VUPD (ID 88.2016): Energi- og emmissionsoptimering ved anvendelse af deammonificationsprocesser i hoved- og sidestrøm

Final report from Aarhus University
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In the framework of the VUDP project *Energi- og emmissionsoptimering ved anvendelse af deammonificationsprocesser i hoved- og sidestrøm*, Aarhus University **(1)** developed a highly specific method to measure anammox and denitrification in activated sludge of wastewater treatment plants (WWTP) at different temperatures, and **(2)** applied the method to measure anammox and denitrification rates in the DEMON (anammox sidestream) and biological tanks (mainstream) of Marselisborg WWTP (Aarhus, Denmark) at different temperatures to make statements on the contribution of anammox to the N-removal processes at Marselisborg WWTP. Main focus was on the potential of anammox in the mainstream.

In addition, Aarhus University **(3a)** organized *in situ* measurements to look for a potential nitrite shunt in the biological tanks of Marselisborg WWTP and **(3b)** analyzed the obtained datasets.

(1) Method to measure anammox and denitrification in activated sludge of a wastewater treatment plants at different temperatures

Aarhus University measured anammox and denitrification (Thamdrup, 2012) with ^{15}N-labelling experiments (stable isotopes) in untreated, freshly sampled activated sludge from the main- and sidestream of Marselisborg WWTP. Labelling experiments are highly specific and sensitive (detection limit in the nanomolar range). For anammox rate measurements, ^{15}N-labelled NH_4^+ was used, which becomes converted to ^{29}N-labelled N_2 by anammox bacteria; for denitrification rate measurements, ^{15}N-labelled NO_2^- was used, which becomes converted to ^{30}N-labelled N_2 by denitrifying bacteria. Labelled N_2 was measured via gas chromatography - isotope ratio mass spectrometry (for details see Holtappels et al., 2011).

In detail, the experiments on anammox and denitrification rates at different temperatures were performed as follows:

Sampling and experimental set up for ^{15}N-labelling experiments

For anammox and denitrification rate measurements, activated sludge samples were collected from approximately 0.5 - 1 m water depth in the DEMON (anammox sidestream) and biological tanks (mainstream) at Marselisborg WWTP (Aarhus, Denmark) in Mai 2018.

Rate measurements were done at 10˚C, 20˚C and 30˚C. For each temperature experiment, 400 mL activated sludge was transferred into a 500 mL incubation bottle (Duran) which was placed in a temperature-controlled water bath (**Figure 1**). The sludge was gently mixed during the entire incubation using a magnetic stirrer below the water bath. The incubation bottle was sealed with a gas tight rubber stopper containing inflow- and outflow ports to introduce anoxia with He, and to connect a 50 mL glass syringe for pressure adjustments (Fortuna Optima, wet glass piston prevents gas loss). The total headspace volume was 240 mL at t0 (bottle plus pressure adjustment syringe). Anoxia was

confirmed via O_2 measurements with an optode inside the closed bottle using a bare optical fiber (PyroScience).

Start of ^{15}N-labelling experiments and sub-sampling in the laboratory

To start the ^{15}N-labelling experiments for the anammox rate measurements, 1mM $^{15}NH_4^+$ (99 atom %, Cambridge Isotope Laboratories) and 200 µM $^{14}NO_2^-$ (to prevent substrate limitation) were added to the activated sludge from the DEMON and biological tanks, respectively. To start the ^{15}N-labelling experiments for the denitrification rate measurements, 200 µM $^{15}NO_2^-$ (98 atom %, Cambridge Isotope Laboratories) was added to the activated sludge from DEMON and biological tanks, respectively.

For ^{15}N-labeled N_2 determination, 5 ml headspace from each incubation bottle was taken with a gas tight syringe (SGE Syringe) through the rubber stopper directly after adding ^{15}N-label (t0) and thereafter every 10 minutes for a total of 1 hour (t1-t6). One mL of the headspace in the sampling syringe was discarded and the remaining 4 mL were transferred to 6 mL exetainers (Labco), prefilled with He-flushed 0.5% $ZnCl_2$ solution, while the excess solution was expelled. Gas samples were stored upside down until analysis. Prior each sampling the headspace of the pressure adjustment syringe was gently mixed with the headspace of the incubation bottle by moving the piston of the pressure adjustment syringe several times a few millimetres up and down. After each sampling the piston of the pressure adjustment syringe lowered automatically and negative pressure was thus prevented.

