Effect of temperature on denitrifying methanotrophic activity of ‘Candidatus Methylohirabilis oxyfera’

Christel Kampman, Laura Piai, Tim L. G. Hendrickx, Hardy Temmink, Grietje Zeeman and Cees J. N. Buisman

ABSTRACT

The activity of denitrifying methanotrophic bacteria at 11–30 °C was assessed in short-term experiments. The aim was to determine the feasibility of applying denitrifying methanotrophic bacteria in low-temperature anaerobic wastewater treatment. This study showed that biomass enriched at 21 °C had an optimum temperature of 20–25 °C and that activity dropped as temperature was increased to 30 °C. Biomass enriched at 30 °C had an optimum temperature of 25–30 °C. These results indicated that biomass from low-temperature inocula adjusted to the enrichment temperature and that low-temperature enrichment is suitable for applications in low-temperature wastewater treatment. Biomass growth at <20 °C still needs to be studied.

Key words | anaerobic methane oxidation, anaerobic wastewater treatment, denitrification, Methylohirabilis oxyfera, temperature optimum

INTRODUCTION

Wastewater treatment plants (WWTPs) can become (close to) self-sufficient through a combination of energy savings and energy recovery. To achieve this in temperate zones, the main focus is on the enhanced pre-concentration of wastewater organic matter (chemical oxygen demand (COD)) and subsequent anaerobic digestion of the obtained primary sludge to produce biogas (Akanyeti et al. 2010). An alternative would be direct anaerobic municipal wastewater treatment. This can be achieved in, for example, a combination of an upflow anaerobic sludge bed reactor and a sludge digester (UASB-digester) (Alvarez et al. 2004; Mahmoud et al. 2004; Zhang et al. 2013). The main disadvantages of this system are that nutrients are not removed and that a large fraction of the generated methane is dissolved in the effluent.

To overcome the aforementioned disadvantages, Kampman et al. (2012) have proposed a novel concept for municipal wastewater treatment at low temperatures. This concept consists of a UASB-digester, a reactor for nitrite-dependent anaerobic methane oxidation and a nitrification reactor. By applying this concept, energy can be saved because of lower aeration requirements than conventional activated sludge treatment. In addition, the recovery of chemical energy contained in the wastewater COD as biogas can be realized. After anaerobic treatment, no readily available carbon sources remain to sustain heterotrophic denitrification. However, when direct anaerobic wastewater treatment is applied at low temperatures, the effluent contains a considerable amount of dissolved methane, which has to be removed from the effluent to achieve a reduction in greenhouse gas emission as compared with conventional activated sludge treatment (Cakir & Stenstrom 2005). Theoretically, the concentration of dissolved methane would be 15–20 mg/L (calculated for atmospheric pressure, 20 resp. 10 °C and 70% methane in the biogas assuming Henry’s law). However, effluent dissolved methane concentrations of 43.5–86.5 mg/L have been determined for municipal wastewater treatment at a temperature range of 8–18 °C (Hartley & Lant 2006).

In the proposed concept, nitrite-nitrogen and methane are concomitantly removed (Equation (1)) (Raghoebarsing
et al. 2006) through the activity of denitrifying methanotrophic bacteria (Kampman et al. 2012)

\[3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O}\]  

(1)

The concept is especially suited for temperate zones where, at lower temperatures, effluent from anaerobic treatment contains enough methane to sustain methanotrophic denitrification (Kampman et al. 2012). The process might also be applied when lower amounts of dissolved methane are present, for example, in summer conditions or in tropical and sub-tropical regions. This, however, may require the addition of biogas to provide sufficient methane. In these cases, a UASB-digester combined with the anammox process could also be used (Hendrickx et al. 2012).

Application of the UASB-digester and the nitritation process at low temperatures has been studied before. In a UASB-digester, 73% COD removal was achieved at a temperature of 15 °C and a short hydraulic retention time of 6 h (Mahmoud et al. 2004). Stable nitritation at low temperatures and low nitrogen concentrations was successfully achieved by aeration duration control: Blackburne et al. (2008) have achieved 80% nitritation at an average nitrogen concentration of 43 mg N/L and a temperature of 18–25 °C, Yang et al. (2007) have obtained >95% nitritation at an average nitrogen concentration of 60 mg N/L and a temperature of 11.9–26.5 °C.

