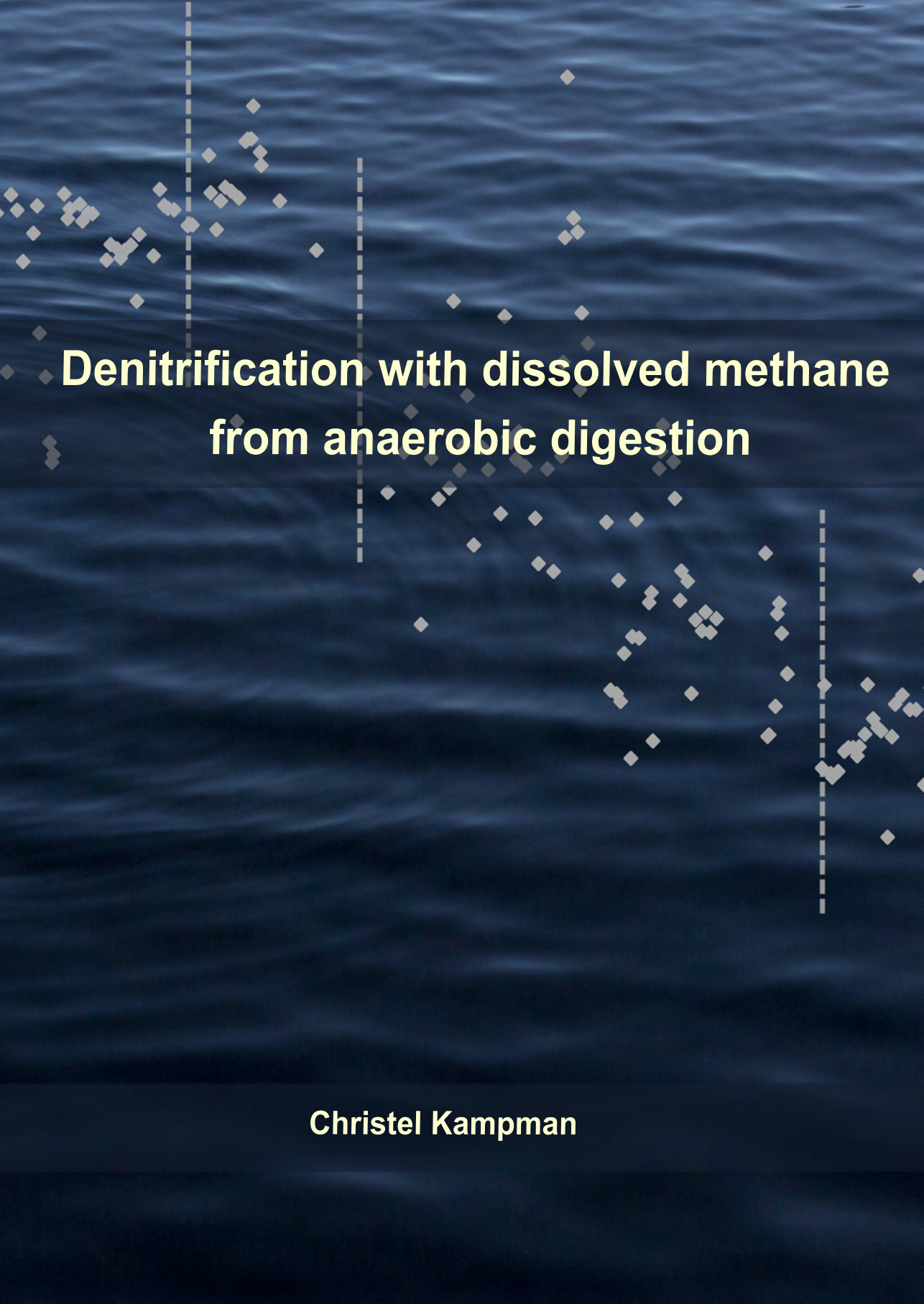

Denitrification with dissolved methane from anaerobic digestion

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Denitrification with dissolved methane from anaerobic digestion

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Contents

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General introduction

1.1 History of municipal wastewater treatment

Wastewater treatment emerged in the beginning of the 20th century. Governments obliged municipal wastewater to be collected and treated to prevent environmental pollution. If wastewater would be discharged without treatment, receiving waters would become deprived of oxygen due to oxidation of organic compounds and nutrient accumulation would occur, resulting in e.g. algae blooms. Treatment processes have become more advanced since then (Lofrano and Brown, 2010). First only solids were removed, later also organic compounds were degraded by biological oxidation and removal of nitrogen and phosphorus has started in the 1970s (Tchobanoglous et al., 2003). At the larger wastewater treatment plants, excess sludge is used for the production of biogas applying anaerobic digestion. Conventional wastewater treatment efficiently removes organic compounds and nutrients. However, treatment requires a lot of energy and the energy demand has increased as wastewater treatment has become more advanced. Approximately 1.2 kWh is used to treat 1 m^3 of wastewater. About 60 % of this energy is used for aeration (CBS), which is required for nitrication and oxidation of organic matter to carbon dioxide.

1.2 New perspectives on wastewater treatment

In response to fossil fuel depletion and climate change, there is a trend towards recovery of energy and resources from wastewater. Biology-based processes that produce energy or chemicals while treating wastewater are getting more attention. Anaerobic wastewater treatment is an established technology for the recovery of the chemical energy that is contained in the organic pollutants in industrial wastewater and municipal wastewater in (sub)tropical climates as biogas. For temperate zones, anaerobic municipal wastewater treatment is still in development. In addition, biological hydrogen production (Li and Fang, 2007; Rozendal et al., 2006), electricity production using microbial fuel cells (Rabaey and Verstraete, 2005; Ter Heijne et al., 2011) and biofuel production from lipids accumulated by heterotrophic lipid-accumulating algae (Tian-Yuan et al., 2013) and waste activated sludge (Siddiquee and Rohani, 2011) are studied. Production of biochemicals, e.g. enzymes, glycerol, dyes, acetic acid,

polyhydroxyalkanoates and lactic acid from various types of wastewater is investigated (Angenent et al., 2004). For municipal wastewater treatment, research focusses on the production of polyhydroxyalkanoates (Salehizadeh and Van Loosdrecht, 2004) and biogas (Chernicaro, 2006).

When energy or chemicals are produced from municipal wastewater, an effluent with a low C/N ratio, a low nitrogen concentration and, in moderate climates, a low temperature will be produced. An additional treatment step will be required to remove the nitrogen. In this chapter, concepts for nitrogen removal at low temperatures and low C/N ratios are compared amongst each other and to conventional nitrification-denitrification. The most appropriate concept, denitrification coupled to anaerobic methane oxidation, was studied in more detail. The outline of the research on this process is presented in section 1.5.

1.3 Anaerobic wastewater treatment

In the research presented in this thesis, nitrogen removal from the effluent of low-temperature anaerobic municipal wastewater treatment was studied. By applying anaerobic treatment, municipal wastewater treatment plants (WWTPs) can become (close to) energy self-sufficient. In both industrialized and developing countries, municipal wastewater is generally treated aerobically (Zeeman and Lettinga, 1999). In (sub)tropical countries also anaerobic treatment of municipal wastewater is applied (Chernicaro, 2006). Anaerobic treatment has many advantages over aerobic treatment. In temperate zones, research mostly focuses on enhanced pre-concentration of wastewater organic matter (chemical oxygen demand, COD) and subsequent anaerobic digestion of the obtained primary sludge to produce biogas, (e.g. Akanyeti et al. 2010). An alternative would be direct anaerobic municipal wastewater treatment. This has many advantages over conventional activated sludge treatment, such as energy production as biogas instead of energy consumption, reduced sludge production and a smaller footprint (Lema and Omil, 2001; Lettinga, 1995). The upflow anaerobic sludge bed (UASB) reactor is the most commonly applied reactor for direct anaerobic wastewater treatment (Lettinga and Hulshoff-Pol, 1991). The reactor is successfully applied in tropical regions, both for (agro)industrial and municipal wastewaters (Zeeman and Lettinga, 1999). In temperate zones, winter temperatures may drop to 5–10 °C (Lettinga et al., 2001). Application of the UASB, or other anaerobic systems, for complex wastewaters such as municipal wastewater at such low temperatures is a challenge, in particular because hydrolysis of suspended COD becomes rate limiting and long solid retention times (SRT) would be required. Moreover, at a temperature $< 15\,^{\circ}C$ an SRT of more than 100 days is required to retain sufficient methanogenic activity (Zeeman and Lettinga, 1999). At low temperatures, reactor systems are required with SRTs long enough to sustain hydrolysis and growth of methanogens,

but can still be operated at a hydraulic retention times (HRT) short enough for an economically feasible application.

