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Abstract

In specific municipal and industrial cases, thermophilic wastewater treatment (>45°C) might bring cost advantages over commonly applied mesophilic processes (10-35°C). To develop such a novel process, one needs sound parameters on kinetics, sludge yield and sludge settleability of three heterotrophic conversions: aerobic carbon removal, denitritation and denitrification. These features were evaluated in acetate-fed sequencing batch reactors (30, 40, 50 and 60°C). Higher temperatures were accompanied by lower sludge production and maximum specific removal rates, resulting mainly from lower maximum growth rates. Thermophilic denitritation was demonstrated for the first time, with lower sludge production (18-26%), higher nitrogen removal rates (24-92%) and lower carbon requirement (40%) compared to denitrification. Acceptable settling of thermophilic aerobic (60°C) and anoxic biomass (50 and 60°C) was obtained. Overall, this parameter set may catalyze the establishment of thermophilic nitrogen removal, once nitritation and nitratation are characterized. Furthermore, waters with low COD/N ratio might benefit from thermophilic nitritation/denitritation.

Keywords: Short-cut nitrogen removal; metabolic flexibility; industrial wastewater treatment; observed yield; decay constant

1. Introduction

One of the key paths for nitrogen to end up in the environment is the discharge of wastewater (Coppens et al., 2016). Untreated wastewaters typically contain organic carbon and ammoniacal nitrogen, which may cause, among others, eutrophication, oxygen depletion and fish mortality (Camargo & Alonso, 2006). To alleviate the environmental burden of nitrogen and organic carbon, biological wastewater treatment is the most widely used manner to treat wastewater prior to discharge, and is typically performed at temperatures between 5 and 35°C (Henze et al., 2008). Thermophilic removal of nitrogen and organic carbon can be an attractive alternative for the current mesophilic practices. Specific applications can be found (i) in regions where the climatic conditions give rise to several types of hot wastewater, (ii) in specific industries delivering wastewater at high temperature, due to the production process or pre-treatment of the wastewater (for example thermophilic digestion), and (iii) on sites where waste heat can be used to warm the wastewater.

Experience with high-temperature aerobic carbon removal identified several advantages, such as a more stable process with higher specific rates (smaller bioreactors), a lower sludge production (lower cost for sludge disposal) and a lower level of contamination at thermophilic conditions (Lapara & Alleman, 1999). However, thermophilic treatment suffers from deteriorated sludge settling due to dispersed growing organisms, only one study reported better settling (Barr et al., 1996). Furthermore, contradictory outcomes regarding the kinetic advantage (LaPara et al., 2000a) and lower sludge production (Vogelaar et al., 2003) warn for caution. In studies on thermophilic aerobic wastewater

treatment, nitrogen removal was assumed to be facilitated by ammonia volatilization and nitrogen assimilation into biomass (Abeynayaka & Visvanathan, 2011a; Abeynayaka & Visvanathan, 2011b; Kurian et al., 2005). As ammonia stripping shifts the problem to the gas phase and the C/N ratio in high-strength nitrogenous wastewaters is too low to obtain sufficient nitrogen removal based on assimilation, it is advisable to remove nitrogen biologically *via*, for example, nitrification/denitrification. Recently, two strategies were successfully implemented to develop lab-scale thermophilic nitrifying bioreactors (Courtens et al., 2016a; Courtens et al., 2016b). The biotechnological feasibility of thermophilic denitrification at 55°C has been demonstrated as well, accompanied by advantages as lower carbon requirement (±23%), reduced sludge production (up to 45%) and improved settling (73% lower sludge volume index) when compared to 34°C (Courtens et al., 2014). Shortcut nitrogen removal *via* nitritation/denitritation could introduce additional economic and ecological benefits in case of low carbon (Fux & Siegrist, 2004; Peng et al., 2017). To the authors knowledge, however, thermophilic denitritation has not been investigated yet.

Since the key conversions for carbon and nitrogen removal at thermophilic temperatures are at hand, the main challenge is now to combine the separate processes. As the biomass concentration is usually kept constant in wastewater treatment systems, a high organic carbon loading rate compared to nitrogen loading rate results in massive growth of heterotrophs. As a consequence, more sludge must be wasted to keep the biomass concentration stable, possibly lowering the SRT below the growth rate of nitrifiers and thus washing them out of the system. To effectively integrate these processes, it is vital to

safeguard nitrification by preventing that heterotrophs outcompete autotrophs. For this, gaps in heterotrophic process parameters need to be filled to attain a sound and elaborate set of data linked to thermophilic kinetics, sludge yield and sludge settleability. Mainly regarding anoxic conversions, data is limited. This study focused on all heterotrophic conversions relevant to conventional and shortcut nitrogen removal applications with activated sludge, being aerobic carbon removal, denitritation and denitrification. In parallel sequencing batch reactors (SBR), the impact of four distinct temperatures (30, 40, 50 and 60°C) was evaluated in depth on kinetic and sludge parameters, along with the retention of metabolic flexibility.

2. Materials and methods

2.1. Reactor set-up and operation

Three parallel sequencing batch reactors (SBR) of 2L, open to the atmosphere, were operated to evaluate aerobic carbon removal, denitritation and denitrification, respectively. The reactors were run at 30, 40, 50 and 60°C using a circulating thermostatic water bath (Julabo MA-4). The transition between temperatures was executed by removing the reactor content and re-inoculating at 2 g volatile suspended solids (VSS) L^{-1} with nitrifying/denitrifying sludge, originating from a landfill leachate wastewater treatment plant with an average temperature of 26.3 ± 3.6°C. Prior to inoculation, the biomass was stored in a fridge at 4°C. The pH was controlled around 7.6 *via* the addition of 0.5M HCl. A 3h cycle was comprised of a 2.5h reaction phase of which 2h pulse feeding, followed by a 15 min settling period and a 10 min draw and 5 minutes idle phase. The biomass concentration in the reactors was kept around 2 g VSS L^{-1} by manually wasting sludge

during the idle phase when necessary. By fixing the biomass concentration at 2 g VSS L^{-1}, the sludge retention time (SRT) was a consequence of the sludge production and was identified by making the sludge balance (in terms of VSS) over the reactors on four consecutive days.

All reactors were fed with synthetic wastewater at a flow rate of 4 L d^{-1}, resulting in a hydraulic retention time of 0.5d. The synthetic wastewater contained sodium acetate as carbon source, NH_4Cl as nitrogen source, KH_2PO_4 as phosphorus source and trace elements dissolved in tap water (Supplementary material). The amount of N and P added to the feed was based on an assumed biomass production of 0.4 g VSS g^{-1} COD, the standard biomass composition (Metcalf et al., 2003) and a safety factor of 2 to ensure excess available nutrients and prevent growth limitation. The safety factor was not used for the anoxic feed, as COD was already present in excess. Oxygen was continuously supplied as electron acceptor to the aerobic carbon removing reactor *via* air pumps connected to diffuser stones, while $NaNO_2$ and $NaNO_3$ (0.35 g N L^{-1}) provided the electron acceptor in the denitritation and denitrification reactor respectively *via* the feed The COD/N ratios in the feed of the denitritation (5.5 ± 0.3) and denitrification (7.3 ± 0.3) reactor were chosen so that the effluent would contain about 1 g COD L^{-1}, the same as the influent of the aerobic reactor similar to the pre-denitrification principle. The food-to-microorganisms rate (F/M) ranged from 0.9-1.2, 1.7-2.7 and 2.0-2.6 g COD g^{-1} VSS d^{-1} in the aerobic reactor, denitritation reactor and denitrification reactor respectively (Supplementary material). The initial COD concentration ranged from 10-22, 497-1049 and 496-1062 mg COD L^{-1} after each feeding pulse in the aerobic reactor, denitritation reactor and denitrification reactor, respectively.