Moreover, 16S rRNA sequences similar to those of the denitrifying methanotrophic bacterium ‘Candidatus Methylophilus oxyfera’ have been detected in sediments and wastewater sludge from temperate zones. From these inocula, M. oxyfera-type bacteria have been enriched (e.g., Raghoebarsing et al. 2006; Luesken et al. 2011; Zhu et al. 2012; Kampman et al. 2014). The bacteria have been enriched at temperatures ranging from 21 to 35 °C (see Table 1). Similar maximum volumetric denitrification rates of 34–38 mg

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Enrichment temperature (°C)</th>
<th>Maximum activity (mg N/L·d)</th>
<th>Enrichment reactor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture of WWTP sludge, The Netherlands</td>
<td>21</td>
<td>16</td>
<td>MBR</td>
<td>Kampman et al. (2014)</td>
</tr>
<tr>
<td>Mixture of WWTP sludge and biomass from enrichments Kampman et al. (2012)</td>
<td>21</td>
<td>37</td>
<td>MBR</td>
<td>Kampman et al. (2014)</td>
</tr>
<tr>
<td>Mixture of WWTP sludge and fresh water sediment, Australia</td>
<td>22</td>
<td>0.91</td>
<td>SBR</td>
<td>Hu et al. (2009)</td>
</tr>
<tr>
<td>Sludge industrial wastewater treatment, The Netherlands</td>
<td>20–23</td>
<td>15</td>
<td>SFBR</td>
<td>Luesken et al. (2011)</td>
</tr>
<tr>
<td>Minerotrophic peatland, The Netherlands</td>
<td>25</td>
<td>14</td>
<td>SFBR</td>
<td>Zhu et al. (2012)</td>
</tr>
<tr>
<td>Freshwater sediment, The Netherlands</td>
<td>25</td>
<td>12</td>
<td>SFBR</td>
<td>Raghoebarsing et al. (2006)</td>
</tr>
<tr>
<td>Freshwater sediment, The Netherlands; synthetic medium</td>
<td>30</td>
<td>34</td>
<td>SFBR</td>
<td>Kampman et al. (2012)</td>
</tr>
<tr>
<td>Freshwater sediment, The Netherlands; medium with 10% wastewater</td>
<td>30</td>
<td>38</td>
<td>SFBR</td>
<td>Kampman et al. (2012)</td>
</tr>
<tr>
<td>Freshwater sediment, The Netherlands</td>
<td>30</td>
<td>36</td>
<td>SFBR</td>
<td>Ettwig et al. (2009)</td>
</tr>
<tr>
<td>Biomass from enrichment Raghoebarsing et al. (2006)</td>
<td>30</td>
<td>11</td>
<td>CSTR</td>
<td>Ettwig et al. (2008)</td>
</tr>
<tr>
<td>Mixture of WWTP sludge and fresh water sediment, Australia</td>
<td>35</td>
<td>24&lt;sup&gt;a&lt;/sup&gt; + 28; 6.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SBR</td>
<td>Hu et al. (2009)</td>
</tr>
<tr>
<td>Biomass from 35 °C enrichment Hu et al. (2009); nitrite fed</td>
<td>35</td>
<td>17</td>
<td>SBR, pulse fed with nitrite</td>
<td>Hu et al. (2010)</td>
</tr>
<tr>
<td>Biomass from 35 °C enrichment Hu et al. (2009); nitrate fed</td>
<td>35</td>
<td>0.54</td>
<td>SBR</td>
<td>Hu et al. (2010)</td>
</tr>
</tbody>
</table>

<sup>a</sup>in a batch test the reactor was fed with both nitrite and nitrate, nitrite consumption was higher; during normal reactor operation the reactor was fed with nitrate only.