Two-stage systems were developed for municipal wastewater treatment at low temperatures. In the first stage solids are entrapped and hydrolyzed, in the second stage methanogenesis takes place. One example is the combination of a hydrolysis upflow sludge bed reactor and an expanded granular sludge bed reactor. In this system, at 12 °C, a COD removal efficiency of 51 % was achieved at HRTs of 2 h and 3 h, respectively (Wang, 1994). Another example is the anaerobic filter combined with an anaerobic hybrid reactor. With this system, at 13 °C, a COD removal efficiency of 71 % was achieved at HRTs of 4 h and 8 h, respectively (Elmitwalli et al., 2002). In both cases, however, a non-stabilized excess sludge was produced. The UASB-digester was successfully applied for pilot-scale anaerobic treatment of municipal wastewater at low temperatures while producing stabilized excess sludge (Mahmoud, 2008; Mahmoud et al., 2004; Álvarez et al., 2004). In the UASB reactor dissolved COD is converted at ambient temperature (5–20 °C). Solids are entrapped in the flocculant sludge bed and a small concentrated sludge stream (up to 12.5 % of the influent flow, Zhang et al., 2012) is recirculated over a digester. In the digester, at elevated temperature (30–35 °C), suspended COD is hydrolyzed and the sludge is enriched in methanogens. The stabilized sludge is returned to the UASB to provide methanogenic activity. With this system a total COD removal efficiency of 66 % was achieved at a temperature of 15 °C and an HRT of 6 h while a long SRT of 21 d was maintained in the digester (Mahmoud et al., 2004). The methanogenic activity, and thereby total COD removal, can be further enhanced by adding co-substrates to the digester (Zhang et al., 2013).

After anaerobic treatment no readily available carbon sources remain to sustain heterotrophic denitrification. In addition, the effluent of low-temperature anaerobic municipal treatment contains considerable amounts of dissolved methane. Theoretically, the concentration of dissolved methane can be 15–20 mg/L assuming Henry's law (calculated for atmospheric pressure, 20 resp. 10 °C and 70 % methane in the biogas). 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 and Lant, 2006). Methane has a high global warming potential (25 times higher than CO2, IPCC, 2007) and therefore emission to the atmosphere should be prevented. Thus, in addition to nitrogen, dissolved methane has to be removed. This is possible by both aerobic and anaerobic oxidation. Removal can also be coupled to nitrogen removal. Systems for physical-chemical recovery of dissolved methane from effluents so far have not been developed (Cookney et al., 2010).

1.4 Nitrogen removal

Nitrogen is removed from wastewater using biological or physical-chemical methods. At concentrations higher than 5 mg NH_4^+-N/L physical-chemical treatment is technically and economically feasible. Biological systems for the removal of high ammonium pagebreak concentrations are in development (Kuntke et al., 2012). At municipal wastewater concentrations (typically 50 mg N/L), biological treatment is preferred (Mulder, 2003). In the following, conventional and novel treatment technologies for the removal of nitrogen at low concentrations, low C/N ratios and low temperatures are compared (table 1.1).

1.4.1 Conventional nitrification-denitrification

Nitrogen is conventionally removed from municipal wastewater by a sequence of nitrification and denitrification. Nitrification is performed by chemolithoautotrophic organisms. Ammonia oxidizing bacteria (AOB), *Nitrosomonas* being the most prevalent in wastewater treatment, oxidize ammonia to nitrite (eq. 1.1). Nitrite oxidizing bacteria (NOB), mainly *Nitrobacter*, oxidize nitrite to nitrate (eq. 1.2). The first reaction requires a theoretical 3.43 g O_2/g NH_4^+-N, the latter requires a theoretical 1.14 g O_2/ NO_2^--N. The process is performed at oxygen concentrations of 1–3 mg O_2/L. Nitrifying bacteria grow slowly and dictate the SRT in conventional activated sludge systems: an SRT of 10–20 d is required for nitrogen removal at 10 °C (Tchobanoglous et al., 2003).

$$2NH_4^+ + 3O_2 \longrightarrow 2NO_2^- + 4H^+ 2H_2O \tag{1.1}$$

$$2NO_2^- + O_2 \longrightarrow 2NO_3^- \tag{1.2}$$

Denitrification, viz. reduction of nitrate to nitrogen gas, is conducted in anoxic zones. A wide variety of facultative aerobic, heterotrophic microorganisms are able to use nitrate as electron acceptor (Tchobanoglous et al., 2003). In pre-anoxic denitrification wastewater COD (represented as $C_{10}H_9O_3N$) is used as electron donor (eq. 1.3), whereas in post-anoxic denitrification an external electron acceptor, mostly methanol (eq. 1.4), but also acetate, glucose, ethanol, lactic acid (Ahn, 2006), hydrolyzed sludge or organic wastes, is added (Ginige et al., 2009).

$$C_{10}H_{19}O_3N + 10NO_3^- \longrightarrow 5N_2 + 10CO_2 + 3H_2O + NH_3 + 10\,OH^- \tag{1.3}$$

$$5CH_3OH + 6NO_3^- \longrightarrow 3N_2 + 5CO_2 + 7H_2O + 6\,OH^- \tag{1.4}$$

One of the most common processes for nitrogen removal is the Modified Ludzack-Ettinger process. This system applies pre-anoxic denitrification, using biodegradable COD from the wastewater as electron donor for denitrification. Effluent recirculation (factor 10–20) is required to supply nitrate. As a result, the effluent typically contains

4–7 mg NO_3^--N/L. Although this method has many advantages (aeration savings since nitrate is used as electron acceptor for part of the COD degradation, production of a well settling sludge, lower space requirements than simultaneous nitrification-denitrification) over simultaneous nitrification-denitrification at low dissolved oxygen concentrations and post-anoxic denitrification (Tchobanoglous et al., 2003), it is not suited for treatment of effluent of anaerobic municipal wastewater treatment. During anaerobic treatment, the biodegradable COD is recovered as biogas. As a result, the effluent has a low C/N ratio. Therefore, for conventional nitrogen removal from the effluent of anaerobic treatment, addition of an external electron donor would be required.

To apply post-anoxic denitrification for the treatment of effluent of anaerobic treatment, the effluent should first be nitrified. In the subsequent anoxic zone, an external carbon source, usually methanol, should be added as an electron donor for denitrification. However, addition of an external electron donor to an effluent from which carbon sources firstly have been recovered as methane is not logical, inefficient, expensive and not considered sustainable (Modin et al., 2007). Still, by post-anoxic denitrification lower effluent nitrate of < 3 mg NO_3^--N/L concentrations can be achieved since no effluent recirculation is applied. Also, using methanol, less excess sludge is produced than when e.g. acetate is added or organic compounds from the wastewater are used as carbon source in pre-denitrification.

1.4.2 Nitrification-denitrification via the nitrite route

Although nitrification-denitrification usually proceeds through nitrate, partial nitrification to nitrite (nitritation; eq. 1.1) offers many advantages. These include a 25 % lower oxygen consumption, resulting in a 60 % reduction of energy required for aeration; 40 % reduced electron donor requirement, which is particularly interesting when treating wastewater with a low C/N ratio; 1.5–2 times higher denitrification rates than with nitrate; 20 % lower carbon dioxide emissions and ca. 30 % lower sludge production in the nitrification process and 55 % lower sludge production in the denitrification process (Kornaros et al., 2008; Peng and Zhu, 2006; Kim et al., 2003). Various strategies can be applied to oxidize ammonium to nitrite (eq. 1.1), while preventing oxidation of nitrite to nitrate (eq. 1.2). NOB can be selectively washed out by control of the SRT (at temperatures > 25 °C) or growth of NOB can be limited by controlling the dissolved oxygen concentration, duration of aeration, phosphate concentration, substrate concentration and load, pH and addition of inhibiting chemicals (Blackburne et al., 2008; Peng and Zhu, 2006).

