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Reference:
Vandekerckhove Tom G.L., Boon Nico, Vlaeminck Siegfried.- Pioneering on single-sludge nitrification/denitrification at 50 °C
Chemosphere - ISSN 0045-6535 - 252(2020), 126527
Full text (Publisher's DOI): https://doi.org/10.1016/J.CHEMOSPHERE.2020.126527
To cite this reference: https://hdl.handle.net/10067/1673240151162165141

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PII: S0045-6535(20)30720-7

DOI: https://doi.org/10.1016/j.chemosphere.2020.126527

Reference: CHEM 126527

To appear in: *ECSN*

Received Date: 14 July 2019

Revised Date: 16 February 2020

Accepted Date: 16 March 2020

Please cite this article as: Vandekerckhove, T.G.L., Boon, N., Vlaeminck, S.E., Pioneering on single-sludge nitrification/denitrification at 50□°C, *Chemosphere* (2020), doi: https://doi.org/10.1016/j.chemosphere.2020.126527.

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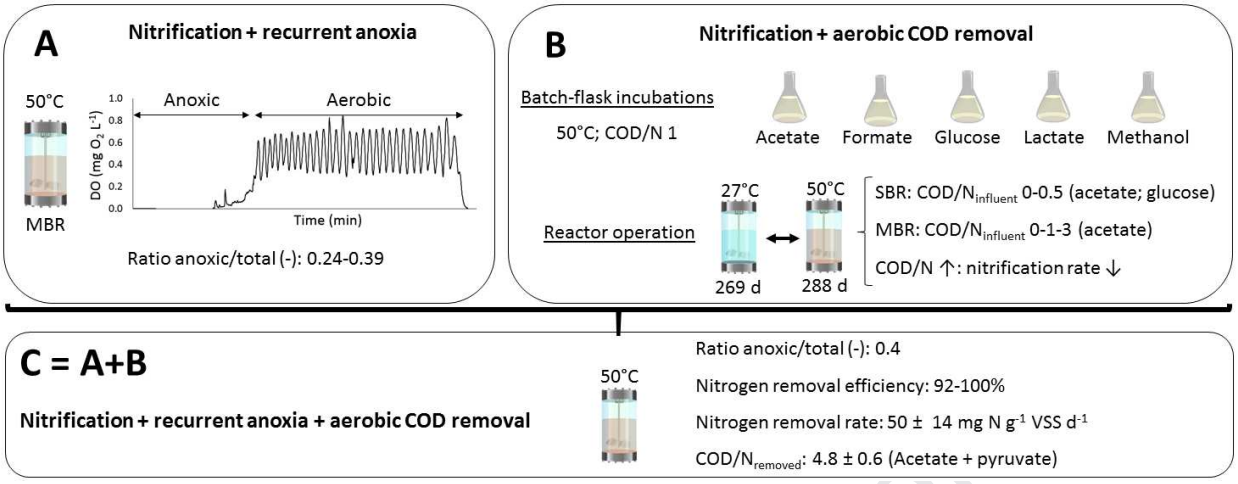

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Pioneering on single-sludge nitrification/denitrification at 50°C

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Abstract

Thermophilic nitrification has been proven in lab-scale bioreactors at 50°C. The challenge is now to develop a solution for thermophilic nitrogen removal, integrating nitrification with denitrification and aerobic carbon removal. This pioneering study aimed at a single-sludge nitrification/denitrification process at 50°C, through exposing nitrification in a step by step approach to anoxia and/or organics. Firstly, recurrent anoxia was tolerated by a nitrifying community during long-term membrane bioreactor (MBR) operation (85 days), with high ammonium oxidation efficiencies (>98%). Secondly, five organic carbon sources did not affect thermophilic ammonium and nitrite oxidation rates in three-day aerobic batch flask incubations. Moving to long-term tests with sequencing batch reactors (SBR) and MBR (>250 days), good nitrification performance was obtained at increasing $COD/N_{influent}$ ratios (0, 0.5, 1, 2 and 3). Thirdly, combining nitrification, recurrent anoxia and presence of organic carbon resulted in a nitrogen removal efficiency of 92-100%, with a $COD/N_{removed}$ of 4.8±0.6 and a nitrogen removal rate of 50±14 mg N g^{-1} VSS d^{-1}. Overall, this is the first proof of principle thermophilic nitrifiers can cope with redox fluctuations (aerobic/anoxic) and the aerobic or anoxic presence of organic carbon, can functionally co-exist with heterotrophs and that single-sludge nitrification/denitrification can be achieved.

Keywords: Ammonia oxidizing archaea; *Nitrososphaera*; *Nitrospira calida*; biological nitrogen removal; soluble microbial products

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1. Introduction

Biological wastewater treatment is widely used to help abate nitrogen pollution through sewage discharge and is commonly practiced at 5-35°C (Henze et al., 2008). Thermophilic removal of nitrogen can be an attractive alternative for the current mesophilic practices. Specific applications can be found in industrial activities delivering warm wastewater, linked to an industrial production process or to pre-treatment of the wastewater, e.g. reject water from thermophilic sludge digestion (50-70°C) (De Vrieze et al., 2016; Gebreeyessus and Jenicek, 2016). Recent research developments demonstrated the separate potential of aerobic nitrification (Courtens et al., 2016a; Courtens et al., 2016c), anoxic denitrification (Courtens et al., 2014) and aerobic carbon removal (Lapara and Alleman, 1999). The major benefit of thermophilic compared to mesophilic treatment was lower heterotrophic sludge production. Since the key conversions for high-temperature nitrogen removal are at hand (Lapara and Alleman, 1999; Courtens et al., 2014; Courtens et al., 2016b; Courtens et al., 2016c; Vandekerckhove et al., 2018; Vandekerckhove et al., 2019b), the main challenge is now to integrate the separate processes. A two-sludge nitrification/denitrification system is likely straightforward to implement, using either an external carbon source or COD from a wastewater bypass over the anaerobic digestion stage. A single-sludge approach, however, would be economically superior to a two-sludge process to limit the capital investment for new treatment plants, enable efficient retro-fitting of existing mesophilic plants, and avoid costs for external carbon dosage or loss of biogas production. Such approach requires the proof that thermophilic nitrification can be paired with conversions requiring recurrent anoxia and presence of organic carbon.

To enable sludge separation and retention through settling, the biomass concentration in activated sludge systems is usually relatively low, and rather constant (3-4 g VSS L^{-1}). Introducing an increasing organic carbon load at constant nitrogen loading rate would result

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in substantial growth of heterotrophs. Compared to nitrifiers, mesophilic heterotrophs are characterized by a higher biomass yield (0.47 g VSS g^{-1} COD vs 0.17 g VSS g^{-1} N) and growth rate (6 vs 0.8 d^{-1}) (Henze et al., 2000). Due to increasing growth of heterotrophic biomass, increasing amounts of sludge must be wasted to keep the mixed liquor concentration stable, possibly lowering the sludge retention time (SRT) below the minimum required for nitrifier growth and thus washing them out of the system. These theoretical expectations have been substantiated in practice. In mesophilic biofilm systems, an exponential decrease in nitrification rate with increasing $COD/N_{influent}$ ratio was reported due to growth of heterotrophs and oxygen competition in the biofilm (Gönenç and Harremoës, 1990; Chen et al., 2006). In suspended systems where the oxygen concentration is controlled, competition for space dictates the microbial community composition. In a previous study with a constant SRT of 25 days, increasing $COD/N_{influent}$ from 0.71 to 3.4 caused a decline in mesophilic nitrification rate, going from 140 to 29 mg N g^{-1} VSS d^{-1}, linked to a concomitant lower nitrifying biomass fraction from 27% to 8% (Carrera et al., 2004). In a MBBR study, an increase in nitrifying biomass fraction from 12% up to 60% was observed when decreasing the $COD/N_{influent}$ from 4 to 0 (Bassin et al., 2015). Furthermore, in a full-scale wastewater treatment plant, it was shown that a quantitative decrease in autotrophic bacteria was related to an increasing organic loading rate (Cydzik-Kwiatkowska et al., 2012). Besides the growth of heterotrophs impacting the retention of nitrifiers, organic carbon (e.g. yeast extract, acetate) might induce an inhibitory effect on thermophilic nitrification, as observed for several ammonia oxidizing archaea (Konneke et al., 2005; de la Torre et al., 2008; Kim et al., 2016).