To determine the mole fractions of $^{15}NH_4^+/^{14}NH_4^+$ and $^{15}NO_2^-/^{14}NO_2^-$ label, respectively, 2 mL subsamples for NH_4^+ and NO_2^- determinations were taken with a syringe through the rubber stopper of the incubation bottle directly before and after adding of ^{15}N-label. Samples were sterile filtered (0.22 µm) and frozen (-20˚C) until further analyses.

For measurements of suspend solids (SS), i.e. total suspend solids (TSS) and volatile suspended solids (VSS), per volume of activated sludge, the sludge was viciously mixed after the experiment, and 40 mL sludge from each incubation bottle was transferred into pre-weight porcelain vials which were heated at 100˚C for 3 days (TSS) and thereafter at 500˚C for 24 h (VSS = TSS - inorganic compounds).

Analyses and calculations for ^{15}N-labelling experiments

The isotopic composition of N_2 was determined via gas chromatography - isotope ratio mass spectrometry (GC-IRMS, SerCom) for calculation of the accumulation of ^{15}N labeled N_2 as $^{29}N_2$ ($^{14}N^{15}N$) and $^{30}N_2$ ($^{15}N^{15}N$). Ammonium was analysed with the salicylate method (Bower and Holm-Hansen, 1980), and NO_2^- was analysed with the Griess reaction using the protocol by García-Robledo et al. (2014). The mole fractions of the ^{15}N-label in the respective NH_4^+ or NO_2^- pools ($F_{N\text{-substrate}}$) were calculated according to Holtappels et al. (2011):

$$F_{N\text{-substrate}} = {}^{15}\text{N-substrate}/({}^{15}\text{N-substrate} + {}^{14}\text{N-substrate})$$

The production of N_2 through anammox (A) was estimated from the $^{29}N_2$ production using the following expression:

$$A = {}^{29}N_2/F_{NH4}$$

and he production of N_2 through denitrification (D) was estimated from the $^{30}N_2$ production using the following expression (Thamdrup and Dalsgaard, 2000, 2002); Thamdrup et al., 2006):

$$D = {}^{30}N_2/(F_{NO2})^2$$

Anammox and denitrification rates (± standard errors) were calculated from the linear regression of the produced N_2 per time ($n \geq 4$ timepoints) and were related to TSS and VSS respectively. The decreasing headspace volume (see above) and the N_2 dissolved in the sludge (bunsen coefficient: $N_{2water}/N_{2gas} = 0.015$) were taken into account for all calculations.