Most of these control strategies can only be applied at high ammonium concentrations and/or high temperatures. A well-established concept is the Single reactor system for High activity Ammonium Removal Over Nitrite (SHARON). This system is applied at 30–40 °C, e.g. for the treatment of reject water from sludge digesters,

and relies on the selective washout of NOB. An SRT is applied, which is shorter than the minimum SRT for NOB, but longer than the minimum SRT for AOB. Below 20 °C, NOB grow faster than AOB and therefore, NOB cannot be selectively washed out (Hellinga et al., 1998). Alternatively, at low temperatures, the aeration duration can be controlled. Using a step-feed sequencing batch reactor and real-time control of the aeration duration based on pH, an average nitrite accumulation above 95 % and a nitrogen removal efficiency above 90 % was achieved at temperatures of 11.9–26.5 °C and an influent nitrogen concentration of 60 mg NH_4^+-N/L (Yang et al., 2007). The oxygen uptake rate can also be applied to control aeration duration and thereby achieve stable nitritation of municipal wastewater. (Blackburne et al., 2008) achieved 80 % nitritation at an average nitrogen concentration of 43 mg N/L and a temperature of 18–25 °C. Moreover, low dissolved oxygen concentrations can be applied to facilitate AOB but limit growth of NOB, since AOB have a higher affinity for oxygen than NOB. At < 0.5 mg O_2/L incomplete denitrification will occur (Tchobanoglous et al., 2003). Hanaki et al. (1990) achieved 75 % nitrite accumulation with a synthetic wastewater with a low influent concentration of 80 mg NH_4^+-N/L, at 0.5 mg O_2/L, yet a temperature of 25 °C. To our knowledge there are no reports of oxygen-limited reactor operation for the treatment of wastewater at low temperature. After nitritation, post-anoxic denitrification using external carbon source could be applied to effluent of anaerobic wastewater treatment. As far as we know, post-anoxic denitrification has not been applied for the post-treatment of effluent from anaerobic municipal wastewater treatment; in tropical regions, usually ponds or trickling filters are applied. This process proceeds as described in section 1.4.1, yet benefits from the advantages described above.

1.4.3 Anaerobic ammonium oxidation

Unlike conventional nitrification-denitrification, or nitrification-denitrification via the nitrite pathway, anaerobic ammonium oxidation (anammox) does not require addition of an organic electron donor and carbon source. Anammox bacteria are autotrophic bacteria, belonging to the group of Planctomycetes. They use nitrite as electron acceptor, ammonium as electron donor (eq. 1.5) and carbon dioxide, which is present in the effluent of anaerobic wastewater treatment, as a carbon source. The latter makes them particularly interesting for applications for streams with a low C/N ratio. They convert nitrite and ammonium to nitrogen gas (eq. 1.5, simplified equation). Also, approximately 10 % of nitrite is converted to nitrate. Without additional nitrate removal, effluent nitrogen concentrations are dictated by nitrate production, which is a disadvantage of the anammox process. Only 55–60 % of ammonium has to be partially nitrified according to eq. 1.1 (Hendrickx et al., 2012b; Ahn, 2006), thus less energy is required for aeration than when applying nitrification-denitrification.

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O \tag{1.5}$$

About 30 full-scale nitritation-anammox reactors have been build (Vlaeminck et al., 2012). However, because of the long doubling times of anammox bacteria, viz. 10–12 d at 35 °C, anammox processes have only been applied for waters containing high nitrogen concentrations and/or at high temperatures. A long SRT is required to achieve high biomass concentrations and thereby sufficiently high volumetric nitrogen removal rates (Kartal et al., 2010). Lab-scale application of anammox for water containing < 100 mg N/L and at temperatures ≤ 20 °C has been studied by Hu et al. (2013), Hendrickx et al. (2012b) and Kartal et al. (2010). Hu et al. (2013) achieved stable nitritation-anammox in a sequencing batch reactor at 12 °C and fed with synthetic wastewater containing 70 mg NH_4^+-N/L. More than 90 % nitrogen removal was accomplished. However, the rate needs to be improved by an order of magnitude to make this process attractive for full-scale application. Hendrickx et al. (2012b) used sludge from an anammox reactor operated at 25 °C to inoculate a gaslift reactor operated at 20 °C and fed with synthetic wastewater containing 69 ± 5 mg (NH_4^+ + NO_2^-)-N/L. After 8 months, it was possible to operate the reactor at a HRT of 5.3 h, which is close to the HRT commonly applied in heterotrophic nitrification-denitrification, viz. 3–4 h. Nitritation at low temperature and low nitrogen concentration, nor the use of actual effluent of anaerobic wastewater treatment, was studied.

Nitritation and anammox can either be performed in two separate reactors (two-stage anammox) or in one reactor. The latter is most commonly applied on full scale (Vlaeminck et al., 2012). It is known by many names, amongst which Completely Autotrophic Nitrogen Removal over Nitrite (CANON), One-stage anammox, Oxygen-Limited Autotrophic Nitrification-Denitrification (OLAND) and DEamMONification (DEMON). The systems retain biomass as flocs in sequencing batch reactors, as granules, or as biofilms. The biomass is layered and consists of AOB and anammox bacteria. In one system, the AOB nitrify ammonium, thereby removing oxygen and producing nitrite for the aerobic bacteria while nitrite and the remaining ammonium are removed by the anammox bacteria (simplified in eq. 1.6).

$$2\,NH_4^+ + 1.5O_2 \longrightarrow N_2 + 2H^+ + 3H_2O \tag{1.6}$$

The same overall conversion rate takes place when applying a nitritation-anammox system. However, a drawback of this system is that two separate process steps are used, resulting in higher capital and operational costs (Toh et al., 2002).

1.4.4 Denitrification coupled to aerobic methane oxidation

Methane is commonly considered a cheap, and on many sites readily available, electron donor for denitrification (Modin et al., 2007; Houbron et al., 1999; Thalasso et al., 1997). In an association between aerobic methane oxidizing bacteria and heterotrophic denitrifying bacteria, methane can serve as the sole carbon source. Soluble

organic compounds such as methanol are excreted by aerobic methane oxidizing bacteria (eq. 1.7). These compounds are subsequently used as electron donors by the denitrifying bacteria (reviewed by Modin et al. (2007); eq. 1.8 and 1.4).

$$CH_4 + O_2 + 2H^+ + 2e^- \longrightarrow CH_3OH + H_2O \tag{1.7}$$

$$CH_3OH + H_2O \longrightarrow CO_2 + 2H^+ + 2e^- \tag{1.8}$$

Denitrification rates competitive with rates in conventional nitrification-denitrification were achieved. However, the theoretical ratio of methane to nitrate consumption for this process is 1.27 (Modin et al., 2007). Due to the presence of oxygen, in practice ratios higher than 2.2 were observed (Modin et al., 2008). The use of nitrite as electron acceptor has not been reported, but heterotrophic denitrifiers are able to use both nitrite and nitrate. To remove 50 mg NO_2^--N/L, a nitrogen concentration typical for municipal wastewater, more than 75 mg CH_4/L would be required. Assuming a ratio of 2.2 molCH_4/molNO_3^- and oxygen equivalents of 2.86 g O_2/g NO_3^--N and 1.71 g O_2/g NO_2^--N it was calculated that a ratio of more than 1.3 mol CH_4/mol NO_2^- can be calculated) would be required. The dissolved methane, nor the total amount of methane produced in low-temperature anaerobic treatment of municipal wastewater, 91 mg total-CH_4/L wastewater), calculated assuming a wastewater concentration of 0.6 g COD/L and 66 % COD removal (Mahmoud et al., 2004), might not be enough to sustain denitrification. Although anaerobic municipal wastewater treatment offers opportunities to recover chemical energy as methane, using denitrification coupled to anaerobic methane oxidation, no methane would remain available for energy production.

1.4.5 Denitrification coupled to anaerobic methane oxidation

Effluent of low-temperature anaerobic municipal wastewater treatment does contain enough methane to sustain denitrification coupled to anaerobic methane oxidation (DAMO). After nitritation of the effluent of low-temperature anaerobic municipal wastewater treatment, nitrite-denitrification coupled to anaerobic methane (eq. 1.9) is theoretically feasible. Denitrifying methanotrophic bacteria consume methane and nitrite in a ratio close to the theoretical ratio of 0.375 (Raghoebarsing et al., 2006). At this ratio only 21 mg CH_4/L is required to remove 50 mg NO_2^--N/L, viz. 75 % of the methane produced during anaerobic treatment is available for energy production.

$$3CH_4 + 8NO_2^- + 8H^+ \longrightarrow 3CO_2 + 4N_2 + 10H_2O \tag{1.9}$$

Methanotrophic bacteria are found in mud, swamps, rivers, rice paddies, streams, oceans, ponds, meadow soils, sediments, deciduous woods, and sewage sludge (Hanson and Hanson, 1996). So were denitrifying methanotrophic bacteria (Shen et al., 2013; Zhu et al., 2012; Ettwig et al., 2009; Hu et al., 2009; Raghoebarsing

et al., 2006). The first denitrifying methanotrophic enrichment culture, inoculated with freshwater sediment, consisted of a bacterium and an archaeon (Raghoebarsing et al., 2006). The archaeon disappeared from the culture during prolonged enrichment (Ettwig et al., 2009, 2008) when nitrite was the electron acceptor. Initially it was assumed that bacteria and archaea cooperated like in anaerobic methane oxidation coupled sulfate reduction (Raghoebarsing et al., 2006). However, denitrifying methanotrophic bacteria, bacteria that carry out the anaerobic oxidation of methane using sulfate and aerobic methanotrophs were shown to use very distinct mechanisms (Murrell and Jetten, 2009). '*Candidatus* Methylomirabilis oxyfera' can catalyze the methane oxidation on its own (Ettwig et al., 2009, 2008), expressing a unique pathway (Ettwig et al., 2010). Recently denitrifying methanotrophic archaea, '*Candidatus* Methanoperedens nitroreducens' have been discovered. These archaea reduce nitrate to nitrite while performing reverse methanogenesis (Haroon et al., 2013) and therefore can contribute to nitrate-denitrification coupled to anaerobic methane oxidation.