NO$_2$-N/L-d are achieved at 21°C (Kampman et al. 2014) and 30°C (Ettwig et al. 2009; Kampman et al. 2012), and doubling times are shorter in membrane bioreactors (MBRs) at 21°C than in sequencing fed-batch reactors (SFBRs) at 30°C (0.9–1.3 months in MBRs versus 1.7–1.9 months in SFBRs) (Kampman et al. 2014). Research progress on the process of nitrite-denitrification coupled to anaerobic methane oxidation is slow due to low growth rates and hence the low availability of biomass. Activity and growth of denitrifying methanotrophic bacteria at temperatures <21°C have not yet been quantified. However, for anammox bacteria which, similar to *M. oxyfera*, are nitrite-reducing autotrophic bacteria (genome and proteome of Ca. ‘*M. oxyfera*’ suggest autotrophy, Wu et al. 2011) with a doubling time in the order of weeks, lower optimum temperatures have been observed for organisms enriched at or adapted to lower temperatures. Hendrickx et al. (2014) have observed a temperature optimum of 20–30°C for anammox enriched at 10°C. Hu et al. (2013) have reported similar maximum activities of a 30°C anammox culture and a 12°C adapted anammox culture. However, anammox bacteria enriched at 12°C have a temperature optimum of 35°C, which is 10°C lower than anammox bacteria enriched at 30°C. If this applies to denitrifying methanotrophic bacteria, they can best be enriched at low temperatures. For application in low-temperature anaerobic wastewater treatment where temperature fluctuates between 10 and 20°C, less biomass (retention) is required if biomass with a lower temperature optimum, yet similar maximum rate, is enriched.

In this work, volumetric denitrification rates of denitrifying methanotrophic bacteria at temperatures in the range of 11–30°C were assessed. The study was performed in two enrichment reactors that had been operated for 2.5 years, respectively, at 21°C and at 30°C. Nitrite and methane consumption rates were measured. Changes in volumetric denitrification rate as a function of temperature and activation energies were determined, and insight in nitrite affinity was obtained. The feasibility of applying reactors with *M. oxyfera*-type bacteria for the treatment of effluent from direct low-temperature anaerobic wastewater treatment is discussed.

**METHODS**

**Setup of activity tests**

Activity tests were performed on two anoxically operated reactors enriched in *M. oxyfera*-type bacteria. Reactor R30, an SFBR (total volume 9.7 L, working volume 5.3–6.7 L) inoculated with freshwater sediment (0.55 ± 0.09 g protein/L) had been operated at 30 ± 1°C for 31 months (Kampman et al. 2012, 2014). Reactor R20, an MBR (total volume 7.7 L, working volume 4.6 L) inoculated with municipal wastewater sludge (0.37 ± 0.05 g protein/L), had been operated at 21 ± 1°C for 30 months. This reactor was spiked with biomass enriched in *M. oxyfera*-type bacteria collected from the effluent of two SFBRs operated at 30 ± 1°C, including the one used in these tests. R20 was spiked after 14 months (0.46 ± 0.09 g protein) and 20 months (1.0 ± 0.52 g protein) of MBR operation (Kampman et al. 2014). Both R30 and R20 were fed with synthetic medium containing nitrite (influent 0.014–0.98 g NO$_2$-N/L; reactor 3–30 mg NO$_2$-N/L) and nitrate, and with a mixture of methane and carbon dioxide (influent 93.6–95.0% CH$_4$, 5.0–6.4% CO$_2$; in reactors in excess at all times) (Kampman et al. 2012, 2014). The temperature of the reactors was controlled by a thermostat bath.

At the start of the activity tests, R30 consumed 14 mg NO$_2$-N/L-d and R20 consumed 18 mg NO$_2$-N/L-d.