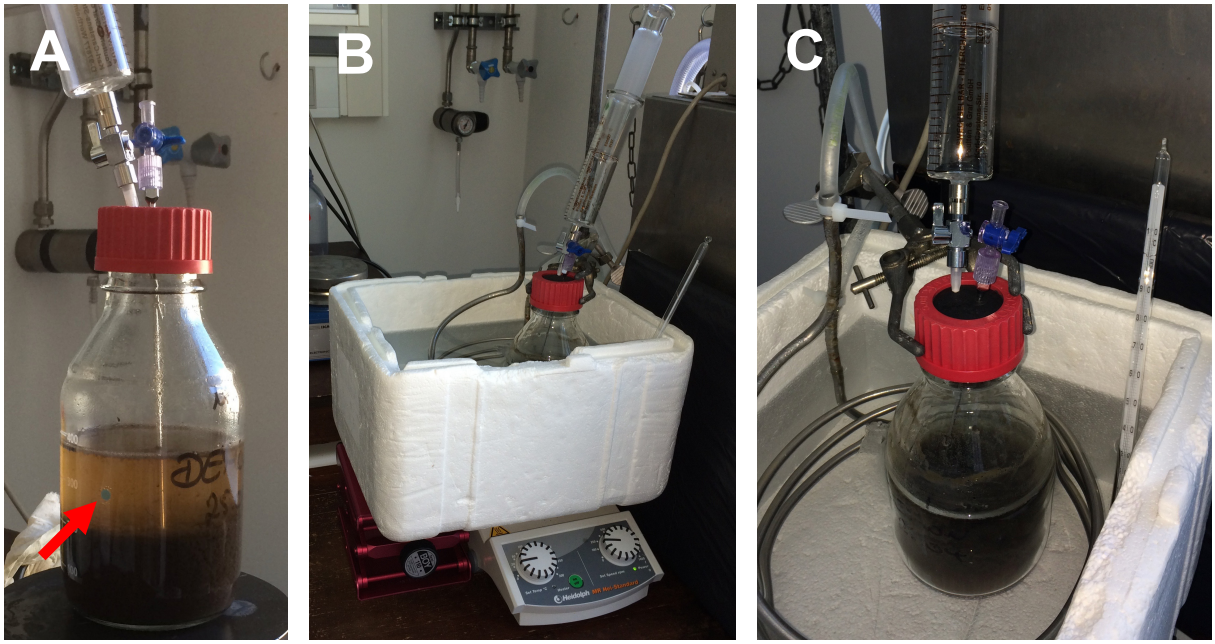


Figure 1: Incubation bottle with activated sludge. Pressure adjustment syringe is connected to the bottle via a port. A) Full view on incubation bottle; red arrow points to the oxygen optode which is placed at the inner wall of the bottle. B) Side view on incubation bottle in temperature bath. A magnetic stirrer for gentle mixing of the sludge is placed below the water bath. C) Top view on incubation bottle in temperature bath.

(2) Results of anammox and denitrification rate measurements in the main- and sitestream of Marselisborg WWTP at different temperatures

Anammox and denitrification rates in the DEMON and biological tanks show a clear temperature dependence with highest rates at 30˚C, medium rates at 20˚C, and lowest rates at 10˚C (**Table 1**).

The temperature dependence of the N-conversion rates especially affects anammox. In the DEMON, anammox rates are 7.7 ± 0.6 and 72.9 ± 7.2 µmol $gVSS^{-1}$ h^{-1} at 10˚C and 30˚C, respectively (mean ± standard error), and thus increased nearly 10 times at higher temperature. Anammox rates also show a strong temperature dependence in the biological tanks. Here, the rates are 0.011 ± 0.002 and 0.082 ± 0.004 µmol $gVSS^{-1}$ h^{-1} at 10˚C and 30˚C, respectively. Also, denitrification shows a distinct temperature dependence. These rates increased from 1.3 ± 0.1 to 2.7 ± 0.2 µmol $gVSS^{-1}$ h^{-1} at 10˚C and 30˚C, respectively, in the DEMON, and from 1.166 ± 0.066 to 6.331 ± 0.410 µmol $gVSS^{-1}$ h^{-1} at 10˚C and 30˚C, respectively, in the biological tanks.

Table 1: Anammox and denitrification rates in the DEMON and biological tank. Rate (± standard error).

	DEMON				**Biological tank**			
	Anammox (µmol N_2-N gSS^{-1} h^{-1})		**Denitrification (µmol N_2-N gSS^{-1} h^{-1})**		**Anammox (µmol N_2-N gSS^{-1} h^{-1})**		**Denitrification (µmol N_2-N gSS^{-1} h^{-1})**	
	TSS	**VSS**	**TSS**	**VSS**	**TSS**	**VSS**	**TSS**	**VSS**
10°C	3.926 (0.307)	7.658 (0.599)	0.656 (0.054)	1.305 (0.108)	0.006 (0.001)	0.011 (0.002)	0.692 (0.039)	1.166 (0.066)
20°C	14.469 (0.821)	30.458 (1.729)	1.050 (0.028)	2.214 (0.059)	0.016 (0.001)	0.027 (0.002)	2.771 (0.098)	4.739 (0.167)
30°C	34.556 (3.423)	72.924 (7.224)	1.273 (0.081)	2.696 (0.172)	0.049 (0.002)	0.082 (0.004)	3.746 (0.242)	6.331 (0.410)