The research progress on denitrification coupled to anaerobic methane oxidation is slow due to a limited number of enrichment cultures (Hu et al., 2009). Despite their highly exergonic reaction ($\Delta G^{0'}$ −928 kJ/mol CH_4, Raghoebarsing et al., 2006), denitrifying methanotrophic bacteria grow slowly. '*Ca.* M. oxyfera'-like bacteria have a doubling time of 1–2 months (Kampman et al., 2012). The maximum volumetric nitrite consumption rate of enrichment cultures coupling denitrification to anaerobic methane oxidation reported is 36 mg NO_2^--N/L d (Ettwig et al., 2009, 2008). This rate would translate to an HRT of 1.4 d. Volumetric denitrification rates have to be increased by an order of magnitude, to be able to compete with conventional nitrification-denitrification, which commonly has an HRT of 3 h–4 h. However, a stagnating rate was observed in two enrichment cultures (Ettwig et al., 2009, 2008). It was hypothesized this could be due to production of an inhibiting compound, or absence of an unknown growth factor. Since a completely stirred tank reactor with external settler and sludge recirculation, and a sequencing batch reactor were applied, inefficient biomass retention may also have been a cause for the stagnating conversion rates.

1.4.6 Simultaneous anammox and DAMO processes

To remove ammonium, nitrite and/or nitrate and methane simultaneously, combination processes containing DAMO bacteria and archaea, and anammox bacteria have been proposed (Shi et al., 2013; Luesken et al., 2011a). Shi et al. (2013) developed a membrane biofilm reactor for simultaneous removal of nitrate and ammonium. Nitrate was converted to nitrite by DAMO archaea, using gaseous methane which was supplied through the membrane; nitrite and ammonium were converted to nitrogen gas and nitrate by anammox bacteria. Though the process is more complicated, i.e. requiring an association of three types of organisms and supply of both ammonium

and nitrate, the ratio methane and nitrogen (ammonium+nitrate) consumption to nitrogen production was similar to the theoretical ratio of nitrite-dependent DAMO, viz. 3 : 8 : 4 (Shi et al., 2013). Therefore, DAMO is preferred.

Luesken et al. (2011a) developed a coculture of DAMO and anammox bacteria. In this process, methane and nitrite were always present in excess, while ammonium was limiting. This way, a culture developed that used consumed methane, nitrite and ammonium. However, nitrate is produced, resulting in a relatively high nitrate concentration in the effluent. It is likely that in this process, DAMO archaea would convert nitrate to nitrite while oxidizing methane, however, this has not been reported. Similarly, in an anammox reactor fed with effluent containing dissolved methane, DAMO archaea might consume nitrate and methane. However, this has not been demonstrated yet. Since part of the nitrite is reduced with ammonium, instead of methane, a substantial part of dissolved methane would remain present in the effluent. Therefore this process is not considered suitable for effluent of anaerobic municipal wastewater treatment at low temperatures; it may be suitable for treatment of effluent in (sub)tropical regions, where less methane is dissolved in the effluent.

1.4.7 Other processes

In addition to aforementioned processes, algal ponds, wetlands and duckweed ponds can be used to remove nitrogen. These systems are either natural or man-made ponds and are commonly applied for wastewater treatment in areas where a lot of sunshine is available and land is cheap. In the systems nutrients, organic matter, suspended solids and pathogens are removed. The retention time is in the order of 1 - 2 weeks. Alhough these processes consume hardly any energy, the requirement for large surface areas seriously restricts application. Moreover, the processes require light and the diurnal and seasonal light cycles and the effect thereof on the nutrient uptake will determine the required area.

1.4.8 Comparison of technologies for nitrogen removal

This section compares the technologies for nitrogen removal from effluent of low-temperature anaerobic treatment of municipal wastewater discussed in sections 1.4.1 through 1.4.6. An overview is presented in table 1.1.

Conventional nitrification-denitrification and nitrification-denitrification via the nitrite route both require addition of an electron donor and carbon source. However, using anaerobic municipal wastewater treatment, COD is recovered as biogas and not available for heterotrophic denitrification. Addition of an external carbon source is not preferred since costs are high and this is not environmentally sustainable. Nitritation-anammox does not require addition of an external electron and already is

an established process for warm wastewaters containing high nitrogen concentrations. However, removal of dissolved methane in nitritation-anammox systems has not been studied. The process requires an aerobic step for nitritation of 55–60 % of wastewater ammonium (typically 50 mg N/L), while for the DAMO process (section 1.4.5) all ammonium has to be oxidized. This means that nitritation-anammox consumes 53–63 mg O_2/L less for nitritation, but 60–80 mg O_2/L is required for removal of 15–20 mg CH_4/L, adding to a total requirement of 134–183 mg O_2/L. Although methane can be used as electron donor for denitrification, denitrification coupled to aerobic methane oxidation is not feasible, since it would consume (almost) all the methane that is produced in anaerobic treatment. Dissolved methane has to be removed from the effluent of anaerobic treatment only and suffices to drive denitrification coupled to anaerobic methane oxidation. Nitritation combined with DAMO would theoretically require 128–168 mg O_2/L, i.e. less than nitritation-anammox with aerobic methane oxidation, while it would solve both problems associated with anaerobic municipal wastewater treatment at low temperatures, viz. removal of nitrogen and dissolved methane. Nitrite is required as an electron acceptor and although oxidation of ammonia to nitrite at low nitrogen concentrations and low temperatures, needs further study, control of aeration duration and dissolved oxygen concentration show promising results with respect to nitrite accumulation. Therefore, this research studied the feasibility of applying denitrification coupled to anaerobic methane oxidation for treatment of the effluent of low-temperature anaerobic municipal wastewater treatment.

1.5 Thesis outline

The objective of this research was to investigate the feasibility of denitrification coupled to anaerobic methane oxidation for the removal of nitrogen and methane from the effluent of low-temperature anaerobic municipal wastewater treatment. Denitrifying methanotrophic bacteria were enriched from freshwater sediment and wastewater sludge. The effects of reactor biomass retention, process conditions and additives on the enrichment rates and volumetric denitrification rates were determined.

The enrichment of denitrifying methanotrophic bacteria from freshwater sediment is described in chapter 2. Sequencing fed-batch reactors were operated at 30 °C; a strategy that had been proven successful for enrichment of *M. oxyfera*-type bacteria. To increase volumetric denitrification rates a reactor was fed with medium prepared with effluent of activated sludge treatment. Also biomass washout from the reactors was monitored and it was shown that washout could have considerably delayed enrichment. Therefore a membrane was placed in one of the reactors, and the effect on denitrification rates was monitored.

In chapter 3, enrichment of denitrifying methanotrophic bacteria from wastewater

sludge, in membrane bioreactors at 20 °C, which is 5–15 °C lower than in most enrichments described, was reported. The bacteria were successfully enriched. To further increase denitrification rates several additives (effluent containing *M. oxyfera*-type bacteria from the reactors described in chapter 2, 100 % effluent of aerobic municipal wastewater treatment, ammonium, increased copper concentration, autoclaved sludge and filtrate from autoclaved sludge) and a shorter HRT were tested with the aim of increasing the denitrification rates. The operation of the two reactors described in chapter 2 was continued and performance was compared with membrane bioreactor performance.

To remove nitrogen and methane from the effluent of anaerobic municipal wastewater treatment at low temperatures, also a denitrifying methanotrophic process should be operated at low temperatures. However, the activity of denitrifying methanotrophic bacteria < 20 °C had not been determined before. Therefore in this research the volumetric denitrification rates of denitrifying methanotrophic bacteria at 11 and 16 °C was measured and compared to rates at 20, 25 and 30 °C. This has been described in chapter 4.