**Procedure for activity tests**

When measuring activity, the reactors were operated in batch mode. During the activity tests, no liquid or gas was brought into or removed from the system; gas recirculation was continued. During 16 d, both reactors were operated at temperatures covering the range from enrichment temperatures to temperatures representative of wastewater treatment in temperate zones. R20 was operated at 29.7°C (day 0.0–2.3), 24.9°C (day 12.9–15.2), 20.4°C (day 2.3–4.3), 15.9°C (day 7.0–8.3) and 11.4°C (day 9.0–11.3). R30 was operated at 29.9°C (day 0.0–2.3), 25.1°C (day 12.9–15.2), 20.4°C (day 2.3–4.3), 15.8°C (day 7.0–8.3) and 11.1°C (day 9.0–11.3). In between the activity tests at different temperatures, nitrite was added or the influent 93.6–95.0% CH$_4$, 5.0–6.4% CO$_2$; in reactors in excess at all times) (Kampman et al. 2012, 2014). The temperature of the reactors was controlled by a thermostat bath.

Nitrite concentration and methane pressure were measured 3 to 4 times per day. In two tests, nitrite was consumed to concentrations below detection limits (0.015 mg NO$_2$-N/L) and in one test, the nitrite concentration was consumed to 0.46 mg NO$_2$-N/L. These reactors were spiked with nitrite (NaNO$_2$) to concentrations of 15–40 mg NO$_2$-N/L, after which the tests were continued. Methane was present in excess (>42%) at all times. During the tests, the pH was buffered at 7.4 ± 0.1 due to the presence of HCO$_3$ and CO$_2$. 
Analyses

Nitrite and nitrate were measured by ion chromatography. Methane was measured by gas chromatography (Kampman et al. 2012). Temperature was logged using a temperature sensor (Pt100) connected to a FieldPoint module and LabVIEW 7.0 (National Instruments, Austin, Texas, USA).

After setting a new experimental temperature, a minimum equilibration time of 1.5 hours was applied to allow redistribution of gases between the gas and liquid phase before analyses were started.

Calculations

Zero-order nitrite and methane consumption rates were calculated from the decline or increase of concentrations or gas pressures in time, assuming gas–liquid equilibrium.

The amount of biomass in the reactors was unknown since it was present both in suspension and as a biofilm on the reactor walls. Therefore, the activities of the reactors could not be directly compared. To determine the temperature response, activities in one reactor were compared for different temperatures.

The temperature dependence of the reaction rate constants was estimated using the Arrhenius relation (Equation (2)), in which $k$ is the reaction rate constant (mol/d), $A$ is the Arrhenius constant, $E_a$ is the activation energy (kJ/mol), $R$ is the gas constant (mol/J·K), $T$ is the temperature (K).

$$k = A \cdot e^{\frac{E_a}{RT}}$$

According to this relation, an increase in temperature will result in an increase in reaction rate. Typically, reaction rates increase by a factor of 2–3 for each 10 °C increase in temperature.

RESULTS AND DISCUSSION

Conversion rates and temperature optimum

At all temperatures tested, methane oxidation and nitrite denitrification with concomitant nitrogen gas production occurred in both R20 (not shown) and R30 (Figure 1). The results indicate that the optimum temperature was 20–25 °C for biomass enriched at 20 °C (in R20) and 25–30 °C for biomass enriched at 30 °C (in R30).

In R20, the maximum nitrite consumption rate, 41 ± 7 mg NO$_2$-N/L·d, was measured at 25 °C (Figure 2). The methane consumption rate was highest at 20 °C, namely 15 ± 2 mg/L·d. Remarkably, the conversion rates sharply decreased as the temperature was increased to 30 °C; the nitrite consumption decreased to 15 ± 1 mg NO$_2$-N/L·d; at 16 °C, a similar rate of 16 ± 4 mg NO$_2$-N/L·d was measured. In the temperature range of 11–25 °C, the conversion rates increased by a factor of 3.2 for a 10 °C increase in temperature, corresponding to an activation energy of 85 kJ/mol. In R30, the highest rates of 18.7 ± 0.7 mg NO$_2$-N/L·d and 17.5 ± 0.5 mg NO$_2$-N/L·d were measured at 25 °C and 30 °C, respectively (Figure 2). In addition, methane consumption rates were highest at 25 and 30 °C; methane was consumed at a rate of 10.9 ± 0.4 mg/L·d at 25 °C and at 10.8 ± 0.3 mg/L·d at 30 °C. In the temperature range of