As expected, anammox is the dominant dissimilatory N-removal pathway in the DEMON and denitrification is the dominat dissimilatory N-removal pathway in the biological tanks. At 30˚C, which is in the range of the *in situ* temperature of the DEMON (30-35˚C), anammox rates are 30 times higher than denitrification rates. At 10˚C, which covers the *in situ* temperature of the biological tanks during the seasons in Denmark except for the summer, denitrification rates are 115 times higher than anammox rates, and at 20˚C, which covers the *in situ* temperature during summer, denitrification rates are 173 times higher than annamox rates in the biological tanks. Anammox thus contributes with about 1% to the dissimilatory N-conversions in the biological tanks (**Figure 2**).

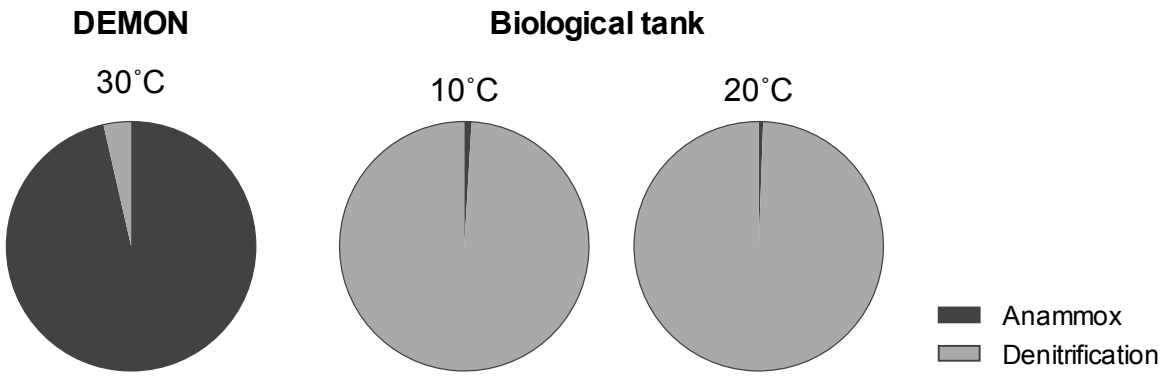


Figure 2: Relative contribution of anammox and denitrification in the DEMON and biological tanks. For the DEMON, the data are shown for 30˚C, which is in the range of the *in situ* temperature, and for the biological tanks, the data are shown for 10˚C and 20˚C, which reflects the *in situ* temperature of the biological tanks for the winter and summer months, respectively.

To exclude to possibility that anammox rates are higher in the bottom sludge than in the surface sludge, e.g. because the sedimentation rate of anammox granules might me higher than the mixing of the water column, we did additional ^{15}N-labelling experiments with activated sludge which was sampled in parallel from 0.5-1 m water depth and 3-4 m water depth in the main- and sitestream of Marselisborg WWTP. For the DEMON the bottom and surface sludge were incubated at 30˚C and for the biological tank, the sludge was incubated at 20˚C. The results confirmed the anammox rates measured in the temperature experiments (**Table 1**) and don't give any indication that anammox rates are higher in the bottom of the tanks.

(3a) *In situ* measurements to address the question on a potential nitrite shunt in the biological tanks of Marselisborg WWTP

Aarhus University organized a measurement campaign in the biological tanks of Marselisborg WWTP to get more information on a potential nitrite shunt. A nitrite shunt is a shortcut nitrogen removal in which NH_4^+ only becomes oxidized to NO_2^- during aeration (first step of nitrification) and the product NO_2^- is taken up directly for denitrification in hypoxic anoxic or conditions. A successful nitrite shunt is cost efficient for WWTPs because it saves energy for aeration during nitrification and lowers the carbon demand during denitrification (Soliman and Eldyasti, 2018).

In detail, the measurements on the nitrite shunt were performed as follows:

Measurements of oxygen dependent nitrogen conversions in the biological tank (mainstream)
Time series measurements of O_2, NO_2^-, NO_3^-, NH_4^+ and pH were done in the biological tank of Marselisborg WWTP close to, within and outside an aeration zone at 1.5 and 3-4 m water depth in August 2018. Date, time, temperature and pH during sampling is shown in **Table 2**.