To the authors' knowledge, this is the first study integrating nitrification, denitrification and aerobic COD removal in a single-sludge system for thermophilic biological nitrogen (and carbon) removal. In a first step, anoxia was introduced in an aerobic, autotrophic nitrifying

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membrane bioreactor (MBR). Secondly, the effect of five organic carbon sources (acetate, formate, glucose, lactate and methanol) on nitrification activity was investigated during three-day batch flask incubations. Moving to aerobic sequencing batch reactors (SBR) and MBR, the impact of increasing $COD/N_{influent}$ ratios (0, 0.5, 1 and 3; acetate and glucose) on the SRT and the concomitant retention of mesophilic (27°C) versus thermophilic (50°C) nitrifying activity was investigated. Thirdly, a single-sludge combination of nitrification, intermittent anoxia and organic carbon was performed in six nitrification/denitrification MBR.

2. Materials and methods

2.1. Nitrification and recurrent anoxia

For single-sludge nitrification/denitrification to be achieved, aerobic and anoxic phases must be alternated to allow for ammonia oxidation and nitrate reduction. Previously obtained thermophilic nitrifying cultures were never imposed to anoxia (Courtens et al., 2016b; Courtens et al., 2016c; Vandekerckhove et al., 2019b). Thus, as a first step in the integration, recurrent anoxia was imposed on a previously described thermophilic (50°C), aerobic, nitrifying mixed community in an MBR (2L) (Courtens et al., 2016a; Vandekerckhove et al., 2019b) (**Figure 1, A**). Six phases of intermittent aerobic/anoxic time intervals with different lengths were imposed (**Table S.1**), with 3 up to 40 minutes of anoxia, comprising between 32 and 65% of the total time interval duration. The anoxic/aerobic time ratios were based on previous research on nitrification and denitrification rates (Courtens et al., 2014; Courtens et al., 2016b; Courtens et al., 2016c; Vandekerckhove et al., 2018; Vandekerckhove et al., 2019b). The actual length of anoxic phase was increased as a precaution. Duration of the phases ranged between 8 and 26 days, or 23 to 76 hydraulic retention times (HRT). Dissolved oxygen concentration during the aerobic time intervals was controlled at 0.5 mg O_2 L^{-1} (Liquiline M CM442). During the anoxic time intervals, aeration was stopped and the

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dissolved oxygen concentration decreased to 0 mg O_2 L^{-1}. Before starting the experiment of recurrent anoxia in the MBR, the dissolved oxygen concentration was controlled at 2 mg O_2 L^{-1} (Courtens et al., 2016b; Vandekerckhove et al., 2019b). The biomass concentration was 3.3 ± 0.3 g VSS L^{-1}. Synthetic medium was continuously fed and consisted of $(NH_4)_2SO_4$ (150 mg N L^{-1}), 12 g $NaHCO_3$ g^{-1} N, KH_2PO_4 (10 mg P L^{-1}) and 0.1 mL L^{-1} trace elements (Kuai and Verstraete, 1998). The pH (7.29 ± 0.18) was controlled *via* the addition of bicarbonate to the medium to buffer the acidification caused by nitrification (12 g $NaHCO_3$ g^{-1} N or 1.8 g $NaHCO_3$ L^{-1}_{medium}). The reactor was operated at a loading rate of 435 ± 38 mg N L^{-1} d^{-1} and liquid samples were taken daily for NH_4^+, NO_2^- and NO_3^- analyses.

At the end of each phase, the *in situ* maximum biomass-specific ammonium oxidation rate (q_{max,NH_4^+-N}) was determined. For this, the pH was controlled between 7.25-7.35 by dosing 0.2 M NaOH/HCl. A few minutes prior to the activity test, 0.2 g $MgSO_4x7H_2O$ L^{-1}, 0.1 g L^{-1} $CaCl_2$ and trace elements (0.1 mL L^{-1}) (Kuai and Verstraete, 1998) were added to the mixed liquor to ensure non-limiting conditions. After stopping the influent flow, ammonium spikes (20 mg N L^{-1}) were added, accompanied by 24 g $NaHCO_3$ g^{-1} N to prevent inorganic carbon limitation. Ammonium concentration was monitored in time to extract the oxidation rate. During these experiments, nitrite slightly accumulated, which was used to calculate q_{max,NO_2^-N}. Experiments were conducted in triplicate in one single reactor and biomass concentration was determined in triplicate to derive biomass-specific oxidation rates. Experiment duration was based on the time needed to oxidize the ammonium. Error propagation was used to calculate the error on the obtained final values.

2.2. Nitrification and aerobic COD removal

The second step in the integration process was to evaluate the competition between thermophilic aerobic heterotrophs and nitrifiers (Figure 1, B). Firstly, short-term incubations

were performed to investigate whether organic carbon as such had an influence on the nitrifying activity and whether heterotrophic activity on easily biodegradable COD affected nitrification activity. Subsequently, long-term bioreactor experiments were executed to study the competition between aerobic heterotrophic and nitrifier growth at different COD/N ratios.

2.2.1. Short-term incubation of nitrification with aerobic COD removal

In a first test, the short-term effect of several organic carbon sources on the nitritation rate was investigated in three-day batch flask incubations (**Figure 1, B**). Five common carbon sources were selected based on industrial use and structural diversity: acetate, formate, glucose, lactate and methanol. A distinction was made between ammonia stripping (measured in an abiotic control experiment), heterotrophic assimilation (based on the COD removal and an assumed yield of 0.53 g VSS g^{-1} COD (Vandekerckhove et al., 2018) and actual nitritation (remaining ammonium removal).

In a subsequent three-day batch flask incubation, nitrite oxidation was monitored in the presence of acetate as a carbon source at different concentrations (0, 5, 10, 25 and 100 mg COD L^{-1}). Acetate was chosen as it was the carbon source used in the reactor experiments combining nitrification, denitrification and aerobic carbon removal. Incubations with a mesophilic nitrifying aquaculture inoculum ABIL (Avecom, Belgium) and a thermophilic nitrifying community (Courtens et al., 2016a) were compared.

More details on these batch flask incubations are provided in Supporting information, Section S.1.2.1.

2.2.2. Long-term incubation of nitrification and aerobic COD removal

Long-term nitrification performance was monitored in SBR and MBR reactors while imposing increasing $COD/N_{influent}$ ratios (**Figure 1, B**). The aim was to control the biomass concentration at ± 2 g VSS L^{-1} and apply a constant biomass-specific loading rate of ± 50 mg

157 N g^{-1} VSS d^{-1}, rendering the increase in $COD/N_{influent}$ the only factor impacting the SRT and,
158 thus, the retention of nitrifiers.