This was calculated based on the influent flow rate, the amount of feed pulses and the influent COD concentration. In the denitritation and denitrification reactor, the remaining COD was taken into account as well, as COD was fed in excess as described above. In the aerobic reactor, effluent acetate concentration was negligible.

At each temperature, the three reactors were run under these conditions for at least 5 times the SRT before the determination of biomass characteristics, biomass kinetics and the metabolic flexibility of the community. Time of operation was 24, 50, 49 and 65 days at 30, 40, 50 and 60°C respectively.

2.2. Biomass production and conversion kinetics

The observed biomass yield (Y_{obs}) was calculated by making the sludge balance over the reactors for 5 consecutive days and by using cumulative terms, as described previously (Courtens et al., 2014). The slope of the ordinary least squares best-fit line and the standard deviation of this slope were taken as Y_{obs} and its error, respectively.

To determine the maximum specific aerobic carbon removal rate ($q_{max,COD,aerobic}$) of the aerobic carbon removing sludge, the reactor content was transferred to a temperature controlled respirometry setup (2L). Dissolved oxygen was monitored and pH was controlled at a setpoint of 7.6 ± 0.2 using 0.5M HCl/NaOH solutions. LabView (National Instruments) software was used as user interface for monitoring the respirometer and controlling the pH. Respirometry is a widely used, indirect, yet validated manner to study aerobic bioprocesses (Vanrolleghem et al., 1999). It tracks substrate removal by continuous, high-frequency online oxygen measurements. The oxygen mass transfer coefficient (k_La) was experimentally determined in triplicate by using the dynamic gassing

out method (Bandyopadhyay et al., 2009). Using the oxygen mass balance (1), the exogenous oxygen uptake rate (OUR_{ex}) was obtained by discretization of the equation. The OUR_{end} was assumed to be constant throughout the short experiment and was accounted for in the aeration term by replacing the theoretical saturation concentration by the actual equilibrium concentration S_0^{eq}.

$$\frac{dS_O}{dt} = k_La\ (S_0^{eq} - S_0) - OUR_{ex} \qquad (1)$$

With S_O the oxygen concentration at time point t. To determine the maximum OUR_{ex}, substrate was added at a concentration of 70, 25, 65 and 60 mg COD L^{-1} at 30, 40, 50 and 60°C, at least three times the substrate affinity constant (23.2, 3.2, 18.7 and 12.3 mg COD L^{-1} respectively). At these concentrations, an equilibrium was established between oxygen uptake and oxygen provision *via* aeration, resulting in a stable oxygen concentration to determine maximum OUR_{ex}.

The OUR_{ex}, however, only gives an indication of the oxidized substrate. Actual substrate removal is both due to oxidation and growth. Therefore, to obtain the $q_{max,COD,aerobic}$, both the maximum OUR_{ex} and maximum biomass yield (Y_{max}) were taken into account. The Y_{max} can be estimated based on respirometry (Strotmann et al., 1999). In short, it is assumed that all dosed substrate is either oxidized or taken up by the biomass for storage or growth. Complete removal of the dosed substrate was reflected by the drop in OUR back to the endogenous level (OUR_{end}). The amount of substrate directed towards respiration can be determined as the cumulative oxygen consumption during the removal of the added substrate. The fraction of COD not directed to respiration was assumed to be incorporated for biomass production, enabling the calculation of Y_{max}. As that the unit of Y_{max} is g COD

g^{-1} COD, a conversion factor of 1.42 g COD g^{-1} VSS based on the empirical formula of organic biomass ($C_5H_7N_2O$) was used to convert Y_{max} to g VSS g^{-1} COD. The respirometric determination of Y_{max} was always performed minimally in quadruplicate. Each $q_{max,COD,aerobic}$ and the biomass concentration were determined in triplicate. Error propagation was used to calculate the error on the obtained final values.

During the respirometry test, the substrate affinity constant for acetate as COD source could be determined. For this, the OUR_{ex} was measured at different COD concentrations. The value of $K_{s,COD}$ could be calculated by plotting the OUR_{ex} as a function of the COD concentration and by fitting a monod-type curve (2).

$$OUR_{ex} = OUR_{ex,max} * \frac{S}{K_{S,COD}+S} \quad (2)$$

With S being the substrate concentration. The affinity constant was estimated in Python, by means of the pyIDEAS package (Van Daele et al., 2015), using the Nelder-Mead algorithm for parameter optimization. To estimate the 95% confidence intervals, the inverse of the Fisher Information Matrix was calculated to obtain a linear approximation of the covariance matrix (Omlin & Reichert, 1999).

The decay rate (k_d) of the aerobic biomass at every temperature could be derived from equation (3), since Y_{obs} stems from the interplay between maximum biomass yield (Y_{max}), decay rate (k_d) and sludge retention time (SRT). The Y_{obs} and SRT were obtained during the reactor experiments, while Y_{max} was derived in the respirometry experiment.

$$Y_{obs} = \frac{Y_{max}}{1+SRT*k_d} \quad (3)$$

The maximum aerobic growth rate ($\mu_{max,aerobic}$) can be calculated as the product of the maximum biomass yield (Y_{max}) and the maximum specific aerobic carbon removal rate ($q_{max,COD,aerobic}$) (Metcalf et al., 2003). Error propagation was used to calculate the error on the obtained final values.

Anoxic batch activity tests were performed using sludge from the anoxic reactors to determine the maximum specific anoxic nitrogen removal rate ($q_{max,N,anoxic}$). Serum flasks of 120 mL were utilized, containing 80 mL of mixed liquor and buffer solution with a final concentration of 2 g P L^{-1} (KH_2PO_4/K_2HPO_4) and a pH of 7.6. Biomass concentration was determined using the concentration in the reactors (determined in triplicate) and the imposed dilution in the serum flasks. Rubber stoppers sealed off the flasks after which they were flushed with N_2 gas. A flushed substrate solution of $NaNO_3/NaC_2H_3O_2$ (COD/N=6.7) or $NaNO_2/ NaC_2H_3O_2$ (COD/N=5.5) was supplemented by means of needled syringes to a final concentration of 50 mg N L^{-1}. All tests were performed in triplicate on a temperature-controlled shaker (120 rpm). Liquid samples were taken, filtered over 0.2 µm filters and stored at 4°C for nitrite, nitrate and COD analysis. To exclude any biomass growth during storage of the COD samples, the pH was adjusted to 11. Error propagation was used to calculate the error on the obtained final values.

2.3. Sludge settleability

Sludge settleability was determined *via* the sludge volume index (SVI) and the initial settling velocity (ISV). SVI was measured by monitoring the sludge height after 30 minutes of settling in-situ in the cylindrical reactors (2L). As such, the test could be performed at the desired temperature. To determine ISV, the time needed to settle over a distance of 5

cm below the air-liquid interface was monitored. Lastly, the particle size distribution (PSD) was determined using the video channel of an EyeTech particle size analyzer (Ankersmid, The Netherlands). The resulting PSDs were projected as absolute number of particles vs. particle size (µm). The D10, D50 and D90 represent the particle size (µm) for which 10, 50 and 90% of the number of particles are smaller. SVI, ISV and biomass concentration were determined in triplicate at each temperature. Error propagation was used to calculate the error on the obtained final values. Microscopic images of the biomass were taken for qualitative comparison of filaments using a Zeiss Axioskop microscope, equipped with a Canon EOS 700D and Image Pro Insight software.

