca.* Scalindua" is an organism that has mainly been recovered from marine environments [51,52]. We detected "*Ca.* Scalindua" also in biomasses treating supposedly less saline pre-treated sewage and reject water, but in relatively lower abundance compared to other genera, and those biomasses were not as active as the marine one. This study is the first to highlight such exceptional metabolic performance under 10–20 °C. Thus, we indicate that implementation of "*Ca.* Scalindua" to N-removal processes treating cold marine streams, and potentially cold streams in general, can be extremely beneficial. This should be considered when choosing appropriate inoculum and reactor design, however challenging.

The exceptional performance of "*Ca.* Scalindua" under low temperatures raises inquiry into their membrane physiology. As described in more detail in Kouba et al. [49], the [3]-ladderanes (Ea15-30 = 51 kJ.mol^{-1}, C20/(C18 + C20) [3]-ladderane ether = 0.11; Fig. 6) and [5]-ladderane esters alkyl moieties in "*Ca.* Scalindua" ladderane phospholipids were exceptionally short, having the highest relative content of C18 compared to C20 alkyls. Reduced length of phospholipid alkyls is typical in cold-adapted bacteria, as a narrower membrane maintains its fluidity at a lower temperature, thus maintaining the function of membrane proteins [53]. "*Ca.* Scalindua" also had an exceptionally high content of bacteriohopanoids, that were hypothesized to maintain membrane viscosity under cold stress [54]. In sum, the membrane of "*Ca.* Scalindua" appears exceptionally suitable to low temperatures.

Conversely, the polar headgroup of ladderane phospholipids was almost exclusively phosphatidylglycerol, while other cultures contained mostly phosphatidylcholine. Phosphatidylglycerol is smaller and thus less disruptive to the membrane packing which makes it less suitable for

low temperatures [53]. This is inconsistent with the hypothesis that larger polar headgroups benefit membrane fluidity and thus enhance anammox activity under low-temperature conditions. Also, it is inconsistent with "*Ca.* Scalindua" analyzed by Rattray et al. [34] that detected also other polar headgroups; such distinction could hypothetically be attributed to the presence of different species.

Further, the adaptation to a saline environment may induce the predisposition of "*Ca.* Scalindua" to low temperatures. Various species of "*Ca.* Scalindua" were reported to be adapted to low temperatures by 'salt-in' strategy, and early evidence points also to one species synthesizing compatible solutes (*i.e.*, glutamate, glutamine, proline) [53]. The freezing point of aqueous solution decreases at elevated content of salts or these solutes, thus appearing as another mechanism making "*Ca.* Scalindua" especially cold-adapted.

4.4. Ladderane composition and anammox performance

Generally, in bacterial membranes, reducing temperature arranges lipids into more compact formations, thus reducing the membrane fluidity/flexibility. However, membrane flexibility is crucial for the function of membrane proteins. Thus, bacteria maintain their membrane fluidity by synthesizing shorter, branched and unsaturated alkyl chains, larger polar headgroups and more terpenoids [53]. However, the data on anammox membrane composition and anammox performance under low temperatures lacked, as the only closely related study restricted itself to the suggestion that cold anammox had more C18 compared to C20 [5]-ladderane esters, while the performance of such cultures remained untested [30].

In our study, the crucial membrane features relating to anammox *Ea* at 10–30 °C were (i) the length of [3]-ladderanes and (ii) polar headgroup size. First, anammox cultures with higher content of C18 compared to C20 [3]-ladderanes chains had lower *Ea* at 15–30 °C (Fig. 6). Interestingly, this did not appear to involve ladderanes with five concatenated cyclobutane rings, and not only ladderane esters as in Rattray et al. [30] and Kouba et al. [31], but also ethers. Second, in mixed cultures dominated by "*Ca.* Brocadia", those with lower *Ea* at 10–15 °C contained more phosphatidylcholine and less phosphatidylglycerol (Fig. 6). As choline is larger than glycerol or ethanolamine, it is thought to introduce additional disruption into membrane lipid packing [53]. Similarly, a larger polar phospholipid head group has been shown to maintain membrane fluidity in barophilic bacteria [55]. Finally, some individual ladderane lipids such as IIId at Fig. 7 correlated extremely well to the *Ea* at 15–30 °C, respectively, providing preliminary information that certain individual anammox lipids could be utilized as biomarkers of anammox performance at low temperatures. Overall, we provide the first evidence on the relation between ladderane phospholipid composition and anammox activity.