159 In a first reactor experiment (**Figure S.1**) (**Table 1**), mesophilic (27°C) and thermophilic
160 nitrification (50°C) in SBR reactors (2L) were subjected to acetate and glucose at
161 $COD/N_{influent}$ ratios of 0.5. The mesophilic and thermophilic SBR were inoculated with a
162 commercial nitrifying inoculum (Avecom NV, Belgium) and a mixture of two previously
163 described thermophilic nitrifying communities respectively (Courtens et al., 2016a; Courtens
164 et al., 2016c). Similar synthetic medium was used as in section 2.1., but with 14-270 mg N L^{-1}
165 $(NH_4)_2SO_4$. During a first period of 120 days, no carbon was fed, after which acetate was
166 added to the feed at a $COD/N_{influent}$ ratio of 0.5 during 49 days. Subsequently, a 63-day period
167 without carbon was again imposed, followed by the addition of glucose at $COD/N_{influent}$ 0.5
168 for 37 days. Hydraulic retention time (HRT) was 0.9 ± 0.2 days. Based on the nitrogen
169 balance, nitrogen loss through ammonia stripping or biomass assimilation in the SBR reactors
170 was limited to 6.3 ± 9.5 and 9.2 ± 11.6% of total influent nitrogen at 27 and 50°C
171 respectively, indicating that nitrification was the main process of ammonia removal. Due to
172 biomass washout via the effluent, the biomass concentration decreased during SBR operation.
173 Therefore, the specific loading rate was not constant and the increasing $COD/N_{influent}$ was not
174 the only factor impacting the SRT and related retention of nitrifiers. Therefore, the reactor
175 configuration was changed to MBR in a second experiment.

176 In the second reactor experiments with the same feed medium and inocula, mesophilic and
177 thermophilic MBR were compared (**Table 2**). Acetate as carbon source was consecutively
178 dosed at $COD/N_{influent}$ ratios of 0, 1 and 3, corresponding to 99 days in both reactors (COD/N
179 0), 149 and 176 days in the mesophilic and thermophilic MBR respectively (COD/N 1) and
180 40 and 57 days in the mesophilic and thermophilic MBR respectively (COD/N 3). All MBR
181 were operated at fixed biomass concentration (1.7-2.3 g VSS L^{-1}) and loading rate (51-64 mg

182 N g^{-1} VSS d^{-1}), making the increasing $COD/N_{influent}$ responsible for the change in sludge
183 retention time (SRT) and the related retention of autotrophic activity. Based on the nitrogen
184 balance, nitrogen loss through ammonia stripping or biomass assimilation in the MBR
185 reactors was limited to 12.4 ± 12.9 and 8.6 ± 13.8% of total influent nitrogen at 27 and 50°C
186 respectively, indicating that nitrification was the main process of ammonia removal.

187 More details on the reactor operation, process control and sampling can be found in
188 Supporting information, section S.1.2.2.

189 2.2.3. Conversion kinetics and substrate affinity

190 During the long-term integration of nitrification and aerobic carbon conversion in the SBR
191 and MBR reactors, the conversion kinetics and substrate affinities were monitored at each
192 $COD/N_{influent}$ ratio tested. The *in situ* maximum biomass-specific ammonium and nitrite
193 oxidation rate (q_{max,NH_4^+-N} and q_{max,NO_2^-N}) at each $COD/N_{influent}$ ratio was determined *in situ* as
194 described above (Section 2.1.). In case nitrite did not accumulate ($q_{max,NH_4^+-N} < q_{max,NO_2^-N}$),
195 NO_2^- was spiked to determine q_{max,NO_2^-N}. The maximum specific aerobic carbon removal rate
196 ($q_{max,COD,aerobic}$) was determined by means of respirometry, executed *in situ*, along with the
197 substrate affinity index for NH_4^+ (K_{s,NH_4^+-N}), NO_2^- (K_{s,NO_2^-N}) and acetate ($K_{s,COD}$). Details are
198 provided in Supporting information, section S.1.2.3.

199 2.2.4. Sludge settleability

200 Sludge settleability during the SBR reactor experiments was determined *via* the sludge
201 volume index (SVI) and the initial settling velocity (ISV), details on the methods and
202 equipment used are provided in Supporting information, section S.1.2.4.

203 **2.3. Nitrification, denitrification and aerobic COD removal**

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After integrating nitrification with recurrent anoxia (step one) and with aerobic COD removal (step two), the final step was to combine all processes, i.e. nitrification and aerobic and anoxic COD removal, in a single-sludge nitrification/denitrification reactor.

The integration of nitrification, recurrent anoxia (denitrification) and organic carbon was investigated in 2L reactors at 50°C (**Figure 1, C**). Seven different reactor runs were operated, of which one SBR and 6 MBR, with different carbon sources (soluble starch, glucose, acetate, pyruvate) and $COD/N_{influent}$ ratios (0.9-6.5) (**Table 4**). Each reactor was inoculated with a mixture of two previously described thermophilic nitrifying bioreactors (Courtens et al., 2016a; Courtens et al., 2016c). Similar synthetic medium was used as in section 2.1., but with 50-350 mg N L^{-1} $(NH_4)_2SO_4$. All reactors were computer controlled as described in Supporting information, section S.1.2.2. Aerobic and anoxic phases followed each other continuously, with feeding during the anoxic phase. The SBR cycle in Run IV (3h) consisted of 2.5 h reaction phase, divided in intermittent aerobic and anoxic phases of 20 and 10 min with feeding in the anoxic phases, 20 min settling phase, 8 min withdrawal phase and 2 min resting phase. In the MBR operation, aerobic (23-70 min) and anoxic phases (7-30 min) were alternated. Effluent withdrawal was enabled *via* a level controller linked to the DaqFactory software. Liquid samples were taken daily for COD, NH_4^+, NO_2^- and NO_3^- analyses and mixed liquor samples for determination of total and volatile suspended solids concentration (TSS and VSS).

2.4. Molecular analyses

Samples were collected for bacterial and archaeal community analysis via qPCR and amplicon sequencing. Samples were taken at the end of every $COD/N_{influent}$ ratio in the SBR and MBR experiments combining nitrification and aerobic COD removal. In the experiments coupling nitrification and denitrification, samples were collected at the start and end of reactor run 3, 4 and 5. Samples were stored at -20°C prior to DNA extraction. DNA

extraction was performed using the ZymoBIOMICS DNA Microprep Kit (Zymo Research, USA) according to the manufacturer's instructions. Quality assessment of the DNA extracts was performed by means of a 2% agarose gel after which the concentration was measured using the QuantiFluor® dsDNA System (Promega, USA). Illumina 16S rRNA gene amplicon libraries were generated and sequenced by BaseClear BV (Leiden, the Netherlands). The DNA extracts were sent to Baseclear B.V. (The Netherlands) for Illumina sequencing on the MiSeq platform for both bacteria and archaea. The sequencing data are deposited at the NCBI (National Center for Biotechnology Information) database, with accession number SRP173879. The total bacteria and archaea was determined by use of qPCR. More information on the amplicon sequencing and data processing and qPCR analysis can be found in Supporting information, section S.1.4. The qPCR results combined with the biomass concentration of the samples yielded total bacteria and archaea in copies g^{-1} VSS, representing the total community when added up. When also taking into account the relative abundance of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in the bacterial community and ammonia oxidizing archaea (AOA) in the archaeal community, as determined by 16S rRNA gene amplicon sequencing, an estimation was made of the relative abundance of each of these microbial groups in the total community. When both AOB and AOA were present in the community, the term ammonia oxidizing organisms (AOO) was used (Bouskill et al., 2012).

2.5. Chemical analyses

Details on the methods applied to determine ammonium, total suspended solids (TSS), volatile suspended solids (VSS), nitrite, nitrate, organic acids, methanol, glucose and soluble COD can be found in Supporting information, section S.1.5.

2.6. Statistical analyses

Statistical analyses were conducted to test for statistically significant differences between q_{max,NH_4^+-N} and q_{max,NO_2^--N} values during the integration of nitrification and anoxia. Also, during the combination of nitrification and aerobic COD removal, statistical differences in SVI, ISV and q_{max,NH_4^+-N} and q_{max,NO_2^--N} were investigated. After screening the data in box plots, the normality was examined and homogeneity of variances was checked. In case of normality and homoscedasticity, a one-way ANOVA and Tukey test was used to test the null hypothesis and determine pairwise differences between parameter values respectively. In case of normality but heteroscedasticity, a one-way Welch ANOVA and Games-Howel Post-Hoc test with Welch's correction was used. When normality could not be assumed, Kruskal Wallis rank sum tests and pairwise Wilcoxon Rank Sum Test with Holm correction for multiple testing were applied. More details on the statistical methods used can be found in Supporting information, section S.1.6.