The effect of the addition of low amounts of oxygen on the volumetric denitrification rates of denitrifying methanotrophic bacteria was tested and described in chapter 5. Possibly, low concentrations would not inhibit these oxygen producing bacteria, but even enhance their growth and enrichment rates. The results of the aforementioned chapters are summarized and discussed in chapter 6. Recommendations are made on how to increase enrichment rates and denitrification rates, to study if treatment of effluent of anaerobic municipal wastewater treatment at low temperature by denitrifying methanotrophic bacteria may become feasible.

Table 1.1: Comparison of processes for removal of nitrogen and methane from the effluent of anaerobic municipal wastewater treatment at low temperature.

	Conventional nitrification-denitrification	**Nitrification-denitrification nitrite route**	**Nitritation-anammox**	**Denitrification-aerobic methane oxidation**	**Denitrification-anaerobic methane oxidation**	**Simultaneous anammox-denitrification-anaerobic methane oxidation**
Energy consumption	very high	high	low	n.d.	low	low
Sludge production	very high	high	low	n.d.	very low	very low
C/N (g COD/g N)	3–6[a]	2–4[a]	-	>2.2	0.375	ca. 0.4
Methane removal	n.d.	n.d.	n.d.	very high	very high	high
External carbon source	very high	high	none	none	none	none
Research status	full-scale	lab-scale	full-scale[b]	lab-scale	lab-scale	lab-scale

[a] Based on the use of methanol as electron carbon source full-scale at high temperatures and high nitrogen concentrations, pilot-scale at low temperatures

[b] n.d. not determined; in aerobic processes methane is likely to be removed by aerobic methane oxidation, however, no numbers are available.

2

Enrichment of denitrifying methanotrophic bacteria for application after direct low-temperature anaerobic sewage treatment

Abstract

Despite many advantages of anaerobic sewage treatment over conventional activated sludge treatment, it has not yet been applied in temperate zones. This is especially because effluent from low-temperature anaerobic treatment contains nitrogen and dissolved methane. The presence of nitrogen and methane offers the opportunity to develop a reactor in which methane is used as electron donor for denitrification. Such a reactor could be used in a new concept for low-temperature anaerobic sewage treatment, consisting of a UASB-digester system, a reactor for denitrification coupled to anaerobic methane oxidation, and a nitritation reactor. In the present study denitrifying methanotrophic bacteria similar to '*Candidatus* Methylomirabilis oxyfera' were enriched. Maximum volumetric nitrite consumption rates were 33.5 mg NO_2^--N/L d (using synthetic medium) and 37.8 mg NO_2^--N/L d (using medium containing effluent from a sewage treatment plant), which are similar to the maximum rate reported so far. Though the goal was to increase the rates, in both reactors, after reaching these maximum rates, volumetric nitrite consumption rates decreased in time. Results indicate biomass washout may have significantly decelerated enrichment. Therefore, to obtain higher volumetric consumption rates, further research should focus on systems with complete biomass retention.

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2.1 Introduction

Anaerobic sewage treatment has many advantages over conventional activated sludge treatment. These include energy recovery as biogas instead of energy consumption, reduced sludge production and a smaller footprint (e.g. Lema and Omil, 2001; Lettinga, 1995). Despite these advantages and successful application of anaerobic sewage treatment in tropical regions it has not yet been applied in temperate zones (Seghezzo et al., 1998). For lower temperatures reactor systems with solid retention times (SRT) long enough for hydrolysis and growth of methanogens, but still relatively short hydraulic retention times (HRT) are required. Also, to comply with discharge standards, effluent from anaerobic treatment requires further treatment. This is required for remaining chemical oxygen demand (COD), but especially for nitrogen and phosphorus, which are largely conserved during anaerobic treatment. In addition, the effluent from a low-temperature anaerobic sewage treatment system contains a considerable amount of dissolved methane (Cookney et al., 2010; Uemura and Harada, 2000). The concentration of dissolved methane can be 20 mg/L assuming Henry's law (calculated for atmospheric pressure, 10 °C and 70% methane in the biogas), and frequently methane supersaturation occurs (Cookney et al., 2010; Uemura and Harada, 2000). If effluent containing dissolved methane would be discharged, methane would be released to the atmosphere. As it is a gas with a high global warming potential, dissolved methane has to be removed to reduce the gas emissions of low-temperature anaerobic sewage treatment compared with conventional treatment.

Pilot-scale application of the combination of an upflow anaerobic sludge bed (UASB) reactor and a sludge digester, referred to as UASB-digester system, was successful for anaerobic sewage treatment at low temperatures (Mahmoud, 2008; Mahmoud et al., 2004; Álvarez et al., 2004). In the UASB reactor (at 10–20 °C) dissolved COD is converted; solids are entrapped in the flocculent sludge bed and transported to the digester. In the digester (at 35 °C) suspended COD is hydrolyzed and the sludge is enriched in methanogens. The sludge is recirculated to the UASB reactor to provide methanogenic activity. With this system a total COD removal efficiency of 66% was achieved at a temperature of 15 °C and an HRT of 6 h while a long SRT of 21 d was maintained in the digester (Mahmoud et al., 2004). Although conventional technologies can be applied to remove remaining COD and phosphorus from the effluent, conventional nitrogen removal is not a preferred option. Effluent from an anaerobic system contains ammonium, which is usually removed by a sequence of nitrification to nitrate and heterotrophic denitrification. However, during anaerobic treatment the readily available carbon sources are removed and addition of an external electron donor, e.g. methanol, would be required to sustain heterotrophic denitrification. Anaerobic ammonium oxidation, an autotrophic process, would be an alternative (Hendrickx et al., 2012b). However, this process will not remove dissolved methane. Instead, a new treatment concept is proposed, in which dissolved methane

is used as electron donor for denitrication via nitrite. Such a system would solve two problems, viz. removal of nitrogen and dissolved methane. To provide nitrite a nitritation reactor is required. To conserve methane for denitrification and to save on aeration energy this reactor is positioned after the reactor for denitrification coupled to anaerobic methane oxidation. Combined, the UASB-digester, a reactor for denitrification coupled to anaerobic methane oxidation, and a nitritation reactor, to supply nitrite required for the denitrifying methanotrophic bacteria, offer a new opportunity for energy-efficient wastewater treatment with a reduced carbon footprint (fig. 2.1).

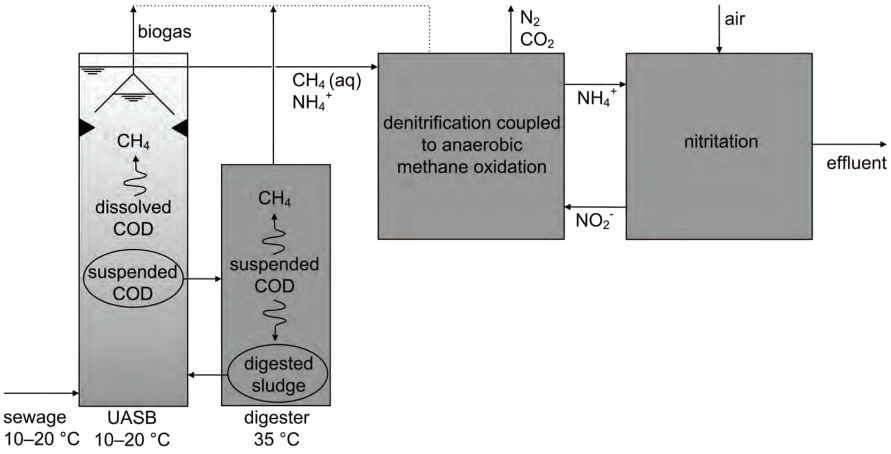

Figure 2.1: New concept for sewage treatment at low temperatures, consisting of anaerobic sewage treatment for removal of organic matter, complemented with denitrification coupled to anaerobic methane oxidation and nitritation for nitrogen and dissolved methane removal.

Though denitrification coupled to aerobic methane oxidation was studied extensively (reviewed by Modin et al., 2007), the progress on denitrification coupled to anaerobic methane oxidation is slow due to a limited number of enrichment cultures (Hu et al., 2009). However, denitrification coupled to anaerobic methane oxidation (eq. 2.1), would have several advantages over aerobic processes. These include that no oxygen is required for partial methane oxidation and methane is used more efficiently. This implies that more nitrogen can be removed using the methane dissolved in the effluent from UASB-digester systems.

$$3\,CH_4 + 8\,NO_2^- + 8\,H^+ \longrightarrow 3\,CO_2 + 4\,N_2 + 10\,H_2O \tag{2.1}$$

A few years ago a denitrifying methanotrophic culture consisting of a bacterium and an archaeon was obtained under anaerobic conditions (Raghoebarsing et al., 2006). Further research has shown that the process also proceeds without the archaea,

indicating that the dominant bacterium, '*Candidatus* Methylomirabilis oxyfera' (*M. oxyfera* hereafter) can catalyze the methane oxidation on its own (Ettwig et al., 2009, 2008), expressing a unique intra-aerobic pathway (Ettwig et al., 2010).