O_2 and pH were measured *in situ* with a HQ40d Multi Meter (Hach). Samples for NO_2^-, NO_3^- and NH_4^+ were taken from the respective water depths with a pump, sterile filtered (0.22 µm),

immediately frozen and kept at -20˚C until further processing. NO_2^- and NO_3^- were analysed with an NOx analyser connected to a reaction chamber (CLD 66s plus a Liquid NO Setup; EcoPhysics) using the VCl_3 reduction method (Braman and Hendrix, 1989), and NH_4^+ was measured with the salicylate method (Bower and Holm-Hansen, 1980).

Table 2: Dates and real times for sampling sides (A) close to, (B) within, and (C) outside an aeration zone as well as temperature and pH (mean ±SD) during sampling of the biological tank.

Water depth (m)	Date	Time (CET) Side (A)	Time (CET) Side (B)	Time (CET) Side (C)	Temp. (˚C)	pH
1.5	14.08.18	10:38-12:02	13:09-14:49	15:11-16:33	19.0	6.7 (±0.1)
3-4	30.08.18	10:52-12:45	13:46-15:40	16:28-18:04	21.5	7.1 (±0.1)

(3b) Nitrite shunt analyses

The data analyses on the potential nitrite shunt in the biological tanks of Marselisborg WWTP show that a nitrite shunt can become largely excluded during the time of our sampling in August 2018.

The time series data of O_2, NH_4^+, NO_2^- and NO_3^- concentrations (**Figure 3**) are providing detailed information on the N-conversions during alternating aeration/non-aeration. Our negative statement on the nitrite shunt is mainly based on the fact that the NH_4^+ oxidation is largely mirrored by the production of NO_3^- (black diamonds and blue squares). If a nitrite shunt would play an important role, NH_4^+ would only become oxidized to NO_2^-, and NO_2^- might either accumulate for later denitrification in anoxic conditions, or immediately be taken up by e.g. simultaneous nitritation/denitrification. However, NO_2^- wouldn't become further oxidized to NO_3^-, as it is the case in our study.

To stress our statement on the nitrite shunt, we further used the time series data of NH_4^+, NO_2^- and NO_3^- during aeration to calculate the consumption and production rates, i.e. we did linear regressions of NH_4^+, NO_2^- and NO_3^- during the time of aeration and calculated the standard errors of the respective slopes (**Figure 4**). The bar below the zero line shows the consumption rate of NH_4^+ during aeration (dark grey) and the divided bar above the zero line shows the production rates of NO_2^- (light grey) and NO_3^- (middle grey). Consumption rates of NH_4^+ and productions rates of NO_3^- are in the same range, which again stresses that a nitrite shunt in the biological tanks of Marselisborg WWTP can become largely excluded. The NO_2^- turnover to NO_3^- is so immediate that it cannot be captured, or might be partially intracellular (comammox).

However, though we largely exclude a nitrite shunt, we cannot fully exclude a partial nitrite shunt at 3-4 m water depth close to an aeration zone (bottom water). Here, the consumption of NH_4^+ is not completely mirrored by the production of NO_3^- (**Figure 3**) and also the consumption and production rates are not in the same range (**Figure 4**). Interestingly, in this area of the biological tank, the O_2 concentration is also during aeration so low (O_2 turnover faster than O_2 supply) that simultaneous nitrification/denitrification might play an important role, which might favor a (partial) nitrite shunt. Oher (micro)habitats of the biological tank might show comparable microbial activities. Daily and seasonal variations of the wastewater nutrient composition, microbial composition of the activated sludge and other parameters like pH, O_2 concentration and retention time can also influence the impact of a nitrite shunt. Soliman and Eldyasti (2018) nicely summarizes parameters which influence AOB vs. NOB growth. For example, a temperature > 24-25˚C favors AOB growth, whereas a temperature between 10-20˚C favors NOB growth. The temperatures during our sampling days were 19.0 and 21.5˚C, respectively, which neither strongly favors AOB nor NOB. A decreasing temperature during other seasons than summer in Denmark would be, however, rather more unfortunate for a nitrite shut in the mainstream of Marselisborg WWTP.