3. Results & Discussion

3.1. Nitrification and recurrent anoxia

During the integration of nitrification and recurrent anoxia, high nitrification performance was preserved in a long-term MBR experiment (85 days), with ammonium oxidation >98% and maximum ammonium and nitrite oxidation rates of 390-636 and 338-473 mg N g^{-1} VSS d^{-1} respectively (**Figure S.2**). The conversion rates remained in the same order of magnitude, despite some significant differences during phase IV and V ($p<0.05$). Complete ammonium and nitrite conversion to nitrate was retained despite lowering the aerobic time, and thus the aerobic SRT.

3.2. Nitrification and aerobic COD removal

3.2.1. Short-term incubation of nitrification with aerobic COD removal

The presence of organic carbon could elicit an inhibitory effect on nitrification, as observed for several ammonia oxidizing archaea cultures (Konneke et al., 2005; de la Torre et al., 2008; Kim et al., 2016). In this study, however, similar or higher ammonium oxidation rates were observed in the presence of up to 100 mg COD L^{-1} of several organic compounds compared to the absence of COD during a three-day exposure in aerobic batch flask tests (**Figure S.3**). Also, nitrite oxidation was relatively unaffected by acetate and even showed an increased rate at 100 mg COD L^{-1} (**Figure S.4**). As nitrification was not inhibited by organic carbon in short-term incubations, the next step was to test the long-term co-existence of nitrifiers and aerobic heterotrophs in reactor experiments.

3.2.2. Long-term incubation of nitrification and aerobic COD removal

Long-term (>250 days) aerobic exposure of thermophilic nitrification to increasing $COD/N_{influent}$ ratios rendered overall good nitrification performance in the SBR and MBR experiments, leading to a successful combination of aerobic ammonium and organic carbon oxidation (**Table 1 and 2**). The shift from no organic carbon addition to dosing acetate and glucose at $COD/N_{influent}$ 0.5 (phase II and IV) shortly led to a reduced nitrification performance in the thermophilic SBR, with a concomitant accumulation of ammonium (**Figure S.5 and S.6**). However, rapid recovery of nitrification rate and subsequent relatively stable operation was observed in phase III and at the end of phase IV. This seemingly inhibitory effect when introducing organic carbon did not occur in the successive MBR experiments (**Figure S.9, S.10, S.11 and S.12**). Each short period of ammonium/nitrite accumulation in the MBR operation was related to technical issues regarding pH and/or DO control, with a fast recovery of ammonium oxidation. Nitrite oxidation, on the other hand, was not as easily restored. This was most likely related to the inhibitory effect of nitrite/free nitrous acid to thermophilic nitrite oxidizing bacteria (NOB) (Courtens et al., 2016a), as sludge washing to remove the accumulated nitrite led to a recovery of NOB activity.

In the SBR and MBR reactors, AOA were the most dominant ammonia oxidizers for mesophilic and thermophilic ammonium oxidation, with species related to "*Ca*. Nitrosocosmicus exaquare" and "*Ca*. Nitrososphaera gargensis, respectively. The mesophilic biomass also contained some Nitrosomonas-related AOB (6 to 22111 times lower abundance than AOA). *Nitrospira*- and *Nitrobacter*-related NOB representatives were present in the mesophilic biomass, whereas the thermophilic communities were comprised only of *Nitrospira*-related organisms (**Table S.5**). Detailed information on the qPCR and 16S rRNA gene amplicon sequencing data can be found in Supporting information section S.3.2.3.

The impact of increasing $COD/N_{influent}$ ratios on the SRT and the concomitant retention of mesophilic versus thermophilic nitrification activity was investigated. In the SBR reactors, biomass concentration dropped throughout the operation and remained mostly below the target of 2 g VSS L^{-1} due to biomass washout (**Figure S.7**). The SRT in the thermophilic SBR was consistently higher than in the mesophilic SBR (**Table 1**). The decreasing SRT resulted in decreasing absolute abundance of AOO and NOB in the mesophilic SBR, whereas the AOA and NOB absolute abundance in the thermophilic SBR were hardly affected (**Figure S.1**). Biomass washout via the effluent during SBR operation resulted in decreasing biomass concentration. Also, the specific loading rate was not constant. Due to these factors, the increasing $COD/N_{influent}$ was not the only factor impacting the SRT and related retention of nitrifiers (**Table 1**). Therefore, no sound conclusions could be drawn from the SBR operation.

In subsequent experiments, the reactor configuration was changed to MBR, enabling an easier control of the biomass concentration and loading rate (**Table 2**). At $COD/N_{influent}$ 0 and 1, no sludge was wasted besides the daily sampling, yielding a SRT of ±250 d. At $COD/N_{influent}$ 3, 0.48 ± 0.07 and 0.36 ± 0.08 g VSS d^{-1} was wasted in the mesophilic and thermophilic MBR respectively, resulting in a SRT of 8.7 ± 0.9 and 11.8 ± 2.9 d respectively.

This lower thermophilic sludge production corroborates the often reported advantage of thermophilic wastewater treatment (Lapara and Alleman, 1999; Courtens et al., 2014; Vandekerckhove et al., 2018). Lower sludge production and the resulting higher SRT yielded a smaller decrease of q_{max,NH_4^+-N} and q_{max,NO_2^-N} with increasing $COD/N_{influent}$ ratio when comparing the thermophilic (50°C) and mesophilic (27°C) reactor. At $COD/N_{influent}$ 0 and 1, the mesophilic maximum nitrification rate was higher than its thermophilic counterpart, whereas similar rates were obtained at $COD/N_{influent}$ 3 (50-60 mg NH_4^+-N g^{-1} VSS d^{-1}) (**Figure 2**). This might indicate that higher $COD/N_{influent}$ ratios could be attained at thermophilic temperatures without washing out the nitrifiers, thus possibly increasing the range of treatable wastewater types. However, the absolute and relative abundance of AOA at 50°C decreased more when increasing the $COD/N_{influent}$ from 1 to 3 compared to the AOO at 27°C (**Figure S.15 and S.16**). In general, the relative abundance of nitrifiers at the different $COD/N_{influent}$ values in this study (15-98%) was higher compared to previous studies on the effect of $COD/N_{influent}$ on nitrifier abundance (8-60%) (Carrera et al., 2004; Bassin et al., 2015).

In-situ activity measurements were used to determine q_{max,NH_4^+-N} and q_{max,NO_2^-N} in the SBR and MBR reactors, which were combined with the relative abundance of AOO, AOA and NOB based on qPCR and 16S rRNA gene amplicon sequencing, yielding the group-specific rates $q_{max,AOO}$ and $q_{max,NOB}$ (**Figure S.17 and S.18**). Also, based on previously determined AOA and NOB yields, $\mu_{max,AOO}$ and $\mu_{max,NOB}$ was estimated (Vandekerckhove et al., 2019a; Vandekerckhove et al., 2019b). The $q_{max,AOO}$, $q_{max,AOA}$, $\mu_{max,AOO}$ and $\mu_{max,AOA}$ were mostly lower than literature values for AOA (**Table S.6**) and could have been underestimated. Activity measurements were performed on the total biomass, whereas the molecular analyses were based on cell numbers. These differences and assumptions made could have influenced

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the results and introduced uncertainty. For example, it was assumed that each cell was equal in size and contained an equal amount of 16S rRNA gene copies when going from biomass- to group-specific rates. In addition, DNA extraction efficiencies could have differed from organism to organism, primer selectivity could have caused under- or overestimation of certain species abundances and the active fraction of the biomass was not known or taken into account. Overestimation of AOA abundance was likely, as the estimated minimum required SRT to retain the AOA (estimated as average μ_{max}^{-1} instead of μ_{AOA}, neglecting decay) was higher than the actual SRT in the reactor at $COD/N_{influent}$ 3 in the MBR at both 27 and 50°C (**Table S.8**), whereas nitrification was retained even after operating more than 4 times the SRT, suggesting that the actual growth rate was higher than experimentally estimated.