Typically effluent from anaerobic sewage treatment plants contains 50 mg N/L. Using the 20 mg/L of dissolved methane, 47 mg N/L could be removed according to the stoichiometry presented in eq. (2.1). The maximum volumetric nitrite consumption rate of enrichment cultures coupling denitrification to anaerobic methane oxidation reported is 36 mg NO_2^--N/L d (Ettwig et al., 2009). This rate would translate to an HRT of 1.4 d. Conventional denitrification typically has an HRT of 3–4 h. Thus, for a practical application of denitrification coupled to anaerobic methane oxidation for sewage treatment, volumetric nitrite consumption rate needs to be increased by an order of magnitude. However, a stagnating rate was observed in two enrichment cultures (Ettwig et al., 2009, 2008). It was hypothesized this could be due to production of an inhibiting compound, or absence of an unknown growth factor. Since a completely stirred tank reactor with external settler and a sequencing batch reactor were applied, inefficient biomass retention may also have been a cause for the stagnating conversion rates.

The objectives of this study were (1) to enrich denitrifying methanotrophic cultures and (2) to increase the volumetric conversion rates of the enrichment cultures, so eventually the process can be integrated in the proposed concept for anaerobic sewage treatment at low temperatures.

Denitrifying methanotrophic bacteria were enriched for a period of 651 d in two sequencing fed-batch reactors. To increase maximum volumetric conversion rates, a long settling time was applied to improve biomass retention and effluent from a sewage treatment plant was fed to one of the reactors as a source of potential growth factors. The reactors were mixed by gas recirculation, providing sufficient transfer of methane. In both reactors, nitrite consumption rates were followed in time and whole culture batch tests were performed to measure denitrifying methanotrophic activity. Washout of biomass with the effluent was quantified to evaluate biomass retention of the systems. The practical applicability of a process with denitrifying methanotrophic bacteria for nitrogen and methane removal after direct low-temperature anaerobic sewage treatment is discussed.

2.2 Materials and methods

2.2.1 Inoculum

Two sequencing fed-batch reactors (SFBRs) were inoculated with sediment (3.7 ± 6.0 g protein each) from ditches in Ooijpolder, The Netherlands, similar to (Ettwig et al., 2009). Prior to inoculation the sediment was sieved (1.0 mm) and diluted with ditch water to obtain a homogeneous slurry.

2.2.2 Medium

Medium contained ($g\,L^{-1}$) 0.1–1.0 $KHCO_3$, 0.05 KH_2PO_4, 0.30 $CaCl_2 \cdot 2\,H_2O$, 0.22 $MgSO_4 \cdot 7\,H_2O$, 0.069–4.83 $NaNO_2$ (0.014–0.980 NO_2^-–N), 0.085–0.765 $NaNO_3$ (0.014–0.126 NO_3^-–N), 0.6 mM HCl, 0.5 mL/L acidic trace element solution and 0.2 mL/L alkaline trace element solution (adapted from Ettwig et al., 2009). The acidic trace element solution contained (g/L) 2.085 $FeSO_4 \cdot 7\,H_2O$, 0.068 $ZnCl_2$, 0.12 $CoCl_2 \cdot 6\,H_2O$, 0.5 $MnCl_2 \cdot 4\,H_2O$, 0.32 $CuSO_4$, 0.048 $NiCl_2 \cdot 6\,H_2O$ and 100 mM HCl. The alkaline trace element solution contained (g/L) 0.067 SeO_2, 0.05 $Na_2WO_4 \cdot 2\,H_2O$, 0.284 $Na_2MoO_4 \cdot 2\,H_2O$ and 10 mM NaOH.

One reactor, referred to as SFBR-, was fed with this synthetic medium. The other reactor, referred to as SFBR+, was fed with medium containing 10 % (v/v) filtered effluent from the aerobic sewage treatment Bennekom, The Netherlands, as a source of potential growth factors. Effluent from aerobic treatment, i.e. low in residual COD, was selected to prevent enrichment of heterotrophic denitrifying bacteria, which might compete with, and thereby hamper, enrichment of denitrifying methanotrophic bacteria. At this treatment plant sewage is treated by means of an activated sludge process, including biological nitrogen and phosphorus removal. Effluent from the activated sludge process is treated in a sand filter in which remaining phosphate is removed by means of iron precipitation. On average the effluent contained 1.3 mg biochemical oxygen demand/L, 24 mg COD/L, 2.1 mg Kjeldahl-N/L and 3.8 mg (NO_2^-+NO_3^-)-N/L. Effluent was filtered over a 0.2 µm filter to remove colloidal and suspended matter.

2.2.3 Setup of sequencing fed-batch reactors

The enrichments were performed in two anaerobically operated SFBRs, in a setup as shown in fig. 2.2. The SFBRs each had a volume of 10 L, with a working volume of 5.3–6.7 L, and were operated in cycles of 1.0–11.5 d of continuous medium supply, followed by a settling period of 2 h and a decanting period of 1 h (effluent removed at 20–25 mL/min). During the supply period 5.0–10 mL/min CH_4/CO_2 (93.6-95.0% CH_4, 5.0 %–6.4 % CO_2) was supplied and gas was recirculated to provide mixing and sufficient gas transfer. Gas, both supplied gas and recirculated gas, was added from the bottom of the reactor, through a glass diffuser producing small bubbles. During decanting 25 mL/min CH_4 /CO_2 was supplied to counteract the effluent removal and to prevent air from entering the reactor. After 623 d in SFBR- an ultrafiltration membrane (VFU250, Memos Membranes Modules Systems GmbH) was placed and liquid was pumped off via the membrane. Cyclic operation was controlled and data (pH and temperature) were acquired using FieldPoint modules and LabVIEW 7.0 (National Instruments). Reactor temperature was controlled at 30 ± 1 °C. Though higher than applied in sewage treatment in temperate zones, this temperature was

selected for faster enrichment of denitrifying methanotrophic bacteria.

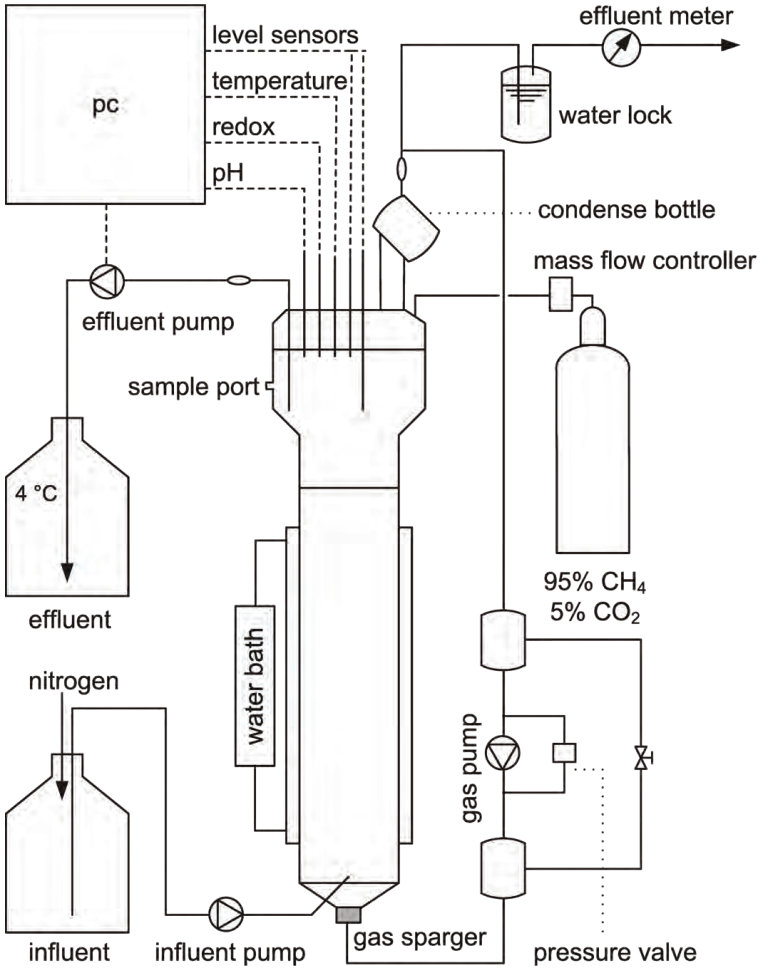

Figure 2.2: Setup of sequencing fed-batch reactors.