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**Figure 1** | Coupling of methane (○) oxidation and nitrite (▪) denitrification in R30 during whole culture batch tests at temperatures of (a) 30 °C, more nitrite was added after 1.3 d, and (b) 11 °C.
11–25 °C, the conversion rates increased by a factor of 2.8 for a 10 °C increase in temperature, corresponding to an activation energy of 75 kJ/mol. Thus, temperature changes affected biomass in R20 slightly more than in R30.

Both R20 and R30 were inoculated with biomass originating from low-temperature environments. However, biomass enriched at 30 °C had a 5–10 °C higher temperature optimum than biomass enriched at 21 °C. While the activity of the biomass in R30 was highest at 25–30 °C, the activity of the biomass enriched at 21 °C dropped when the temperature was increased to 30 °C. This indicated that biomass in R30 had changed due to the higher reactor temperature over the 31 months of enrichment. Whether a culture enriched at 30 °C can adjust to lower temperatures was not investigated. R20 was spiked with biomass from two SFBRs at 30 °C after 14 and 20 months of operation. As a result, consumption rates increased (Kampman et al. 2014). This indicated that long-term (>3 months) activity of the biomass at lower temperatures is possible and that biomass might have changed due to the lower temperature after spiking. However, enrichment of denitrifying methanotrophic bacteria at 30 °C is not of interest for low temperature applications. At 21 °C, similar maximum denitrification rates, shorter doubling times (Kampman et al. 2014) and a lower temperature optimum were observed; therefore, for temperate zones, biomass should be enriched at ≤20 °C.

Except for stopping reactor feeding (both gas and liquid) during the activity tests, the reactors were operated in the same way as during continuous operation. During the activity tests, in R30 and especially in R20, the activity at the enrichment temperatures (30 °C and 21 °C, respectively) was higher than during continuous operation (14 mg NO₂-N/L·d vs. 17.5 ± 0.5 mg NO₂-N/L·d at 30 °C in R30 and 18 mg NO₂-N/L·d vs. 41 ± 7 mg NO₂-N/L·d at 21 °C in R20). After the tests, continuous operation was resumed and nitrite was consumed at a rate of 14 mg NO₂-N/L·d in both R20 and R30, indicating that changes in temperature had little or no influence on the stability of the process. Nitrite was not limiting during continuous operation, thus could not have resulted in the apparent low nitrite consumption rates. What could have caused the difference in rate between activity tests and continuous operation is unknown.

Nitrite affinity

In most activity tests, the nitrite consumption rates did not decrease at decreasing nitrite concentrations, even if nitrite was consumed to below 0.015 mg NO₂-N/L (R30 at 30 °C Figure 1(a)). This indicated that the nitrite affinity of the denitrifying methanotrophic culture was high, which is in good agreement with the high affinity of 1 μg NO₂-N/L reported by Ettwig et al. (2008). The affinity was higher than the affinity reported by He et al. (2013).

Conversion ratios

The molar consumption ratio CH₄ : NO₂⁻ was in quite good agreement with the theoretical ratio of 3 CH₄ : 8 NO₂⁻ (Equation (1)) at temperatures of 16–30 °C in R20 and 20–30 °C in R30 (Table 2).

At lower temperatures, methane consumption rates were higher than could be explained from nitrite consumption rates. Although anaerobic methane oxidation is sometimes coupled to nitrate denitrification (Hu et al. 2009), in this research no

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Temperature (°C)</th>
<th>CH₄:NO₂⁻</th>
<th>Theoretical ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>R20 11</td>
<td>6.0</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>3.8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3.3</td>
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<td>30</td>
<td>3.6</td>
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<tr>
<td>R30 11</td>
<td>12.0</td>
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<td>16</td>
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<tr>
<td>30</td>
<td>4.4</td>
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</table>

Table 2: CH₄:NO₂⁻ molar ratios during activity tests at temperatures of 11–30 °C in R20 and R30.
significant nitrate consumption occurred. It is possible that at lower temperatures a change in metabolism occurred; however, it is as yet unknown what such a mechanism could be.