2.4. Retention of metabolic flexibility

The original inoculum, containing both aerobic and anoxic heterotrophs, was subjected to one specific condition for several SRT with either oxygen, nitrite or nitrate as electron acceptor. After stabilization, the metabolic flexibility or the ability to perform the different functionalities (aerobic carbon removal, denitritation and denitrification) was evaluated. Ex-situ anoxic batch activity tests were performed with sludge from the aerobic carbon removing reactor as described above (Section 2.2), using nitrite (as $NaNO_2$) or nitrate (as $NaNO_3$) as electron acceptor. For the denitritation reactor, an anoxic batch activity test was performed using $NaNO_3$ as electron acceptor, as well as an aerobic respirometric test as described earlier (Section 2.2) to determine $q_{max,COD,aerobic}$. For the denitrification reactor, the same tests were carried out as for the denitritation reactor, but with nitrite as electron acceptor in the anoxic batch activity test. Prior to the aerobic activity tests with sludge from the anoxic reactors, the remaining COD was first oxidized biologically through aeration

before performing the actual test. Maximum specific removal rates and the required biomass concentration were determined in triplicate and error propagation was used to calculate the error.

2.5. Chemical analyses

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (APHA, 1992). Nitrite and nitrate were determined on a 761 Compact Ion Chromatograph (Metrohm, Switzerland) equipped with a conductivity detector and a calibration curve between 0.05-100 mg L^{-1}. Acetate was measured using a 930 Compact IC Flex (Metrohm, Switserland), equipped with an organic acid column (Metrosep 250/7.8, Metrohm), a protection column (Metrosep Dual 4/4.6, Metrohm) and a 850 IC conductivity detector. To calculate COD concentrations, the measured acetate concentration and a conversion factor of 1.084 g COD g^{-1} acetate was used.

2.6. Statistical analysis

Statistical analysis was applied to determine whether values of certain parameters were statistically significantly different at the tested temperatures. Details can be found in the Supplementary material. Correlations between different parameters were tested using the non-parametric Spearman's correlation test.

3. Results and discussion

3.1. Overall performance of the reactors

Aerobic carbon removal was characterized by a fast start-up in each run, obtaining 100% acetate removal efficiency at the imposed loading rate after the first day of operation. For both anoxic processes, a start-up period of less than one week was necessary. Actual pH in the reactors resided close to the setpoint and for each process no significant difference ($p>0.05$) was observed in pH between the tested temperatures (Supplementary material). A biomass concentration of 2 g VSS L^{-1} was aimed for by wasting sludge manually during the idle phase when necessary. From 50°C onwards, no sludge wasting was necessary for the denitritation reactor, while for the aerobic carbon removal and denitrification, sludge disposal was no longer needed from 60°C. Nonetheless, sludge proliferated and stable biomass concentrations were obtained (Supplementary material). The average SRT for each process at 30 and 40°C remained between 3.3 and 4.6 d. From 40°C onwards, the average SRT increased, ranging between 5.8 and 6.2 d at 50°C and between 9.3 and 10.8 d at 60°C (Supplementary material).

It is unknown whether the elevated temperatures selected for thermophilic organisms or adapted mesophilic bacteria. However, previous research on thermophilic aerobic treatment has shown a shift in microbial community towards a lower microbial diversity compared to mesophilic conditions (LaPara et al., 2000a; Tripathi & Allen, 1999), with mostly certain species of *Bacillus* and *Thermus* thriving under these conditions (Lapara & Alleman, 1999). Even short-term (±10 days) perturbations in reactor temperature (30 to 45°C) resulted in a different community compared to the 30°C control, with a high degree of variability in

community structure between replicate systems (Nadarajah et al., 2007). Also for denitrification, imposing thermophilic conditions to a mesophilic culture rendered a different community compared to common mesophilic operation (Courtens et al., 2014). Considering the obtained $\mu_{max,aerobic}$, a correction factor of 0.8 for the change in μ_{max} in anoxic conditions (Henze, 2000) and the time of operation under each temperature in this study, the amount of doubling times ranged between 253 and 879 over the temperatures and conditions tested. Even though the in-situ growth rates were most likely lower because substrate was limiting (COD in the aerobic and nitrite/nitrate in the anoxic reactors), it is reasonable to assume that the duration was long enough to cause a considerable community shift.

Besides a community shift, physiological adaptations may also occur at thermophilic conditions. Thermophilic communities have displayed a more selective physiology and were limited in the range of COD sources that could be oxidized (Tripathi & Allen, 1999). It is, thus, possible that the decrease in diversity and the limitations in COD-types renders different physiological pathways in high temperature cultures. Additionally, mesophilic conditions that are not stressful either select for fast growing organisms with a low substrate affinity (r strategists) or for slow growing organisms with a high substrate affinity (K strategists). Extreme conditions, i.e. thermophilic temperatures, may render micro-organisms implementing a third strategy, called the L-strategy, which is invoked by organisms that are well adapted to such extreme conditions and may render different physiological pathways (Golovlev, 2001).

3.2. Aerobic carbon removal

Some contradictory results exists when comparing maximum aerobic carbon oxidation rates between mesophilic and thermophilic temperatures. Higher oxidation rates at elevated temperatures can be up to a factor 3-10 (Lapara & Alleman, 1999), while others stated no difference (Jahren et al., 2002; LaPara et al., 2000a; LaPara et al., 2000b). In this study, conducted under stable conditions and using synthetic feed, $q_{max,COD,aerobic}$ decreased at elevated temperatures (**Fig. 1, A**). The $q_{max,COD,aerobic}$ at 30°C and 40°C was 16.5 ± 0.4 and 20.6 ± 1.1 g COD g^{-1} VSS d^{-1} but decreased significantly ($p<0.05$) from 50°C onwards. At 50 and 60°C the maximum activity was 8.7 and 9.5 g COD g^{-1} VSS d^{-1}, being only 53 and 58% of the activity at 30°C. Taking into account the inorganic fraction of the biomass, and thus the total sludge concentration, the $q_{max,COD,aerobic}$ at 50 and 60°C was 7.6 and 4.3 g COD g^{-1} TSS d^{-1} respectively. These values are quite low compared to literature, reporting thermophilic rates between 5.7 and 31.6 g COD g^{-1} TSS d^{-1} at temperatures of 45 to 65°C, calculated as the product of $\mu_{max,aerobic}$ and Y_{max} (Couillard et al., 1989; Couillard & Zhu, 1993; Lapara & Alleman, 1999; Surucu et al., 1975). At 40°C, a low affinity constant for acetate of 3.2 ± 2.7 mg COD L^{-1} was found, compared to slightly higher values for the other temperatures (23.2 ± 5.3, 18.7 ± 8.4, 12.3 ± 7.0 mg COD L^{-1} at 30, 50 and 60°C respectively) (**Fig. 1, D**). These relatively high substrate affinities are in accordance with another study at 55°C using acetate as carbon source, reporting a K_s value of 3 ± 2 mg COD L^{-1} (Vogelaar et al., 2003). Higher affinity constants have been reported (Couillard et al., 1989; LaPara et al., 2000b; Surucu et al., 1976), but could have been caused by a non-biodegradable fraction of the organic carbon or by soluble microbial products regarded as non-biodegraded substrate (Vogelaar et al., 2003).