Further, we detected higher content of monoalkyl ether phospholipids in cultures with higher *Ea* at 10–15 °C (Fig. S2). However, monoalkyl ether phospholipids and monoether could be not only one of the final membrane building blocks, but also perhaps an intermediate of lipid biosynthesis, or a by-product of cell lysis. Importantly, hydrocarbons and polar head attached to the glycerol backbone can be cleaved off by a phospholipase enzyme [56].

Anammox cultures are known to contain triterpenoids such as various bacteriohopanoids [57] and squalene [34], and these were suggested to play a role in maintaining membrane fluidity [54]. In contrast to ladderane lipids, these triterpenoids are not exclusively synthesized by anammox bacteria, so we analysed them only in highly enriched cultures, detecting bacteriohopanetetrol cyclitol ether, bacteriohopanetetrol and squalene in enrichments of "*Ca.* Scalindua", "*Ca.* Brocadia", and "*Ca.* Kuenenia". Their signal intensity was correlated to *Ea* at 10–30 °C, suggesting that their abundance may also contribute to maintaining anammox membrane fluidity at low temperatures.

The importance of shorter ladderane ethers, not only esters, suggests that future studies on anammox adaptation to different temperatures should not restrict their focus to any particular ladderane group. Rather, we advise a thorough assessment of the whole ladderane content, including ether-bound ladderane alkyl moieties.