2.2.4 Operation of sequencing fed-batch reactors

During the reported 651 d of enrichment, the nitrite loading rate (NLR; calculated as the daily nitrite addition per maximum reactor volume, viz. 6.7 L) was controlled to match the consumption rate.

The nitrite concentration, which was estimated 3–5 times per week, was maintained at 3–30 mg NO_2^--N/L. When the nitrite concentration was < 3 mg NO_2^--N/L the nitrite loading rate was increased. When the nitrite concentration exceeded

30 mg NO_2^--N/L medium supply was stopped until concentration decreased to < 15 NO_2^--N/L. NLR was adjusted by adjusting cycle duration (1.0–11.5 d) or medium concentration (0.014–0.980 g NO_2^--N/L). The nitrite concentration in the medium was increased in time as the nitrite consumption rates increased. To control the pH between 7.0 and 8.0, the bicarbonate concentration in the medium was decreased in time (from 1.0 to 0.1 g/L), while the denitrification rateand thereby the proton consumption rate increased.

Every 7–20 d and when changes were made to reactor operation, nitrite and nitrate concentrations and gas composition (methane, nitrogen, carbon dioxide and oxygen) were measured. Activity measurements were performed regularly to measure biomass activity. Protein concentration was measured to estimate biomass concentrations in inoculum and effluent (in SFBR- from day 556 to day 621 of the enrichment, in SFBR+ from day 551 to day 621 of the enrichment). Molecular analyses were performed to determine the microbial composition and monitor the enrichment of denitrifying methanotrophic bacteria in time.

2.2.5 Activity measurements

To measure the nitrite and nitrate consumption rate of the biomass in the reactors, medium supply was stopped and nitrite and nitrate concentrations were measured 5–8 times during 1–2 d. In three additional tests on each reactor, methane consumption rate and nitrogen gas production rate were measured. Gas supply was stopped, and the gas phase of the reactors was flushed with nitrogen. Methane concentration was then adjusted to 5–10% and carbon dioxide concentration was adjusted to 3–5%. Gas composition was measured, in duplicate, simultaneously with nitrite and nitrate concentration. Before gas measurements started an equilibration time of 2 h was deployed.

2.2.6 Analytical methods

Nitrite and nitrate concentrations were estimated using test strips (Merckoquant, Merck chemicals) and measured according to APHA standard method 4110 B (APHA et al., 1998) using ion chromatography (Metrohm IC Compact 761). The mobile phase was an aqueous solution of 3.2 mM sodium carbonate, 1 mM sodium bicarbonate and 1 % (v/v) acetone. The chemical suppressor was regenerated using 50 mM sulfuric acid and 1 % (v/v) acetone.

Methane, nitrogen, carbon dioxide and oxygen were measured by gas chromatography (Shimadzu GC-2010). The gas chromatograph was equipped with two columns (Porabond Q (50 m x 0.53 mm; 10 µm, Varian, part no. CP7355) and Molsieve 5A (25 m x 0.53 mm; 50 µm; Varian; Part.no. CP7538)) connected in parallel. Standards and samples (50 µL) were injected into an injector at 120 °C. The column was at

1.7 bar and 65 °C. Gases were detected by means of a thermal conductivity detector at 150 °C. The carrier gas was helium at 82.5 mL/min.

Samples (1–25 mL) for protein determination were centrifuged (5 min, 1–2 mL samples at 9300 g, samples > 2 mL at 5000 g) and supernatant was removed. The pellets were resuspended in 0.5 mL 1.0 M sodium hydroxide and the cells were hydrolyzed for 30 min at 50 °C. After hydrolysis, samples were neutralized with 0.5 mL 1.0 M hydrochloric acid. Next, protein concentration was measured according to the Hartree-Lowry method (Hartree, 1972).

2.2.7 Molecular analyses

Inoculum and reactors were sampled (2 mL) for molecular analyses. After centrifugation (5 min at 9300 g) the supernatant was discarded and the pellets were stored at −18 °C for DNA isolation. DNA was isolated according to Ettwig et al. (2009). The isolated DNA was used as a template for polymerase chain reaction (PCR) for amplification of the 16S rRNA gene using a combination of primer 202F (Ettwig et al., 2009) and the general bacterial primer 1545R (Juretschko et al., 1998). The obtained amplicons were used as a template for nested PCR using 'NC10' specific primers qP1F and qP2R (Ettwig et al., 2009). Thermal cycling, for both PCRs, was carried out with an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and elongation at 72 °C for 3 min; cycling was completed by a final elongation step at 72 °C for 10 min. Cloning of the PCR products and sequence analysis was performed as described by Ettwig et al. (2009). ChromasLITE (version 2.01) was used to check the quality of the obtained sequences. BLAST search analysis was performed to identify newly obtained sequences and to obtain related sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). Sequences were aligned in MEGA4 software using CLUSTALW (Tamura et al., 2007). Phylogenetic analysis was performed in MEGA4 using the neighbor-joining method with pairwise deletion of gaps. The tree topology was tested by bootstrap analysis (1000 replicates). Representative sequences were submitted to GenBank (accession numbers JF803475-JF803482, JQ362447 and JQ362448).

Fluorescence in situ hybridization (FISH) was performed as described by Ettwig et al. (2008), however samples were stored at −18 °C and the hybridization buffer contained 50 % formamide. The probes used were S-*-DBACT-0193-a-A-18 (DBACT193) and S-*-DBACT-1027-a-A-18 (DBACT1027), targeting bacteria affiliated with the 'NC10' phylum (Raghoebarsing et al., 2006), the EUB mix for almost all bacteria, EUB338, EUB338II, EUB338III (Daims et al., 1999) and the DNA stain DAPI.

2.3 Results and discussion

2.3.1 Nitrite loading and consumption rates

The operation of the SFBRs was controlled based on the microbial activity. The activity was represented by the volumetric nitrite consumption rate; i.e. an increase in microbial activity was characterized by a higher consumption rate. Activity tests performed throughout reactor operation (fig. 2.3) confirmed that the NLR corresponded well with the nitrite consumption rate and was therefore a good measure of microbial activity.

The nitrite consumption rates in both reactors increased in time (fig. 2.3). The NLR applied to SFBR- was exponentially increased to 25.1 mg NO_2^--N/L d on day 364 (phase I in fig. 2.3a) and to a maximum of 33.5 mg NO_2^--N/L d on day 457. Prior to day 361 almost all supplied nitrite was consumed (reactor concentrations averaged 2.1 mg NO_2^--N/L, ranging from 0.0 and 7.8 mg NO_2^--N/L). The period thereafter (phase II in fig. 2.3a) the reactor appeared to be overloaded (up to 66.4 mg NO_2^--N/L d at day 364) and operational problems (influent pump failure; problems with level sensor resulting in undesired settling) occurred. Consequently, a lower NLR of 12.0 mg NO_2^--N/L d was applied. As a result, the nitrite concentration in the reactor decreased and was below the detection limit from day 406 onwards. Since then, the NLR was increased to an eventual new maximum of 33.5 mg NO_2^--N/L d. Starting on day 457 (phase III in fig. 2.3a), the nitrite consumption rate decreased to a lower NLR of about10 mg NO_2^--N/L d from day 609 to 623. In an attempt to increase the NLR again, a membrane was used from day 623 onwards to remove effluent and achieve complete biomass retention. In the following 30 d this led to an increase in NLR (phase IV in fig. 2.3a).

The NLR in SFBR+ also increased exponentially (phase I in fig. 2.3b) to a maximum of 37.8 mg NO_2^--N/L d on day 372. Subsequent operational problems (too high influent flow rate, influent pump failure, problems with level sensor resulting in undesired settling) in phase II (fig. 2.3b), interrupted further NLR increase. Up until the start of phase III (day 374), nearly all supplied nitrite was consumed (reactor concentrations averaged 1.3 mg NO_2^--N/L, ranging from 0.0 and 7.4 mg NO_2^--N/L, except for a short increase from day 85 to day 121 caused by overloading, resulting in a continuous accumulation of nitrite to 18.1 mg NO_2^--N/L d at day 107). From the start of phase III, the nitrite consumption rate decreased and NLR had to be adjusted frequently (phase III in fig. 2.3b). This was followed by an increase in NLR (phase IV) and finally a stabilization of the NLR around 16 mg N/L d in phase V.

The nitrate consumption rates in both reactors were much lower than the nitrite consumption rates. After 4 months of enrichment the nitrate consumption rate decreased to below 2 mg NO_3^--N/L d (data not shown) and the nitrate concentration in the medium was set to 14 mg NO_3^--N/L.