Sludge handling and disposal can be a significant cost, amounting up to 40% of the operational cost for sewage treatment (Zessner et al., 2010). Applying thermophilic conditions to the system can bring about a reduction in sludge production and possibly renders the treatment more cost-effective. In this study, aerobic carbon removal at 60°C produced up to 54 ± 7% less sludge compared to 30°C (**Fig. 1, B**). The obtained Y_{obs} values at 50 and 60°C, 0.16 and 0.11 g VSS g^{-1} COD respectively, were within the range of reported values of 0.05 and 0.3 g VSS g^{-1} $COD_{removed}$ (Suvilampi & Rintala, 2003). Although thermophilic sludge contained more inorganics, total sludge production (TSS) was still lower compared to mesophilic temperatures (**Fig. 1, B**). The observed biomass yield factor is a result of biomass growth and decay and its value is a function of the maximum biomass yield (Y_{max}), the sludge decay rate (k_d) and the sludge retention time (SRT). In literature, thermophiles are often reported to have higher growth rates, but also have a higher energy requirement for maintenance and a higher decay rate than mesophiles, resulting in a lower net microbial growth and sludge production (Lapara & Alleman, 1999; Vogelaar et al., 2003). However, the kinetic values determined in this study do not support this statement and point in another direction. At every tested temperature, Y_{max} did not vary beyond the range of 0.53 and 0.60 g VSS g^{-1} COD (**Fig. 1, B**), which corroborates a previous statement that Y_{max} is hardly impacted by temperature (Vogelaar et al., 2003). Maximum growth rate ($\mu_{max,aerobic}$) decreased from 9.1 and 12.2 d^{-1} at 30 and 40°C to 4.6 and 5.7 d^{-1} at 50 and 60°C onwards. The decay rate on the other hand, did not increase in the same proportion and ranged between 0.38 and 0.49 d^{-1} (**Fig. 1, E and C**). These findings suggest that the reduction in biomass production resulted mainly from decreasing maximum growth rate and increasing SRT and less from increasing decay rate at higher

temperatures. Lower yields, however, can result in increased aeration requirement as less organic carbon is directed towards biomass.

It is often reported that thermophilic aerobic wastewater treatment suffers from deprived sludge settling (Lapara & Alleman, 1999). The main reason is poor floc formation, resulting in a higher biomass concentration in the effluent (Liao et al., 2011; Suvilampi & Rintala, 2003). In this study, however, good floc formation at 40, 50 and 60°C was reflected in higher effluent quality in terms of biomass concentration (Supplementary material). The average SVI increased with temperature from 67 ± 1 mL g^{-1} VSS at 30°C to 191 ± 6 mL g^{-1} VSS at 50°C and decreased back to 82 mL g^{-1} VSS at 60°C (**Fig. 4, A**). The considerable rise in SVI in the aerobic reactor between 30 and 50°C might be attributed to a higher level of filamentous organisms rather than poor floc formation. Although filaments were not quantified, a higher amount was visible when comparing under a microscope at 30 and 50°C (Supplementary material). Literature reports that the amount of filamentous bacteria is positively correlated with the SVI, with a higher levels of filaments in thermophilic compared to mesophilic systems (Liao et al., 2011). The enhanced settling and lower SVI at 60°C could be due to the higher inorganic content of the sludge that brought about a higher specific biomass density. Higher initial settling velocities (ISV) can facilitate faster effluent withdrawal and lowers the risk of biomass washout. The ISV did not show a correlation with increasing temperature (0%, $p>0.05$) and ranged between 0.59 and 3.78 m h^{-1} (**Fig. 4, B**).

No clear difference in PSD was found in function of temperature, except at 40°C when there was a bigger share of larger particles, reflected in a higher D50/D90 value, which

could explain the elevated ISV at 40°C (Supplementary material). At 60°C, a drop in VSS/TSS ratio was accompanied by an increase in ISV, implying a higher inorganic content which is related to higher density and higher settling velocities (Schuler & Jang, 2007; Vlyssides et al., 2008). Settling velocity thus relies on multiple factors and may be affected by particle size and inorganic content in the biomass. Overall, at 30 and 60°C, practically acceptable settling was obtained, meaning SVI values below 100 mL g^{-1} VSS (67 ± 1 and 82 ± 5 mL g^{-1} VSS respectively).

The ratio of volatile over total suspended solids (VSS/TSS) of the biomass was negatively correlated with increasing temperature (>71%, $p<0.05$) (**Fig. 4, C**).The increasing inorganic fraction of the aerobic sludge at 60°C (**Fig. 4, C**) could partly be explained by the slightly higher decay rate and the significantly longer SRT rendering more accumulation of inorganic lysis products, but also by the higher accumulation of precipitated inorganic compounds as calcite and dolomite (Supplementary material). The precipitation of inorganics was simulated using PHREEQC, a hydrogeochemical modeling software (Parkhurst & Appelo, 1999), details can be found in the Supplementary material. Furthermore, at 60°C, the effluent VSS/TSS (±92.0%) was higher than the mixed liquor VSS/TSS (±46.5%), meaning the less dense biomass (VSS) was more prone to washout, also contributing to an increasing fraction of inorganics in the reactor (Supplementary material).

3.3. Denitritation and denitrification

To our knowledge, few studies have investigated thermophilic denitrification and no studies exist on thermophilic denitritation. One experiment inoculated an upflow sludge blanket

reactor at 55°C with thermal mud from a hot spring and obtained a maximum denitrification rate ($q_{max,N,anoxic}$) of 51 mg N g^{-1} VSS d^{-1} (Laurino & Sineriz, 1991). A more recent study, comparing mesophilic and thermophilic denitrification (34 vs. 55°C), stated a lower $q_{max,N,anoxic}$ at thermophilic conditions (922 vs 435 mg N g^{-1} VSS d^{-1}) and a 45% reduced sludge production (Courtens et al., 2014). This is in accordance with the findings of this study. The specific denitrification rate at 30°C was 2.5 g N g^{-1} VSS d^{-1} (**Fig. 3, A**). Increasing the temperature to 50 and 60°C resulted in a decrease in activity to 1.6 ± 0.1 and 1.5 ± 0.2 g N g^{-1} VSS d^{-1} respectively. The specific denitritation rate significantly ($p<0.05$) declined at 60°C to 1.9 ± 0.3 g N g^{-1} VSS d^{-1}, compared to 3.3 ± 0.2 g N g^{-1} VSS d^{-1} at 30°C (**Fig. 3, A**). The obtained values for $q_{max,N,anoxic}$ at thermophilic temperatures were higher than ever reported. The $q_{max,N,anoxic}$ in terms of TSS was lower than in terms of VSS due to the inclusion of the inorganic fraction of the sludge (**Fig. 3, B**). As for carbon requirement for denitrification, no significant reduction ($p>0.05$) in $COD/N_{removed}$ was observed between 30, 40 and 50°C. At 60°C, however, the $COD/N_{removed}$ was significantly lower than 30°C ($p<0.05$), yielding a 22.8 ± 5.2% lower carbon requirement. This substantiates an earlier study, where a 23% diminished carbon requirement was reported for denitrification at 55°C compared to 34°C (Courtens et al., 2014). No significant reduction ($p>0.05$) was observed in carbon requirement for denitritation (**Fig. 3, C**).

Sludge production decreased remarkably with up to 77 ± 17 and 79 ± 5% when comparing 60°C to 30°C for denitritation and denitrification respectively (**Fig. 2, A**). In the denitrification reactor, sludge production at 50 and 60°C, 0.12 and 0.04 g VSS g^{-1} $COD_{removed}$, respectively, was lower than reported in a previous study at 55°C (0.16 g VSS

g^{-1} $COD_{removed}$) (Courtens et al., 2014). The reduction in TSS production was less pronounced due to a higher inorganic content at elevated temperatures and amounted up to 46 ± 7 and 50 ± 10% when comparing 60°C to 30°C for denitritation and denitrification respectively (**Fig. 2, B**). Finally, anoxic sludge production in terms of g VSS g^{-1} COD was 5.6 and 59.5% lower than aerobic sludge production.