Thermophilic *Nitrospira* activity (2.7-12.1 g N g^{-1} VSS d^{-1}) and growth rates (0.11-0.50 d^{-1}) were similar to previously obtained kinetics at 50°C (Vandekerckhove et al., 2019b) (**Table S.7**). In the first phase without carbon in the mesophilic SBR and MBR reactor, $q_{max,NOB}$ and $\mu_{max,NOB}$ were disproportionately high, almost a factor 10 higher than literature values, mainly caused by very low NOB relative abundances (**Figure S.14 and S.16**). The NOB kinetics during all other phases ($q_{max,NOB}$: 2.8-17.3 g N g^{-1} VSS d^{-1}; $\mu_{max,NOB}$: 0.11-0.86 d^{-1}) were similar to literature values ($q_{max,NOB}$: 3.3-72 g N g^{-1} VSS d^{-1}; $\mu_{max,NOB}$: 0.23-2.6 d^{-1}).

These results introduce a need for caution when interpreting AOO- and NOB- specific kinetics obtained by combining experimental kinetic data of the total biomass with qPCR- and 16S rRNA gene amplicon sequencing -based relative abundance. Overall, changing $COD/N_{influent}$ ratios rendered declining nitrification activity of the total biomass, accompanied by fluctuating specific AOO and NOB q_{max} and μ_{max} that remained in the same order of magnitude.

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As expected, the increase in organic carbon was accompanied by growth of heterotrophs and concomitant increasing $q_{max,COD,aerobic}$ in the SBR and MBR reactors (**Figure S.8 and 2**). Interestingly, rates at 50°C were consistently lower compared to 27°C. Although literature often states higher aerobic carbon degradation rates at elevated temperatures (Lapara and Alleman, 1999), this study is in accordance with those reporting no kinetic advantage or even lower rates at thermophilic conditions (LaPara et al., 2000a, b; Jahren et al., 2002; Vandekerckhove et al., 2018).

Substrate affinity and settling is discussed in supporting information section S.3.2.6 and S.3.2.7 respectively.

3.3. Nitrification, denitrification and aerobic COD removal

After the stepwise integration of nitrification with recurring anoxia and aerobic COD removal, a single-sludge combination for nitrification/denitrification was attempted in seven bioreactor runs (Run I to VII) (**Table 4**). Taken together, the combination of intermittent aerobic/anoxic phases, nitrification and heterotrophic carbon removal (aerobic/anoxic) seemed to elicit some inhibitory effect on nitrification in Run I to V that was not related to the separate combinations or ROS production (Supporting information, section S.4.1). qPCR analysis revealed that the absolute and relative AOA and NOB abundance decreased by 1-3 orders of magnitude when comparing the start and end of operation in Run III, Run IV and Run V (**Figure S.27 and S.28**). Considering the short operation time and the high SRT (76-195 days), it is unlikely that the nitrifiers were washed out of the system. In case of washout, nitrifiers actively grow and perform nitrification, but the SRT is lower than the nitrifier growth rate, by which they are washed out of the system. Simulations with previously obtained nitrifier decay rates demonstrated that an increased decay in the absence of activity/growth could be the possible cause the complete loss of nitrifiers (Supporting information, Section S.4.2). The loss of nitrification seemingly occurred when the

399 $COD/N_{influent}$ ratio was higher than 3. Additionally, in case of nitrification failure, the COD
400 loading rate, influent and effluent COD concentrations were consistently higher compared to
401 the aerobic combination of nitrification and carbon conversion (**Table 1**). Also, during the
402 batch-flask incubations (see section 3.2.1.), organic carbon never exceeded 252 mg COD L^{-1}.
403 In Run III and Run IV, the lower COD loading rate (<300 mg COD g^{-1} VSS d^{-1}), influent
404 COD concentrations (<800 mg COD L^{-1}) and $COD/N_{influent}$ ratios <3 not causing inhibition
405 were only maintained for short periods (<1 week).

406 In Run VI, the impact of these factors was more thoroughly investigated by implementing a
407 slower $COD/N_{influent}$ increase and a lower loading rate of N and COD. In a first step, acetate
408 (80% of $COD_{influent}$) and pyruvate (20% of $COD_{influent}$) were dosed for 15 days at COD_{tot}/N
409 ratio 3.37 ± 0.37, COD influent concentration of 391 ± 37 mg COD L^{-1} and loading rate of
410 260 ± 39 mg COD g^{-1} VSS d^{-1}, similar to the aerobic combination of nitrification and carbon
411 conversion (**Table 1**). Good nitrification performance was observed, with a $CODt_{ot}/N_{removed}$
412 of 5.4 ± 1.2 (**Figure S.26**). In a second step, the nitrogen loading rate was lowered to raise the
413 influent COD_{tot}/N ratio to 5.5 ± 0.7 for 20 days while keeping the influent COD concentration
414 and loading rate similar (**Table 1**). COD removal occurred in the anoxic phase but the
415 $COD_{tot}/N_{removed}$ was high, possibly depicting storage of the acetate. After a few days, the
416 $COD_{tot}/N_{removed}$ stabilized around 4.7 ± 1.6 and the effluent nitrate concentration decreased,
417 indicating denitrification. Nitrification prevailed despite high influent COD_{tot}/N ratios and
418 effluent COD concentrations similar to previous runs when nitrification failed. The previous
419 failure of nitrification was, thus, not a result of elevated influent $COD/N_{influent}$ ratios or SMP.
420 In a third step, the COD_{tot}/N was maintained at 5.8 ± 0.3 and the N and COD influent
421 concentrations and loading rates were increased (**Table 1**). The effluent nitrate concentration
422 decreased further until complete nitrogen removal was achieved at $COD_{tot}/N_{removed}$ of 4.1 ±
423 0.7. However, a technical malfunction prevented long-term operation to confirm this

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observation. Nonetheless, it was proven that complete nitrogen removal was possible at 50°C, at a rate of 50-53 mg N g^{-1} VSS d^{-1} and with an anoxic/total phase ratio of 0.4.

Because complete nitrogen removal was only achieved for a few days, a subsequent 7th MBR run was operated under the same conditions as the end of run VI (**Table 4**). Aeration (17 minutes) and no aeration (13 minutes) were alternated (43% of the time no aeration). In a first phase (day 0 to 22 of operation), a nitrogen removal efficiency of 92-100% was obtained from day 4 onwards, with a $COD/N_{removed}$ of 4.8 ± 0.6 and a nitrogen removal rate of 50 ± 14 mg N g^{-1} VSS d^{-1} (**Figure 3**). These results corroborate the findings of Run VI, that complete nitrogen removal is possible at 50°C. Due to biomass growth, sludge was wasted regularly to maintain a biomass concentration of 2.5 ± 0.3 g VSS L^{-1}, resulting in an estimated SRT between 26 and 46 days based on the VSS balance over the reactor. Concomitantly, the nitrogen loading rate was increased (day of operation 23-53). Sludge wasting resulted in the production of nitrite alongside nitrate from day 23 onwards, indicating that NOB activity could not cope with the increasing loading rate and decreasing SRT. As such, denitrification *via* nitrite and nitrate occurred, with a nitrogen removal efficiency of 86-100%, a $COD/N_{removed}$ of 3.0 ± 0.4 and a nitrogen removal rate of 61-104 mg N g^{-1} VSS d^{-1} (**Table 1**). Based on the nitrogen and VSS balance over the reactor, an observed biomass yield (Y_{obs}) of 0.11 g VSS g^{-1} $COD_{removed}$ or 0.42 g VSS g^{-1} $N_{removed}$ was estimated, which was similar to results from previous experiments on thermophilic denitrification (Vandekerckhove et al., 2018).