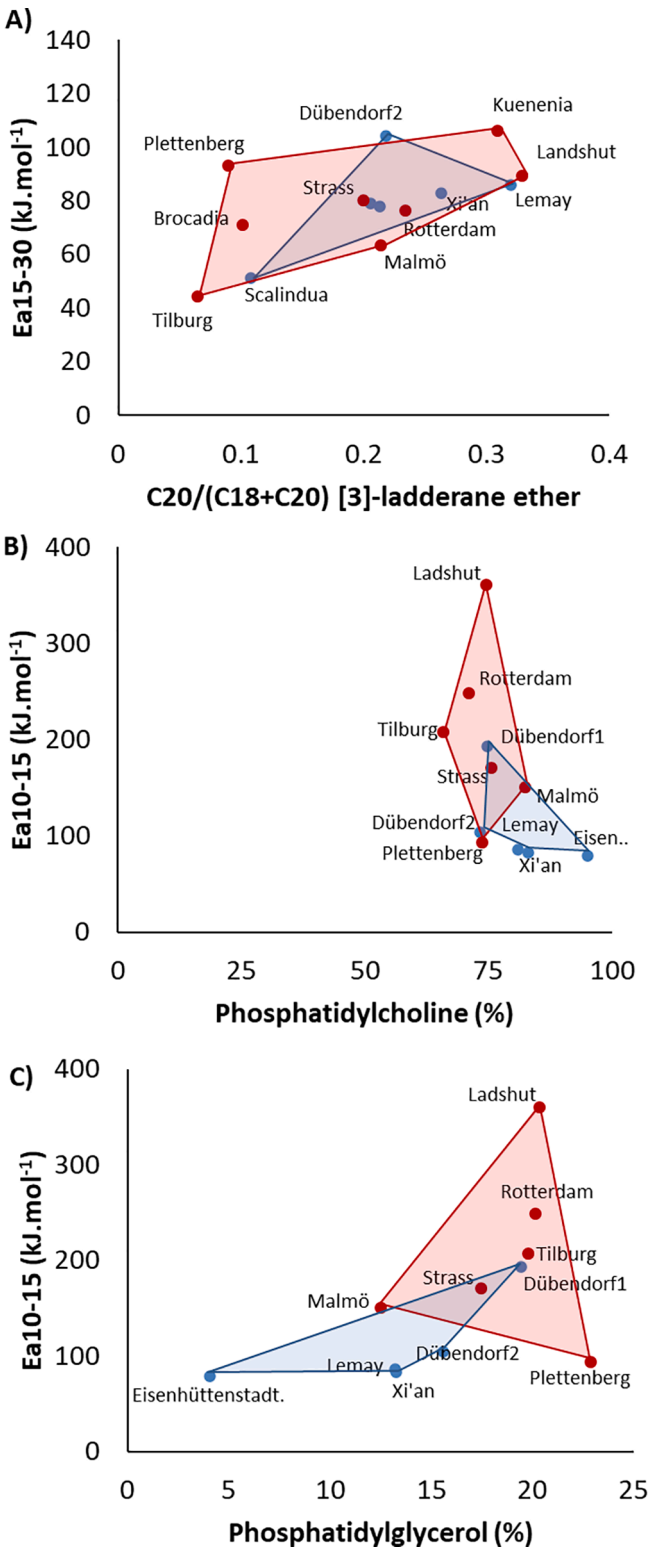

Fig. 6. Relation of anammox cultures ladderane phospholipid composition and activation energy between 15 and 30 °C and 10–15 °C, summarizing our data. Panel A contains all cultures. In B and C, only mixed cultures dominated by "*Ca.* Brocadia" were included. Red – mesophilic cultures, blue – psychrophilic cultures.

4.5. Biomass growth mode and PN/A configuration

In all cultures, the biomass growth mode did not seem to correlate with anammox performance, with one exception. Free-cell anammox cultures consistently exhibited the highest maximum specific activity at 25–30 °C (Fig. S2). This is probably because planktonic cultures contain fewer non-anammox populations and less inactive organic matter (e.g., extracellular polymeric substances), both of which make them more active overall.

In the mixed psychrophilic cultures, the Lemay biomass (Anox-Kaldnes K5 carriers) was six-fold more active than in the Xi'an (MBBR, main-stream) and Eisenhüttenstadt (granules/flocs, side-stream) cultures. We suspect th;at these less active cultures were exposed to more organic carbon in the municipal wastewater and flocculating agent, respectively. Thus, we hypothesize that their lower anammox activity may be due to stronger competition for nitrite from denitrifiers and that biomass growth mode may not have been the main factor. Despite the elevated presence of denitrifiers in Xi'an biomass, our activity assays with this culture evaluated the stoichiometric ratio of nitrite to ammonium removal of 1.29 (average). This compares favorably to 1.32 given by Strous et al. [33] and can be explained by the lack of organic carbon addition during the assays. Nevertheless, more detailed anammox stoichiometry involves the utilization of nitrogenous species for amino acid biosynthesis and biomass growth. As these processes can be affected by the exposure of anammox bacteria to low temperatures [26], their potential impact on anammox stoichiometry deserves further attention.

Nevertheless, certain biomass growth mode properties, such as biofilm depth, may impact substrate or toxin uptake rate, thereby affecting anammox performance.

"*Ca.* Scalindua", the anammox genus highly promising for cold adaptation, can be enriched from marine sediments not only in the form of flocs but also granules [58]; it can be combined with partial denitrification [59] and utilize various reactor set-ups as reviewed by Yokota et al. [60].

4.6. PN/A configuration

Lotti et al. [17] hypothesized that anammox growing in one-stage PN/A may become adapted to oxygen inhibition, and that the mechanism for maintaining homeostasis under oxygen inhibition may also alleviate cold stress. In this study, we did not detect a correlation between one or two-stage PN/A operation and anammox cultures performance at low temperature.