Denitritation is a cost-beneficial alternative for denitrification, in case of low biodegradable organics, bringing about cost savings through a reduced biological sludge production of 40%, a smaller carbon requirement of 40% and the achievement of higher rates and thus smaller bioreactors (Fux & Siegrist, 2004). Demonstrated for the first time at thermophilic conditions, the advantages of denitritation over denitrification were endorsed. The lower sludge production, expressed per unit of nitrogen removed, amounted to 18 and 26% at 50 and 60°C respectively when compared to complete denitrification. Biomass specific denitritation rate was 92% higher at 50°C and 24% at 60°C. Last but not least, carbon requirement dropped sharply with 40%.

As opposed to aerobic settleability, a strong negative correlation was found between SVI and temperature (>97%, $p<0.05$) for both denitrification and denitritation. The SVI in the denitrification reactor decreased from 168 ± 17 mL g^{-1} VSS at 30°C to 31 ± 1 mL g^{-1} VSS at 60°C and in the denitritation reactor from 177 ± 22 to 64 ± 1 mL g^{-1} VSS (**Fig. 4, A**). Both anoxic processes showed a positive correlation between ISV and temperature (>77%, $p<0.05$) and a negative correlation between VSS/TSS ratio and temperature (>89%, $p<0.05$) (**Fig. 4, B and C**). At 40°C, there was a bigger share of larger particles, reflected in a higher D50/D90 value (Supplementary material). The decrease in VSS/TSS ratio is most

likely the dominant factor influencing the settling behavior, as a higher inorganic content is associated with higher density and thus better settling (Courtens et al., 2014; Vlyssides et al., 2008). At 40°C, however, the elevated ISV in both reactors might also be linked to the bigger share of larger particles (Supplementary material). Although a decreasing SVI was observed with increasing temperatures, the SVI at 30°C was high for both denitrification and denitritation (168 ± 17 and 177 ± 22 mL g^{-1} VSS respectively). At 40, 50 and 60°C, on the other hand, practically acceptable settling sludge with SVI values <100 mL g^{-1} VSS and high settling velocity was obtained. At temperatures above 30°C, settling of the anoxic sludge in term of SVI and ISV was superior to the settleability of the aerobic biomass.

3.4. Retention of metabolic flexibility

The inoculum was functionally diverse, able to perform both aerobic and anoxic carbon removal. After five sludge retention times submitted to a specific electron acceptor, the ability of the community to utilize other electron acceptors might be suppressed. This was the case for the aerobic sludge, displaying limited capacity to utilize nitrite or nitrate to oxidize organic carbon during short term incubations between 5 and 26 hours ($q_{max,N,anoxic}$ <0.6 g N g^{-1} VSS d^{-1}) (**Fig. 5, A**). As neither nitrate nor nitrite was present in the feed of the aerobic reactor and more energy can be derived from the use of oxygen, it could be expected that the biomass loses its denitrifying capacity. Both anoxic reactors retained the ability of aerobic carbon oxidation, represented by a $q_{max,COD,aerobic}$ comparable to the aerobic carbon removing reactor (**Fig. 5, B and C**). It is known that denitrification almost exclusively is a facultative anaerobic or microaerophilic trait and the denitrification apparatus and the aerobic electron transport chain have a close relationship (Zumft, 1997).

Because of this specific feature, anoxic conditions likely do not remove the capacity to respire oxygen. Sludge from the denitrification reactor could remove nitrite 7-58% faster than nitrate, with nitrite removal rates (2.1-3.1 g N g^{-1} VSS d^{-1}) in the same order of magnitude as observed with biomass from the denitritation reactor (1.9-3.5 g N g^{-1} VSS d^{-1}). When nitrite was the electron donor in the reactor for 5 SRT, the possibility to use nitrate was not lost entirely but the maximum removal rate of nitrate was always lower compared to nitrite, namely 52 ± 6, 77 ± 21, 32 ± 7 and 90 ± 36% lower at 30, 40, 50 and 60°C respectively. Overall, the facultative behavior of the aerobic biomass was limited after long-term exposure to oxygen, whereas anoxically produced biomass retained the ability to use oxygen as electron acceptor.

3.5. Practical outlook for an integrated process

The size of a wastewater treatment facility, and thus capital cost, is affected by the removal rate and sludge settleability. Removal rate decreased in this study at higher temperatures, whereas (especially in anoxic conditions) settleability increased. For the aimed application of treating nitrogenous wastewater, bioreactor size would mainly depend on autotrophic conversion rates rather than heterotrophic rates, as thermophilic nitrification rates (170-198 mg N g^{-1} VSS d^{-1}) are reported to be considerably lower than denitrification rates obtained in this study (1626 mg N g^{-1} VSS d^{-1} at 50°C) (Courtens et al., 2016a; Courtens et al., 2016b). The substantial decline in sludge production could entail savings in sludge disposal cost, a significant portion of operational cost. Furthermore, for waters with a low COD/N ratio, achieving shortcut nitrogen removal through nitritation/denitritation should be

encouraged as this study for the first time revealed advantages of thermophilic denitritation over denitrification.

An exploratory case study was performed using the data to assess the possible effect of upgrading an existing mesophilic denitrifying reactor to thermophilic temperatures. Details on the parameters and assumptions used for the case study can be found in the Supplementary material. A mesophilic denitrifying reactor (30°C) would result in a denitrification-SRT of 3.6 days, whereas at thermophilic conditions (50°C) a denitrification-SRT of 10.5 days would be obtained. It is thus evident that upgrading a mesophilic treatment facility to thermophilic temperatures would render higher sludge retention times and thus better retention of slower growing nitrifying organisms. When an equally comprehensive parameter set becomes available for autotrophic nitrification, it can be integrated with the obtained heterotrophic parameter set, enabling the development of a detailed process model. Such model can be used as a tool to study the process in-silico to develop the optimal thermophilic process design and control measures for the retention of nitrifying organisms and evaluate the prospective cost benefits of this integrated solution.

4. Conclusions

Aerobic carbon removal, denitritation and denitrification at thermophilic conditions were characterized by a fast start-up (1-7 days). Higher temperatures were accompanied by lower sludge production and maximum specific removal rates, resulting mainly from decreasing maximum growth rates. Thermophilic denitritation was demonstrated for the first time, with lower sludge production (18-26%), higher nitrogen removal rates (24-92%) and lower carbon requirement (40%) when compared to denitrification. Acceptable settling of

thermophilic aerobic (60°C) and anoxic biomass (50 and 60°C) was obtained. Overall, the obtained parameter set may catalyze the establishment of thermophilic nitrogen removal, once nitritation and nitratation are characterized.

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References

Abeynayaka, A., Visvanathan, C. 2011a. Mesophilic and thermophilic aerobic batch biodegradation, utilization of carbon and nitrogen sources in high-strength wastewater. Bioresource Technology, 102, 2358-2366.

Abeynayaka, A., Visvanathan, C. 2011b. Performance comparison of mesophilic and thermophilic aerobic sidestream membrane bioreactors treating high strength wastewater. Bioresource Technology, 102, 5345-5352.

APHA. 1992. Standard methods for the examination of water and wastewater, 18th edition. American Public Health Association/Amercian Water Works Association/Water Environment Federation. Washington DC, USA.