During the reactor runs in which nitrification failed (Run I to V), the community was either immediately subjected to high influent COD concentrations and high $COD/N_{influent}$, or the transition from low to high carbon load was fast (<2 weeks). During Run VI, on the other hand, the organic carbon in the influent was more gradually increased compared to the previous runs, after which complete nitrogen removal was achieved in about 40 days. Also, in

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Run VII, the nitrogen loading rate was gradually increased and the organic carbon concentration was kept relatively low. This might have provided sufficient time for the nitrifiers to adapt to the changing conditions. Microbial adaptations to new environmental conditions or stress occur through regulatory and mutational adaptation (Ryall et al., 2012). Possibly, the fast increase in organic carbon, together with the change in redox, rendered too much stress for the nitrifiers to adapt quickly enough, whereas the more gradual change did. Additionally, an environmental change can either reduce or increase growth rate and viability of microorganisms. A faster increase in organic carbon combined with nitrogen load on a nitrifying community that has never been in the presence of organic carbon, could have resulted in more stressful conditions compared to a gradual increase. This could have induced a much lower nitrifier growth rate and a concomitant increase in decay rate due to the stressful conditions, eventually yielding the observed decrease in nitrifier abundance and activity in Run III, IV and V. Previous research has shown that nitrifiers require sufficient time to adapt to changing conditions (Courtens et al., 2016c). The smaller the temperature increase in a nitrifying bioreactor, the higher the temperature that could be achieved. Overall, it was shown that nitrogen removal was possible at 50°C through nitrification/denitrification over nitrate and/or nitrite for 53 days. The next step is a focus on long-term steady-state reactor operation, further investigating the maximum nitrogen removal rates that can be achieved and to progress towards real wastewater matrices.

4. Conclusions

Insights were provided for the novel integration of autotrophic/heterotrophic and anoxic/aerobic processes in a single-sludge system for thermophilic biological nitrogen removal through nitrification/denitrification. Fluctuations in redox (aerobic/anoxic) did not affect the activity of aerobic thermophilic nitrifiers in the long run (85 days), nor did spikes of organic carbon in three-day batch flask incubations. Furthermore, nitrifiers could

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functionally co-exist with aerobic heterotrophs in long-term reactor experiments ($COD/N_{influent}$ 0-3). When comparing 50 to 27°C under increasing $COD/N_{influent}$ ratio, thermophilic sludge production was lower, resulting in a higher sludge retention time (11.8±2.9 vs 8.7±0.9 d). This gave rise to a lower decrease in maximum nitrification rate, yielding similar conversion rates at $COD/N_{influent}$ 3 (50-60 mg NH_4^+-N g^{-1} VSS d^{-1}). Combining nitrification, recurrent anoxia and organic carbon in MBR experiments ($COD/N_{influent}$ 0.9-6.5) yielded complete nitrogen removal over 53 days at a rate of 50-104 mg N g^{-1} VSS d^{-1}, along with insights on the preservation of nitrifying activity. The next step is a focus on long-term steady-state operation of thermophilic single-sludge nitrification/denitrification and to progress towards real wastewater matrices.

Acknowledgements

The authors acknowledge (i) the Agency for Innovation by Science and Technology (IWT Flanders) [grant number SB-141205] for funding Tom G.L. Vandekerckhove, (ii) Wouter Peleman and Zoë Pesonen for practical support during their master thesis, (iii) Jolien De Paepe for assisting in the reactor operation, and (iv) Jo De Vrieze and Tim Lacoere for their help with qPCR and 16S rRNA gene amplicon sequencing.

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564 **Table and Figures**

565 **Table 1:** Integration of nitrification and aerobic COD removal sequencing batch reactors
566 (SBR): operational parameters. HRT: hydraulic retention time. SRT: Sludge retention time.
567 N/A: not determined.

Parameter	**Mesophilic SBR**	**Thermophilic SBR**
Temperature (°C)	27	50
Inoculum	Commercial mesophilic aquaculture inoculum	Thermophilic nitrifying sludge
Volume (L)	2	
pH (-)	7.25-7.35	
Dissolved oxygen concentration (mg O_2 L^{-1})	2	
HRT (d)	0.9 ± 0.2	
Ammonium concentration influent (mg N L^{-1})	101 ± 63	70 ± 46
Duration operational periods:		
$COD/N_{influent}$ 0 (d)	120	
$COD/N_{influent}$ 0.5 (acetate) (d)	49	
$COD/N_{influent}$ 0 (d)	63	
$COD/N_{influent}$ 0.5 (glucose) (d)	37	
Biomass-specific nitrogen loading rate (mg N g^{-1} VSS d^{-1})		
$COD/N_{influent}$ 0	93 ± 35	68 ± 44
$COD/N_{influent}$ 0.5 (acetate)	65 ± 24	39 ± 11
$COD/N_{influent}$ 0	252 ± 89	119 ± 88
$COD/N_{influent}$ 0.5 (glucose)	141 ± 90	72 ± 17
Biomass concentration (g VSS L^{-1})		
$COD/N_{influent}$ 0	1.7 ± 0.5	1.4 ± 0.4
$COD/N_{influent}$ 0.5 (acetate)	0.9 ± 0.3	1.6 ± 0.5
$COD/N_{influent}$ 0	0.6± 0.2	0.9 ± 0.4
$COD/N_{influent}$ 0.5 (glucose)	0.3 ± 0.2	0.5 ± 0.1
SRT (d)		
$COD/N_{influent}$ 0	33 ± 4	93 ± 38
$COD/N_{influent}$ 0.5 (acetate)	12 ± 2	43 ± 16
$COD/N_{influent}$ 0	N/A	N/A
$COD/N_{influent}$ 0.5 (glucose)	7.5 ± 1.6	33 ± 14

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570 **Table 2:** Long-term integration of nitrification and aerobic COD removal. Operational
571 parameters during the membrane bioreactor (MBR) experiments. HRT: hydraulic retention
572 time. SRT: Sludge retention time.

Parameter	**Mesophilic MBR**	**Thermophilic MBR**
Temperature (°C)	27	50
Inoculum	ABIL	Thermophilic nitrifying sludge
Volume (L)	2	
pH	7.25-7.35	
Dissolved oxygen concentration (mg O_2 L^{-1})	2	
HRT (d)	0.9 ± 0.3	0.9 ± 0.3
Ammonium concentration influent (mg N L^{-1})	80-120	100-150
Duration operational periods:		
$COD/N_{influent}$ 0 (d)	99	99
$COD/N_{influent}$ 1 (acetate) (d)	149	176
$COD/N_{influent}$ 3 (acetate) (d)	40	57**
Biomass-specific nitrogen loading rate (mg N g^{-1} VSS d^{-1})		
$COD/N_{influent}$ 0	64 ± 25	60 ± 29
$COD/N_{influent}$ 1 (acetate)	54 ± 17	60 ± 17
$COD/N_{influent}$ 3 (acetate)	51 ± 6	65 ± 26**
Biomass concentration (g VSS L^{-1})		
$COD/N_{influent}$ 0	1.7 ± 0.3	1.8 ± 0.4
$COD/N_{influent}$ 1 (acetate)	2.0 ± 0.2	2.0 ± 0.4
$COD/N_{influent}$ 3 (acetate)	2.2 ± 0.3	2.3 ± 0.3**
SRT (d)		
$COD/N_{influent}$ 0	250*	250*
$COD/N_{influent}$ 1 (acetate)	250*	250*
$COD/N_{influent}$ 3 (acetate)	8.7 ± 0.9	11.8 ± 2.9**

573 *: No sludge was wasted besides the daily sampling, yielding a SRT of ± 250 d