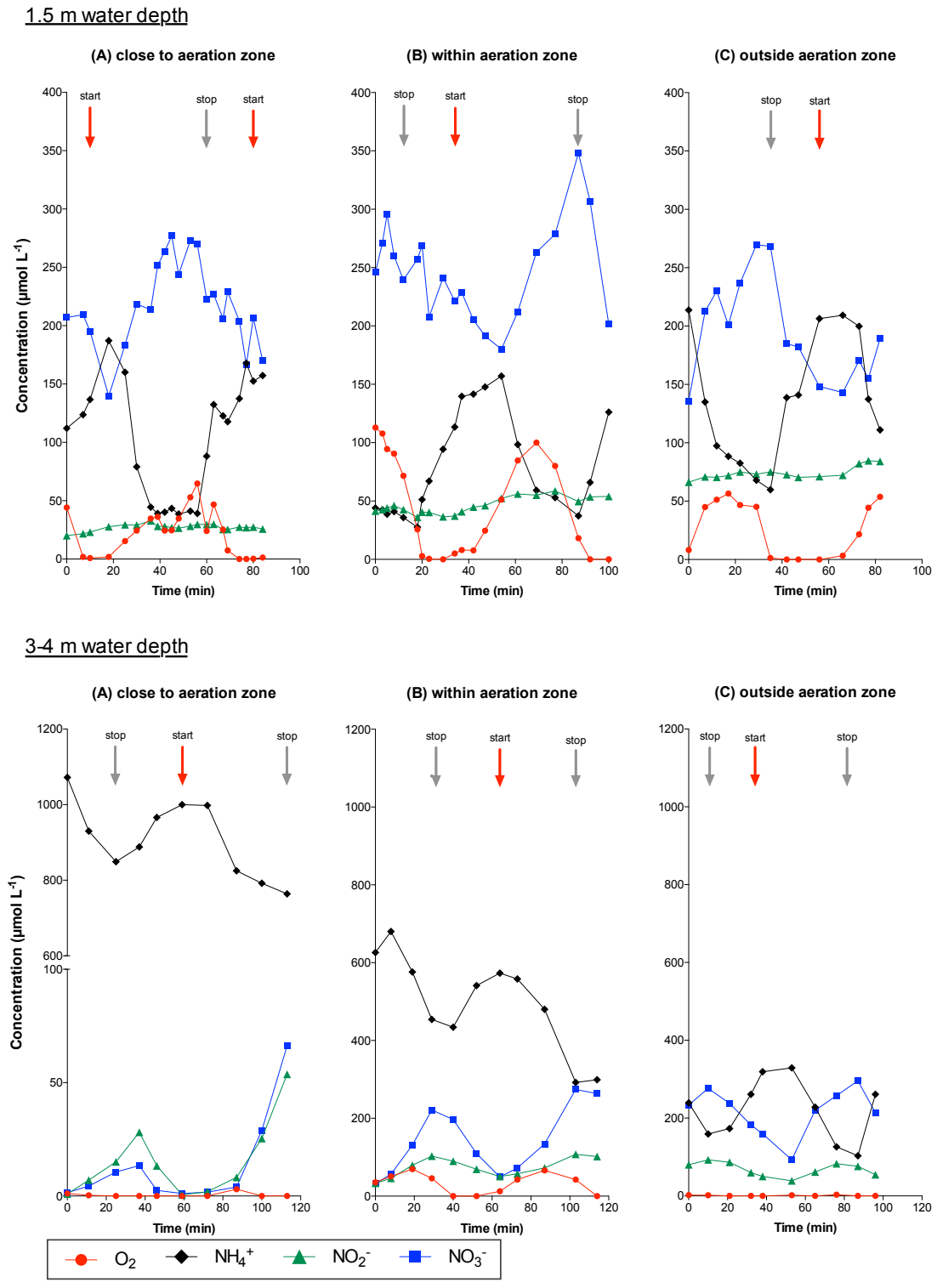
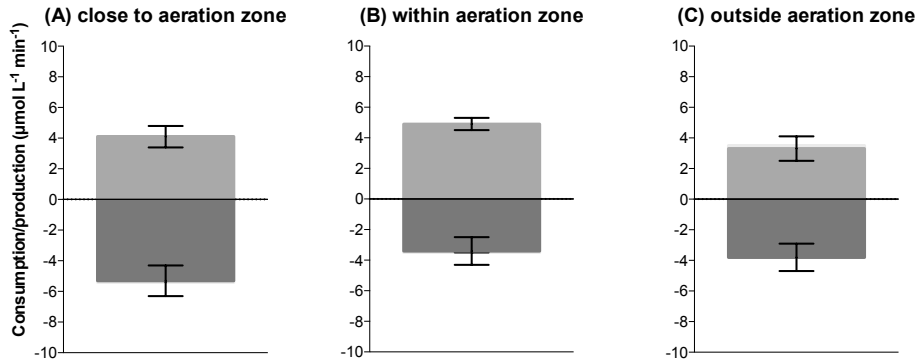