Bandyopadhyay, B., Humphrey, A.E., Taguchi, H. 2009. Dynamic measurement of the volumetric oxygen transfer coefficient in fermentation systems. Biotechnology and Bioengineering, 104, 842-853.

Barr, T.A., Taylor, J.M., Duff, S.J.B. 1996. Effect of HRT, SRT and temperature on the performance of activated sludge reactors treating bleached kraft mill effluent. Water Research, 30, 799-810.

Camargo, J.A., Alonso, A. 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: A global assessment. Environment International, 32, 831-849.

Coppens, J., Meers, E., Boon, N., Buysse, J., Vlaeminck, S.E. 2016. Follow the N and P road: High-resolution nutrient flow analysis of the Flanders region as precursor for sustainable resource management. Resources Conservation and Recycling, 115, 9-21.

Couillard, D., Gariepy, S., Tran, F.T. 1989. Slaughterhouse effluent treatment by thermophilic aerobic process. Water Research, 23, 573-579.

Couillard, D., Zhu, S. 1993. Thermophilic aerobic process for the treatment of slaughterhouse effluents with protein recovery. Environmental Pollution, 79, 121-126.

Courtens, E.N.P., Spieck, E., Vilchez-Vargas, R., Bode, S., Boeckx, P., Schouten, S., Jauregui, R., Pieper, D.H., Vlaeminck, S.E., Boon, N. 2016a. A robust nitrifying

community in a bioreactor at 50°C opens up the path for thermophilic nitrogen removal. ISME J, 10, 2293-2303.

Courtens, E.N.P., Vandekerckhove, T., Prat, D., Vilchez-Vargas, R., Vital, M., Pieper, D.H., Meerbergen, K., Lievens, B., Boon, N., Vlaeminck, S.E. 2016b. Empowering a mesophilic inoculum for thermophilic nitrification: Growth mode and temperature pattern as critical proliferation factors for archaeal ammonia oxidizers. Water Research, 92, 94-103.

Courtens, E.N.P., Vlaeminck, S.E., Vilchez-Vargas, R., Verliefde, A., Jauregui, R., Pieper, D.H., Boon, N. 2014. Trade-off between mesophilic and thermophilic denitrification: Rates vs. sludge production, settleability and stability. Water Research, 63, 234-244.

Fux, C., Siegrist, H. 2004. Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitritation/anammox: environmental and economical considerations. Water Science and Technology, 50, 19-26.

Golovlev, E.L. 2001. Ecological strategy of bacteria: Specific nature of the problem. Microbiology, 70, 379-383.

Henze, M. 2000. Activated Sludge Models-ASM1, ASM2, ASM2d and ASM3. IWA Publishing, IWA Scientific Technical Report 9.

Henze, M., van Loosdrecht, M.C.M., Ekama, G.A., Brdjanovic, D. 2008. Biological wastewater treatment. IWA Publishing.

Jahren, S.J., Rintala, J.A., Odegaard, H. 2002. Aerobic moving bed biofilm reactor treating thermomechanical pulping whitewater under thermophilic conditions. Water Research, 36, 1067-1075.

Kurian, R., Acharya, C., Nakhla, G., Bassi, A. 2005. Conventional and thermophilic aerobic treatability of high strength oily pet food wastewater using membrane-coupled bioreactors. Water Research, 39, 4299-4308.

Lapara, T.M., Alleman, J.E. 1999. Thermophilic aerobic biological wastewater treatment. Water Research, 33, 895-908.

LaPara, T.M., Konopka, A., Nakatsu, C.H., Alleman, J.E. 2000a. Effects of elevated temperature on bacterial community structure and function in bioreactors treating a synthetic wastewater. Journal of Industrial Microbiology & Biotechnology, 24, 140-145.

LaPara, T.M., Konopka, A., Nakatsu, C.H., Alleman, J.E. 2000b. Thermophilic aerobic wastewater treatment in continuous-flow bioreactors. Journal of Environmental Engineering-Asce, 126, 739-744.

Laurino, C.N., Sineriz, F. 1991. Denitrification by thermophilic soil bacteria with ethanol as substrate in a UASB reactor. Biotechnology Letters, 13, 299-304.

Liao, B.Q., Lin, H.J., Langevin, S.P., Gao, W.J., Leppard, G.G. 2011. Effects of temperature and dissolved oxygen on sludge properties and their role in bioflocculation and settling. Water Research, 45, 509-520.

Metcalf, amp, Eddy, I. 2003. Wastewater engineering : treatment and reuse. Fourth edition / revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel. Boston : McGraw-Hill, [2003] ©2003.

Nadarajah, N., Allen, D.G., Fulthorpe, R.R. 2007. Effects of transient temperature conditions on the divergence of activated sludge bacterial community structure and function. Water Research, 41, 2563-2571.

Omlin, M., Reichert, P. 1999. A comparison of techniques for the estimation of model prediction uncertainty. Ecological Modelling, 115, 45-59.

Parkhurst, D.L., Appelo, C.A.J. 1999. User's guide to PHREEQC (Version 2) : a computer program for speciation, batch-reaction, one-dimensional transport, and inverse geochemical calculations. 99-4259.

Peng, L., Carvajal-Arroyo, J.M., Seuntjens, D., Prat, D., Colica, G., Pintucci, C., Vlaeminck, S.E. 2017. Smart operation of nitritation/denitritation virtually abolishes nitrous oxide emission during treatment of co-digested pig slurry centrate. Water Res, 127, 1-10.

Schuler, A.J., Jang, H. 2007. Density effects on activated sludge zone settling velocities. Water Research, 41, 1814-1822.

Strotmann, U.J., Geldern, A., Kuhn, A., Gendig, C., Klein, S. 1999. Evaluation of a respirometric test method to determine the heterotrophic yield coefficient of activated sludge bacteria. Chemosphere, 38, 3555-3570.

Surucu, G.A., Chian, E.S.K., Engelbrecht, R.S. 1976. Aerobic thermophilic treatment of tigh-strength wastewaters. Journal Water Pollution Control Federation, 48, 669-679.

Surucu, G.A., Engelbrecht, R.S., Chian, E.S.K. 1975. Thermophilic microbiological treatment of high-strength wastewaters with simultaneous recovery of single cell protein. Biotechnology and Bioengineering, 17, 1639-1662.

Suvilampi, J., Rintala, J. 2003. Thermophilic aerobic wastewater treatment, process performance, biomass characteristics, and effluent quality. Reviews in Environmental Science and Biotechnology, 2, 35-51.

Tripathi, C.S., Allen, D.G. 1999. Comparison of mesophilic and thermophilic aerobic biological treatment in sequencing batch reactors treating bleached kraft pulp mill effluent. Water Research, 33, 836-846.

Van Daele, T., Van Hoey, S., Nopens, I. 2015. pyIDEAS: an open source python package for model analysis. 12th International Symposium on Process Systems Engineering (Pse) and 25th European Symposium on Computer Aided Process Engineering (Escape), Pt A, 37, 569-574.

Vanrolleghem, P.A., Spanjers, H., Petersen, B., Ginestet, P., Takacs, I. 1999. Estimating (combinations of) Activated Sludge Model No. 1 parameters and components by respirometry. Water Science and Technology, 39, 195-214.

Vlyssides, A.G., Barampouti, E.M.P., Mai, S.T. 2008. Simple estimation of granule size distribution and sludge bed porosity in a UASB reactor. Global Nest Journal, 10, 73-79.