574 **: New inoculation

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580 **Table 3:** Integration of nitrification, aerobic COD removal and denitrification. Overview of the influent and effluent COD concentrations and
581 the biomass-specific nitrogen and COD loading rate ($B_{X,N}$ and $B_{X,COD}$ respectively) during the operation of the mesophilic and thermophilic
582 aerobic MBR reactors (at $COD/N_{influent}$ 3) and the 7 different reactor runs for nitrification/denitrification. The different lines within the same row
583 designate sequential operational phases within each run. *: removal efficiency during periods before loss of nitrification

	Aerobic	Aerobic	Aerobic/anoxic						
Parameter	**COD/N 3 (27°C)**	**COD/N 3 (50°C)**	**Run I**	**Run II**	**Run III**	**Run IV**	**Run V**	**Run VI**	**Run VII**
Test duration (d)			45	37	1-15 15-24 24-37	0-7 7-10 10-17	0-28	0-15 15-36 36-42	0-22 23-53
$COD/N_{influent}$	3.7 ± 0.5	3.0 ± 0.8	6.5 ± 1.5	5.6 ± 2.9	0 2.7 ± 0.3 4.2 ± 1.1	0.9 ± 0.3 2.3 ± 0.2 3.7 ± 0.3	4.0 ± 0.6	3.4 ± 0.4 5.5 ± 0.7 5.8 ± 0.4	6.1 ± 0.7 4.4 ± 0.5
$B_{X,COD}$ (mg COD g^{-1} VSS d^{-1})	137-267	89-188	434-468	365-517	0 236-272 334-352	51-88 102-193 314-322	336-520	219-316 137-262 294-298	269-488 265-422
$B_{X,N}$ (mg N g^{-1} VSS d^{-1})	42-64	34-90	73-96	29-88	57-117	89-116	80-126	60-105 31-54 50-53	20-67 61-104
$COD_{acetate,\ removed}/N_{removed}$	/	/	/	/		3.0 ± 1.1 5.5 ± 0.3 5.2 ± 0.9	9.4 ± 4.5	5.1 ± 1.0 13.3 ± 6.5 5.2 ± 1.4	5.1 ± 0.7 3.8 ± 0.6
$COD_{removed}/N_{removed}$	/	/	15.4 ± 4.3	10.9 ± 4.7	0 4.2 ± 1.4 /	1.6 ± 0.2 3.1 ± 0.2 3.7 ± 0.3	5.7 ± 3.9	5.4 ± 1.2 6.7 ± 5.6 4.1 ± 0.7	4.8 ± 0.6 3.0 ± 0.4
N removal efficiency (%)*	/	/	42 ± 12	52 ± 10	/ 54 ± 22 /	32 ± 8 45 ± 10 /	46 ± 23	47 ± 7 31 ± 10 87 ± 17	92 ± 15 93 ± 5
$COD_{influent}$ (mg COD L^{-1})	325-412	173-644	1971-2372	1315-1923	0 654-840 1160-1206	281-337 577-566 818-993	802-1327	247-373 217-333 557-636	540-1438 546-857
$COD_{effluent}$ (mg COD L^{-1})	23-28	39-156	273-699	92-160	0 147-447 359-532	48-52 56-62 166-184	160-770	38-143 132-320 198-274	30-194 210-302

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584 **Table 4:** Integration of nitrification, aerobic COD removal and denitrification. Overview of the operation parameters during the 6 thermophilic
585 nitrification/denitrification reactor runs. HRT: hydraulic retention time. MBR: membrane bioreactor. SBR: sequencing batch reactor.
586 $COD/N_{influent}$: ratio of COD and nitrogen concentration in the influent.

Parameter	**Run I**	**Run II**	**Run III**	**Run IV**	**Run V**	**Run VI**	**Run VII**
Inoculum	Thermophilic nitrifiers[a]	Thermophilic nitrifiers[a] + mesophilic denitrifiers[b]	Thermophilic nitrifiers[a]				
Reactor type	MBR	MBR	MBR	SBR	MBR	MBR	MBR
Reactor operation (d)	45	37	37	17	33	42	53
Carbon source	Soluble starch	Soluble starch	Glucose	Acetate	Acetate + pyruvate	Acetate + pyruvate	Acetate + pyruvate
$COD/N_{influent}$	6.5 ± 1.5	5.6 ± 2.9	2.7 ± 0.3 4.2 ± 1.1	0.9 ± 0.3 2.3 ± 0.2 3.7 ± 0.3	4.0 ± 0.6	3.37 ± 0.37 5.5 ± 0.7 5.8 ± 0.3	6.1 ± 0.7 4.4 ± 0.5
HRT (d)	1.5 ± 0.9	1.3 ± 0.2	1.2 ± 0.7	1.3 ± 0.2	1.3 ± 0.2	0.9 ± 0.1	0.9 ± 0.3
Anoxic/total phase ratio (-) and length (min/min)	0.3; 30/90	0.2; 20/90	0.2; 7/30	0.2; 7/30	0.2; 7/30	0.4; 13/30	0.4; 13/30
Ammonium concentration influent (mg N L^{-1})	300	300-350	300	250-300	250-300	100 50 100	75-140 140-220
Biomass concentration (g VSS L^{-1})	2.9 ± 0.5	3.8 ± 0.3	3.3 ± 0.3	2.4 ± 0.07	2.2 ± 0.35	1.6 ± 0.2	2.5 ± 0.3
Biomass-specific nitrogen loading rate (mg N g^{-1} VSS d^{-1})	83 ± 12	73 ± 20	86 ± 21	105 ± 12	103 ± 19	59 ± 23	50 ± 14 77 ± 15

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587 a: From previously described thermophilic nitrifying bioreactors (Courtens et al., 2016a; Courtens et al., 2016b)
588 b: Nitrifying/denitrifying biomass (Avecom, Belgium)

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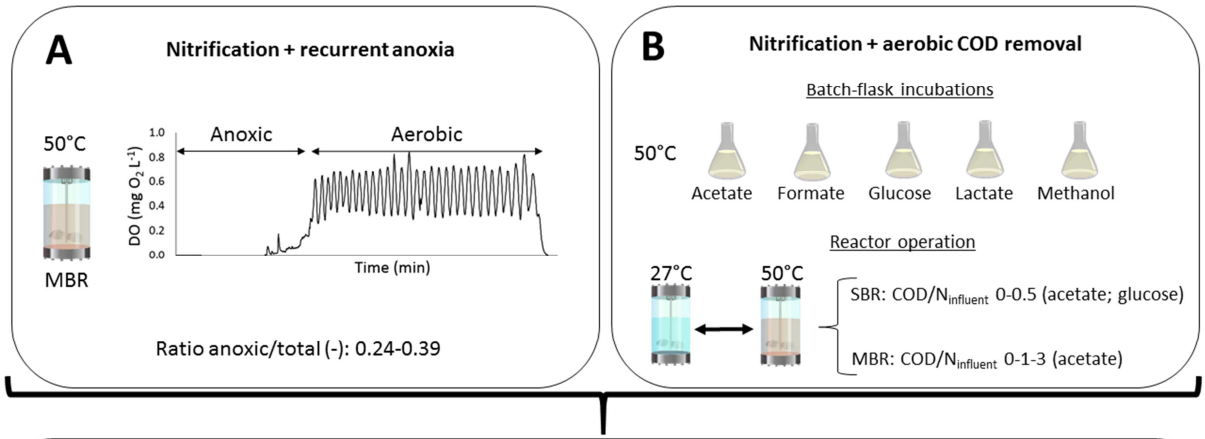

C

Nitrification + recurrent anoxia + aerobic COD removal

	Reactor type	Ratio anoxic/total (-)	$COD/N_{influent}$	COD source
Run I	MBR	0.3	6.5	Soluble starch
Run II	MBR	0.2	5.6	Soluble starch
Run III	MBR	0.2	2.7-4.2	Glucose
Run IV	SBR	0.2	0.9-3.7	Acetate
Run V	MBR	0.2	4	Acetate + pyruvate
Run VI	MBR	0.4	3.4-5.8	Acetate + pyruvate

50°C

Figure 1: Schematic overview of experiments. Nitrification and recurrent anoxia in a membrane bioreactor (MBR) (A). Nitrification and aerobic COD removal in short- and long-term incubations in batch flasks, sequencing batch reactors (SBR) and MBR (B). Nitrification, denitrification and aerobic COD removal in MBR and SBR (C).