Implications

The aim of this study was to study the feasibility of applying *M. oxyfera*-type bacteria for the treatment of effluent from anaerobic municipal wastewater treatment in temperate zones. Biomass enriched at 21°C had an optimum temperature of 20–25°C. This was 5–10°C lower than the optimum of biomass enriched at 30°C. After the tests, denitrifying methanotrophic activity during continuous operation (almost) recovered. A similar effect of enrichment temperature was observed on the optimum temperature for anammox bacteria, which also perform nitrite-denitrification and have a similar low growth rate (Hu et al. 2013; Hendrickx et al. 2014). Considering the long doubling times of denitrifying methanotrophic bacteria, efficient biomass retention is required, for example, as flocs, granules or biofilms. The effect of temperature thereon still needs to be studied.

For low-temperature reactor operation, the use of biomass enriched at low temperatures enables the achievement of higher loading rates at the same sludge retention time as compared with biomass enriched at mesophilic temperatures. Since denitrifying methanotrophic bacteria were enriched from low-temperature inocula, it is expected that a stable process, with possibly an even lower temperature optimum, can be maintained at ≤21°C. However, the duration of the activity tests was too short to observe any growth (doubling time of denitrifying methanotrophic bacteria, 1–2 months). Long-term reactor operation or enrichment at ≤21°C is required to verify this assumption.

Temperature not only affects biological activity, it also influences, for example, gas solubility. At lower temperatures, more methane is dissolved in the effluent from the UASB-digester. In winter, at a wastewater temperature of 10°C, the theoretical dissolved methane concentration is about 20 mg/L (assuming Henry’s law, atmospheric pressure, and 70% methane in the biogas). This is enough to sustain nitrite-dependent anaerobic methane oxidation (Kampman et al. 2012). In summer, at a temperature of 20°C, the theoretical dissolved methane concentration is 16 mg/L and additional biogas may be required for complete denitrification. To determine whether biogas addition is required to sustain denitrification, dissolved methane concentrations should not just be calculated. Instead, concentrations should be determined from mass balance concentrations, or, preferably, be measured. This is because, for municipal wastewater treatment at a temperature range of 8–18°C, effluent dissolved methane concentrations of 43.5–86.5 mg/L have been determined (Hartley & Lant 2006). Even if biogas has to be added to sustain denitrification at 20°C, this is only a small fraction of the biogas produced, namely 5% (assuming 600 mg COD/L, a COD removal of 73% and a production of 0.25 g methane/g COD) and would hardly affect energy production by the proposed treatment system.

This research confirmed that the nitrite affinity of denitrifying methanotrophic bacteria is high, as already reported by Ettwig et al. (2008); nitrite consumption rates did not decrease as nitrite concentrations decreased below 0.015 mg NO₂⁻-N/L. This allows for low nitrogen concentrations in the effluent of a UASB-digester, coupled to a denitrifying methanotrophic reactor and a nitritation reactor as proposed by Kampman et al. (2012).

CONCLUSIONS

- For the first time, the activity of denitrifying methanotrophic bacteria at temperatures <20°C was shown.
- The activity of denitrifying methanotrophic biomass enriched at 21°C was highest at 20–25°C. At a temperature of 30°C, the activity dropped.
- Denitrifying methanotrophic biomass enriched at 30°C showed maximum activity at 25–30°C.
- For future studies on the application of denitrifying methanotrophic bacteria in low-temperature municipal wastewater treatment, enrichment should be conducted at temperatures of 21°C and possibly lower.
- Denitrifying methanotrophic bacteria have a high affinity for nitrite; consumption rates did not decrease as nitrite concentrations decreased below 0.015 mg NO₂⁻-N/L. This means that if denitrifying methanotrophic bacteria are applied in wastewater treatment, low nitrogen effluent concentrations are feasible.

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