Vogelaar, J.C.T., Klapwijk, B., Temmink, H., van Lier, J.B. 2003. Kinetic comparisons of mesophilic and thermophilic aerobic biomass. Journal of Industrial Microbiology & Biotechnology, 30, 81-88.

Zessner, M., Lampert, C., Kroiss, H., Lindtner, S. 2010. Cost comparison of wastewater treatment in Danubian countries. Water Sci Technol, 62, 223-30.

Zumft, W.G. 1997. Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Reviews, 61, 533-+.

Figures

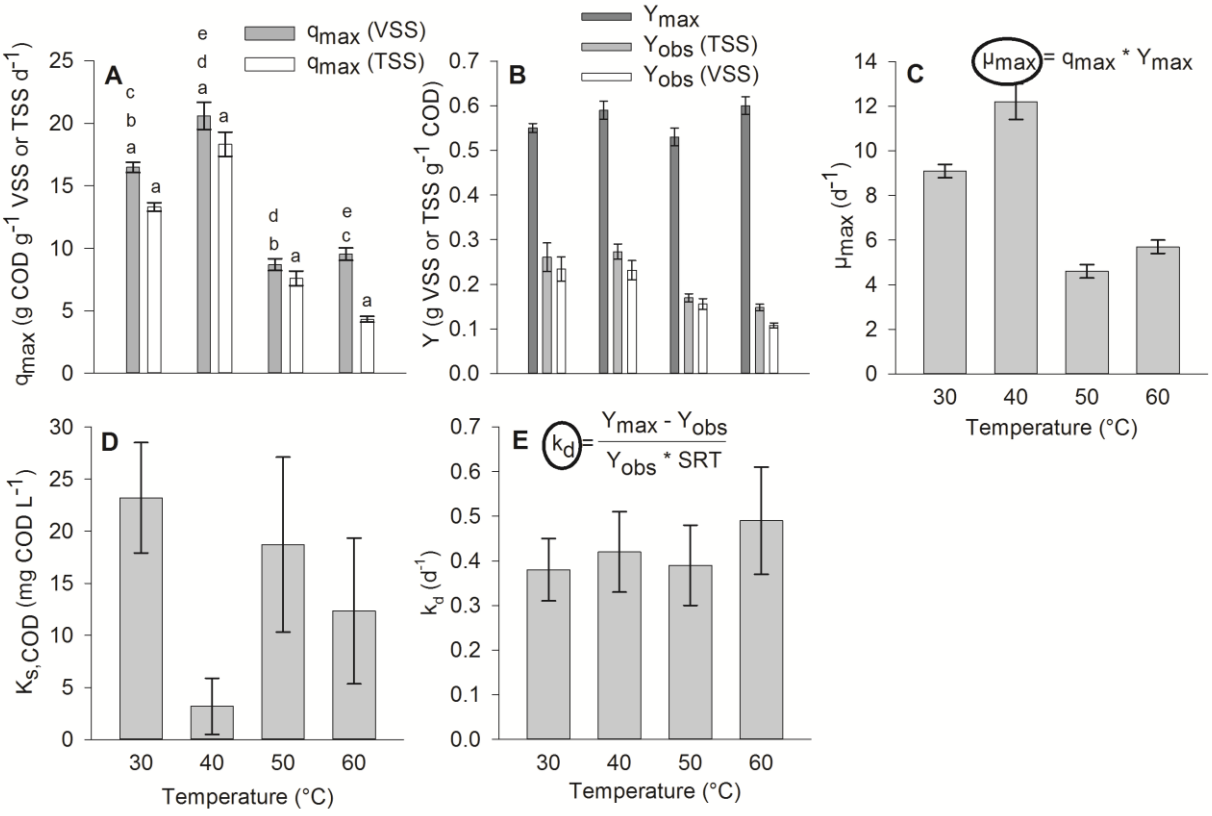
Fig. 1: Kinetics of aerobic carbon removal at the tested temperatures with (A) the maximum specific substrate removal rate in terms of VSS and TSS, (B) the biomass production, (C) the calculated maximum growth rate, (D) the substrate affinity constant for acetate and (E) the calculated decay rate. Significant pairwise differences ($p<0.05$) of parameter values over temperature changes within the same reactor are indicated with letters.

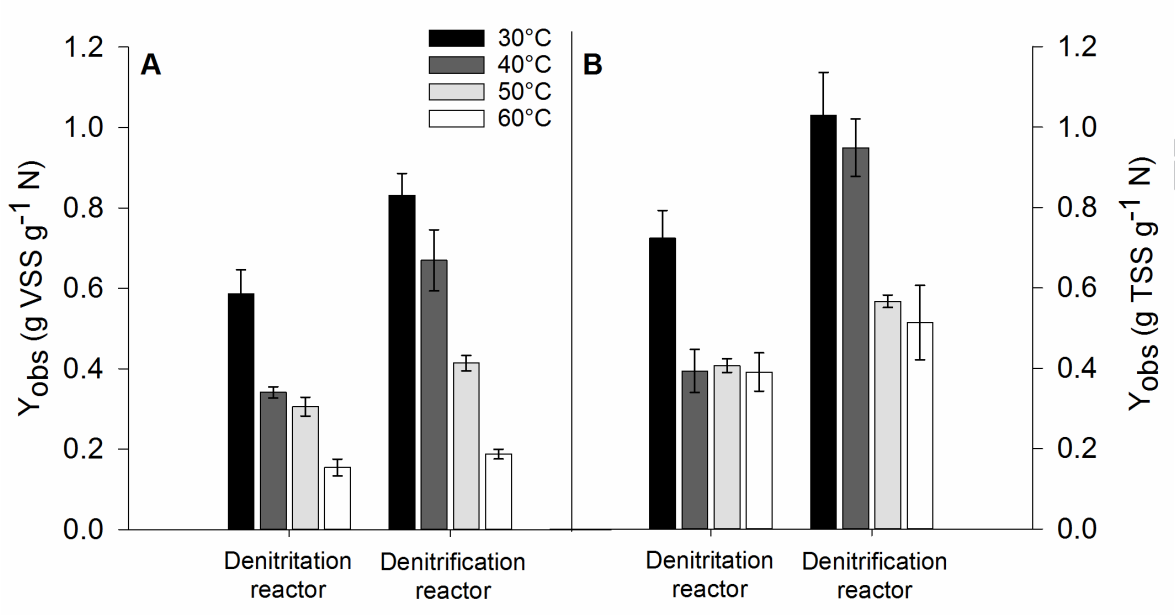
Fig. 2: The observed biomass production of denitritation and denitrification at the tested temperatures in terms of (A) VSS and (B) TSS.