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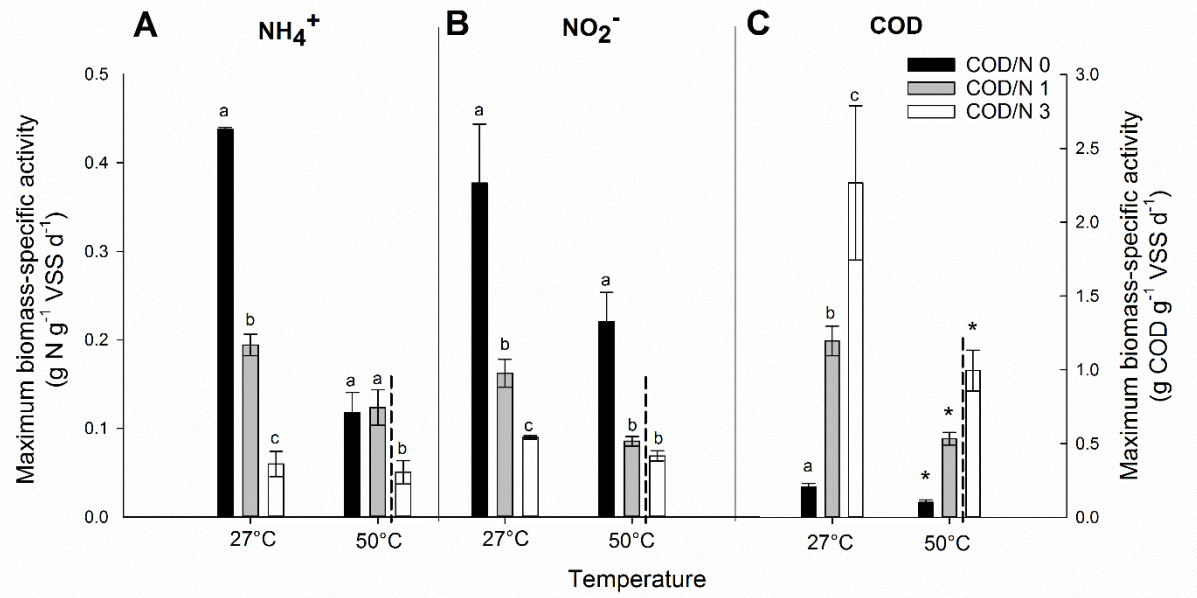

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597 **Figure 2:** Long-term integration of nitrification and aerobic COD removal in MBR.
598 Maximum biomass-specific ammonium (A), nitrite (B) and COD (C) oxidation rates at 27
599 and 50°C at the tested $COD/N_{influent}$ ratios. Significant pairwise differences ($p<0.05$) in
600 q_{max,NH_4^+-N}, q_{max,NO_2^--N} and $q_{max,COD,aerobic}$ at 27 and 50°C are indicated with different **non-**
601 **capital** letters. Vertical dashed line represents the new inoculation of the thermophilic MBR
602 at $COD/N_{influent}$ 3. *: applied statistical method was not able to show pairwise significant
603 difference.

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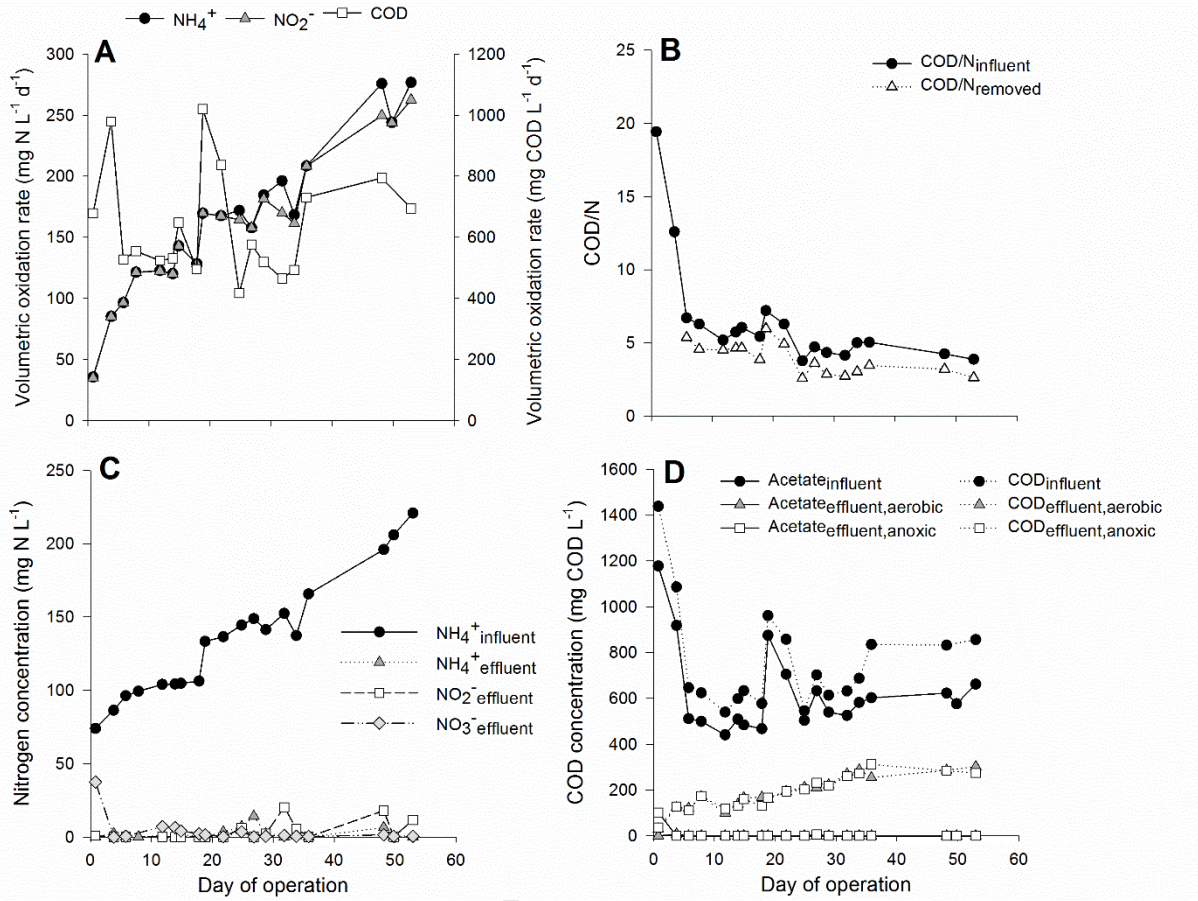

Figure 3: Integration of nitrification, denitrification and aerobic COD removal. Run VII, with (A) the ammonium, nitrite and COD oxidation rates, (B) the COD/N ratio in the influent and the removed COD/N, (C) the influent and effluent nitrogen concentrations and (D) the influent and effluent COD concentrations.

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Highlights (5 bullet points, 85 characters per bullet)

- The concept of single-sludge nitrification/denitrification was demonstrated at 50°C
- Thermophilic nitrifiers can cope with redox fluctuations (aerobic/anoxic)
- Thermophilic nitrifiers can functionally co-exist with heterotrophs
- 92-100% nitrogen removal was achieved at $COD/N_{removed}$ 4.8 ± 0.6

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Author contribution section

Tom G.L. Vandekerckhove performed the experiments, helped in the design of the experiments and wrote the paper

Nico Boon helped design the experiments, provided valuable input for writing the paper and reviewed the manuscript

Siegfried E. Vlaeminck as corresponding author designed the experiment, provided valuable input and helped write the paper.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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