Figure 3: Time series of O_2, NH_4^+, NO_2^- and NO_3^- concentrations in the biological tank, measured (A) close to, (B) within, and (C) outside an aeration zone at 1.5 m water depth (main water column; upper panels) and 3-4 m water depth (bottom water; lower panels). Arrows indicate the start and end of visible bubbling (aeration) in the biological tank. Note different scales for 1.5 and 3-4 m water depth and scale break for (A) at 3-4 m depth.

1.5 m water depth



3-4 m water depth

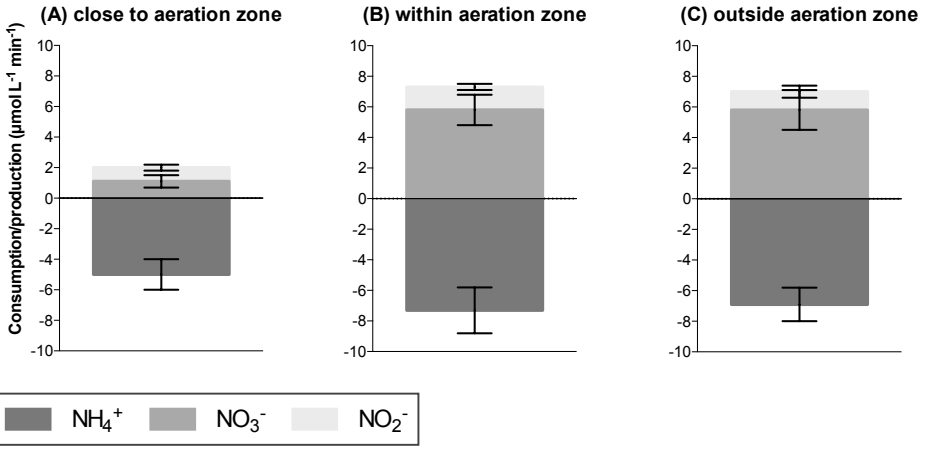


Figure 4: NH_4^+ consumption rates (negative values) and NO_2^- and NO_3^- production rates (positive values) during aeration in the biological tank, measured (A) close to, (B) within, and (C) outside an aeration zone at 1.5 m water depth (main water column; upper panels) and 3-4 m water depth (bottom water; lower panels).

In conclusion:

- **A highly specific method to measure anammox and denitrification in WWTPs was developed**
- A temperature dependence of anammox and denitrification is clearly visible, i.e. higher rates at higher temperatures
- Anammox is the dominating dissimilatory N-removal pathway in the DEMON
- Denitrification is the dominating dissimilatory N-removal pathway in the mainstream
- **N-removal by anammox in the mainstream of Marselisborg WWTP is negligible (approximately 1% of the measured denitrification rates in the mainstream)**
- **A nitrite shunt can become largely excluded in the biological tanks of Marselisborg WWTP during the time of our sampling in August 2018**

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