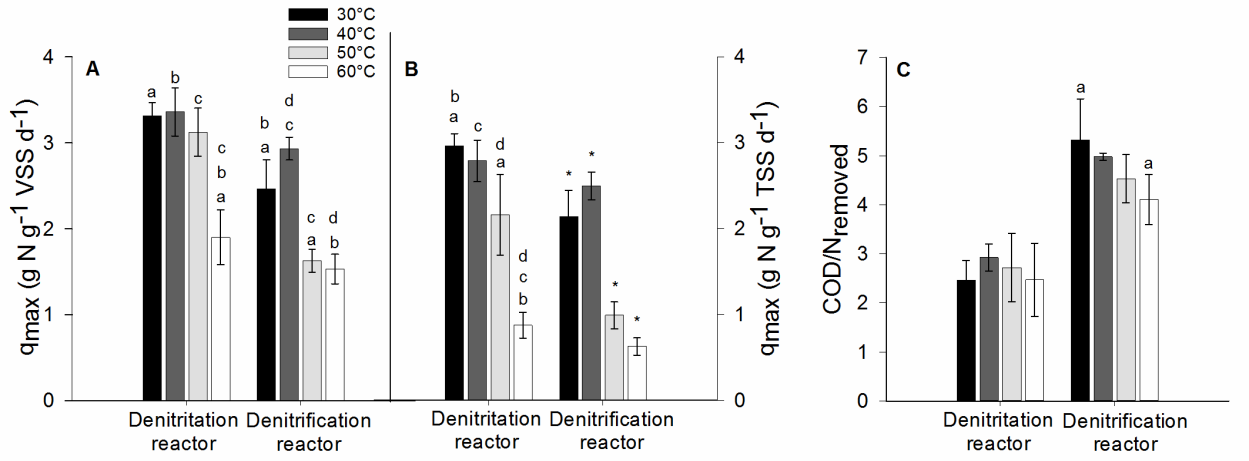
Fig. 3: Kinetics of denitritation and denitrification at the tested temperatures with (A) the maximum specific substrate removal rate in terms of VSS and (B) in terms of TSS and (C) the corresponding $COD/N_{removed}$ ratios. Significant pairwise differences ($p<0.05$) of parameter values over temperature changes within the same reactor are indicated with letters. *: applied statistical method was not able to show pairwise significant difference.

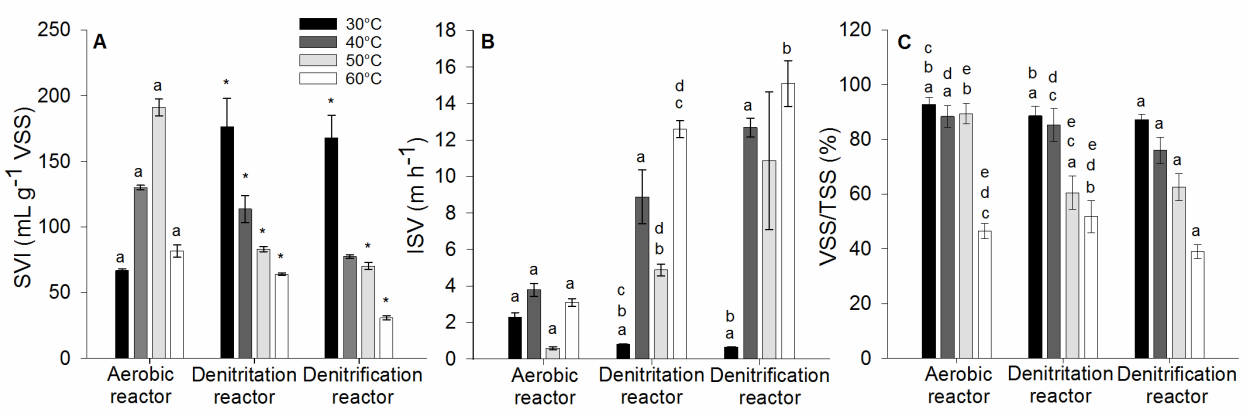
Fig. 4: Settleability of the aerobic and anoxic biomass at the tested differences with (A) the sludge volume index (SVI), (B) the initial settling velocity (ISV) and (C) the ratio of volatile to total suspended solids (VSS/TSS). Significant pairwise differences ($p<0.05$) of parameter values over temperature changes within the same reactor are indicated with letters. *: applied statistical method was not able to show pairwise significant difference

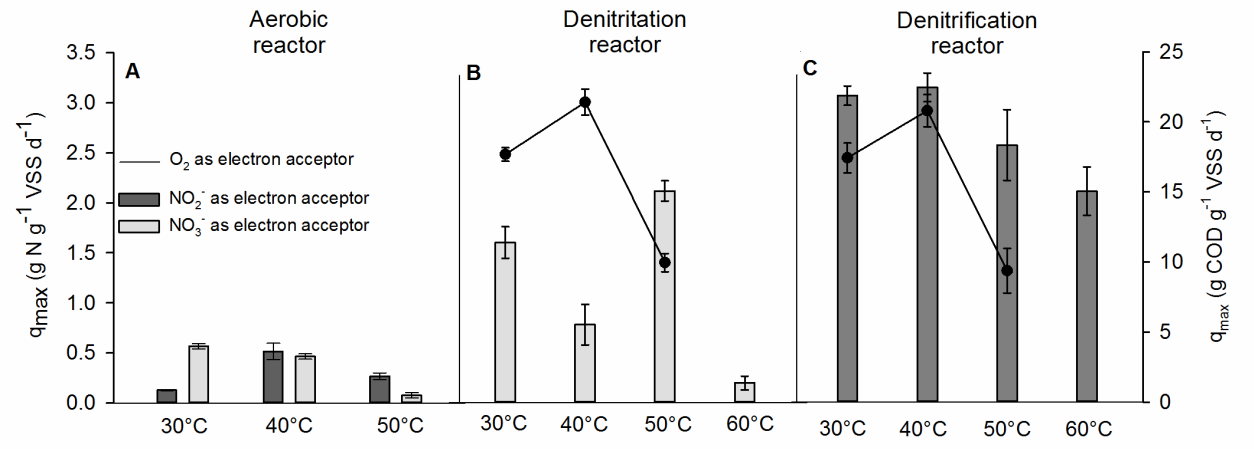
Fig. 5: Maximum specific substrate removal in (A) the aerobic reactor for nitrite and nitrate, (B) the denitritation reactor for oxygen and nitrate and (C) the denitrification reactor for oxygen and nitrite.

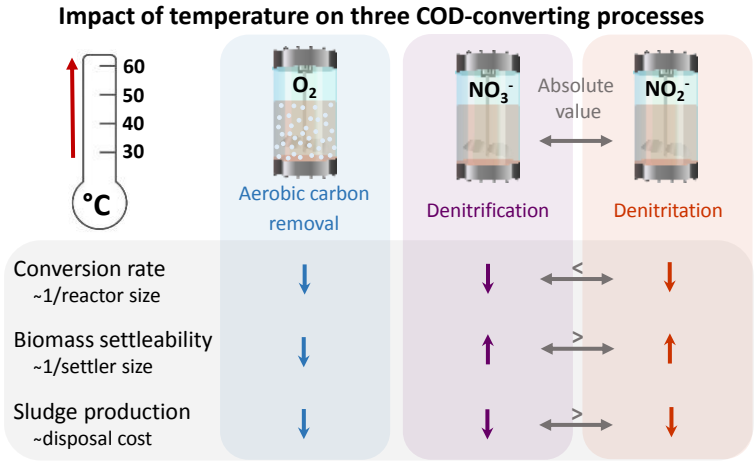

A
qmax (VSS)
qmax (TSS)
qmax (g COD g-1 VSS or TSS d-1)
B
Ymax
Yobs (TSS)
Yobs (VSS)
Y (g VSS or TSS g-1 COD)
C
μmax = qmax * Ymax
μmax (d-1)
Temperature (°C)
D
Ks,COD (mg COD L-1)
Temperature (°C)
E
kd = (Ymax - Yobs) / (Yobs * SRT)
kd (d-1)
Temperature (°C)











Highlights

- Aerobic and anoxic carbon removal were fast in start-up at all temperatures.
- Increasing temperature lowered aerobic sludge yield, mainly due to lower $\mu_{max.}$
- High thermophilic denitritation/denitrification rates up to 3.3 g N g^{-1} VSS d^{-1}.
- Thermophilic denitritation as cost-saving alternative to denitrification.
- Increasing temperature yielded ambiguous trends in aerobic and anoxic settling.