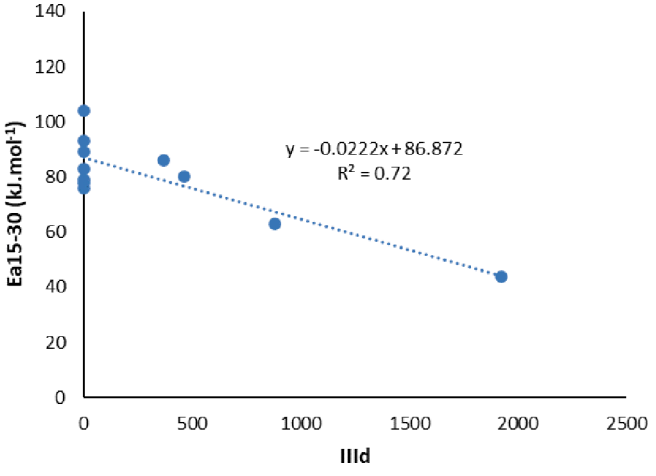

Fig. 7. The relation between signal of individual ladderane lipids (counts per second) in mixed anammox cultures (no enrichments included) and anammox process temperature coefficient between 15 and 30 °C (activation energy Ea15-30).

4.7. Anammox temperature optima

The temperature optimum of all the tested anammox cultures was ≥30 °C, including the long-term psychrophilic enrichments. In contrast, enrichments from arctic conditions had a much lower average optima of 12 °C [35]. Because some long-term lab-scale experiments at 10–20 °C observed temperature optima up to 25–30 °C, which is less than typically reported optima of 35–38 °C [15,41,61], we wondered whether longer exposure to a psychrophilic regime might reduce the optima even further. But this was not the case. Our psychrophilic cultures came from multi-year full-scale and lab-scale operations and contained a variety of anammox populations, including marine "*Ca.* Scalindua" enrichment, so their high-temperature optima may be related to other factors, such as even lower cultivation temperatures (>10 °C, freeze–thaw cycles), or specific ladderane composition.

4.8. Implications for development of anammox process

We provide an exhaustive overview of temperature coefficients (*Ea*) useful for the modelling of anammox activity, and thus anammox reactors, at mesophilic-to-psychrophilic temperatures. We also show that mesophilic anammox biomasses have a broad diversity of *Ea* as well as that anammox genera can have highly distinct aptitude for low temperatures. Concerning the latter, marine "*Ca.* Scalindua" seems to have extremely beneficial predispositions towards low temperatures which is particularly relevant to municipalities using sea water for toilet flushing [62] or WWTPs accepting saline industrial wastewater; this can be realized in various set-ups [60]. Overall, our data can also be used for the design as well as selecting inoculum of psychrophilic anammox installations. This is particularly useful for WWTPs without mesophilic anammox treating reject water that allows for a seamless inoculation of colder main stream of wastewater, e.g., for effluent polishing [63]. Lastly, we establish a link between anammox activity and ladderane composition, which will stimulate use of anammox membrane lipids (ladderanes, bacteriohopanoids) as biomarkers of anammox cultures adaptation to low temperatures that could be of high practical use to operators [26].

5. Conclusions

This study provides preliminary evidence that the performance of anammox biomasses at low temperatures can be related not only to their cultivation temperature but also their unique membrane constituents. Most importantly, we observed that anammox performance at low temperatures was closely related to ladderanes and bacteriohopanoids. Thus, these anammox membrane components appear to be key aspects of cold anammox physiology. Furthermore, long-term operation under psychrophilic conditions, while not always necessarily enhancing absolute activity, consistently improved the anammox temperature coefficient at 10–15 °C (85±49 kJ.mol^{-1}, median ± standard deviation). The *Ea* of mesophilic cultures at 10–15 °C are highly diverse (160±95 kJ.mol^{-1}), stressing the need for individual assessment of such cultures when modelling their activity. In addition, we showed the exceptional performance of a cold-adapted enrichment of marine "*Ca.* Scalindua", highlighting its potential for nitrogen removal from cold and more saline streams, which is crucial when choosing the most appropriate inoculum and reactor set-up. Collectively, these findings, based on a complex assessment of metabolic activities, microbial community structure and membrane lipids in 14 anammox cultures and on a comprehensive literature survey, provide essential knowledge for the more accurate modelling for instance by the inclusion of measured *Ea*, inoculation and set-up of anammox reactors, in particular for the main stream of WWTP.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2022.136712.

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