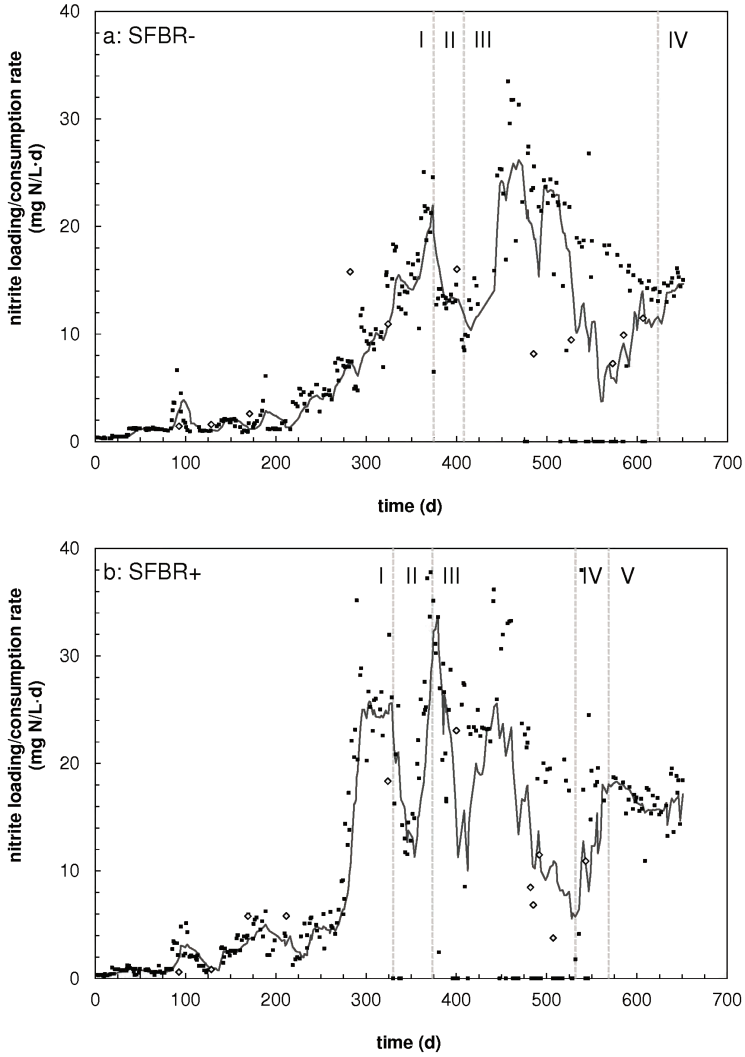

Figure 2.3: Nitrite loading rate (■), weighted average over 10 measurements of NLR (–) and nitrite consumption rate determined in activity tests (◇) in reactors (a) SFBR- and (b) SFBR+ in time. Latin numbers indicate (I) exponential increase in NLR, (II) operational problems, (III) decreasing NLR, (IV) membrane placed in SFBR-, increase in NLR in SFBR+ and (V) stabilization of NLR. An NLR of zero was set to avoid nitrite accumulation or caused by technical problems (such as failing pumps).

The maximum nitrite consumption rates that were achieved (SFBR- 33.5 mg NO_2^--N/L d; SFBR+ 37.8 mg NO_2^--N/L d) were slightly higher than the maximum

nitrite consumption rates reported by most other researchers (Hu et al., 2009; Ettwig et al., 2008; Raghoebarsing et al., 2006) and similar to the maximum rate reported by Ettwig et al. (2009), using similar inoculum and operational conditions. After reaching a maximum, in both reactors the consumption rates decreased and eventually stabilized at lower nitrite consumption rates. Placement of the membrane in SFBR- seemed to stop or even reverse the trend of decreasing nitrite consumption rates, suggesting the importance of efficient biomass retention (section 2.3.3).

Effluent from a sewage treatment plant was added to SFBR+ as a source of potential growth factors, which may previously have limited further increases in NLR. The maximum nitrite consumption rate in SFBR+ was 11 % higher than that for SFBR-. Although it seems addition of effluent did not hamper the enrichment and might even have had a positive effect, several operational aspects (such as described operational problems and changes made to NLR) may have affected enrichment. Therefore it remains to be investigated if the higher NLR that could be applied to SFBR+ was because effluent from the sewage treatment plant contained a missing growth factor. The stagnation and later on decrease of volumetric nitrite consumption rates in SFBR+ could indicate that additional impediment exists, be it an inhibiting compound produced in the reactor or the absence of nutrients or unknown growth factors. The effect of effluent addition may be more pronounced once other limitations have been resolved.

2.3.2 Coupling nitrite and methane consumption

On each reactor, three activity tests were performed to establish, in addition to nitrite consumption, nitrate and methane consumption and nitrogen gas production. Simultaneous nitrite and methane consumption, with concomitant nitrogen gas production could be confirmed. For example, the results of an activity test with SFBR+ after 324 d of enrichment are shown in fig. 2.4. Nitrite, a small amount of nitrate, and methane were consumed and nitrogen gas was produced. The molar conversion ratio of $CH_4 : NO_2^- : N_2$ was $3.0 : 7.9 : 4.5$, which is in good agreement with the stoichiometric ratio of $3 : 8 : 4$ (eq. 2.1). Also in SFBR- after 324 d, 400 d and 485 d of enrichment and in SFBR+ after 400 d and 485 d of enrichment ratios close to expected stoichiometric ratios were measured (table 2.1). This indicated nitrite and methane removal according to eq. 2.1 was the dominant process in the reactors.

2.3.3 Biomass growth and washout

Biomass in the reactors was present both in suspension and attached to the walls. Consequently, representative biomass samples could not be taken and the total amount of biomass in the reactors could not be quantified. Therefore, the increase in NLR applied to the reactors in time (phases I in fig. 2.3) was used to estimate a

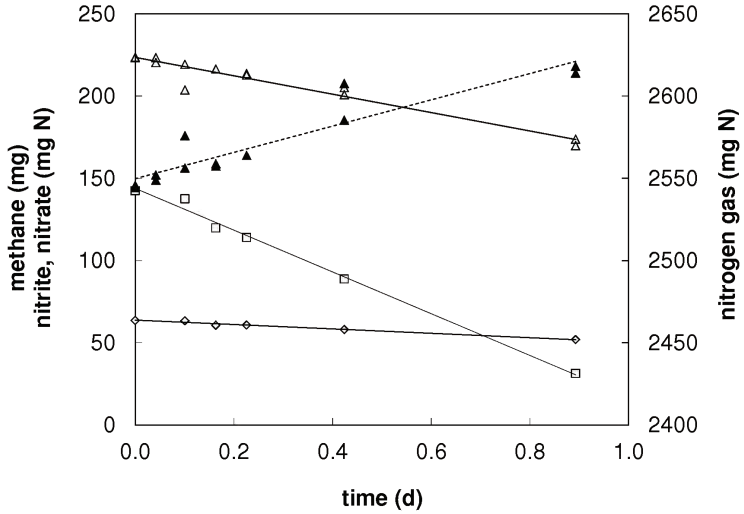

Figure 2.4: Results from a whole culture batch test performed with SFBR+ after 324 d of enrichment. Nitrite (□) and nitrate (◇) on primary y-axis in mg N, methane (△, measured in duplicate) on primary y axis in mg, nitrogen gas on secondary y-axis (▲, measured in duplicate) in mg N. Molar conversion ratio of CH_4 :NO_2^- :N_2 was 3 : 7.9 : 4.5.

Table 2.1: Molar conversion ratios (methane : nitrite : nitrogen gas; theoretical ratio 3 : 8 : 4) of SFBR- and SFBR+ in time.

Time (days)	**Ratio**		
	methane	**nitrite**	**nitrogen gas**
SFBR-			
324	3.0	7.4	4.4
400	3.0	7.6	4.4
485	3.0	9.0	5.1
SFBR+			
324	3.0	7.9	4.5
400	3.0	5.0	3.8
485	3.0	11.9	4.7

doubling time for the amount of bacteria in the reactor. The net doubling time in SFBR- was estimated to be 1.9 months and the doubling time in SFBR+ to be 1.7 months. It remains to be investigated whether the somewhat shorter doubling time in SFBR+ was because effluent from the sewage treatment plant, fed to SFBR+, contained a missing growth factor.

To estimate if a substantial portion of the new cells was lost from the reactors, and if this loss could have contributed to the stagnation and decrease in nitrite consumption rates, biomass washout from each reactor was quantified over a period of three months. The daily growth, based on nitrite consumption, and expected biomass yield were compared. This provides an estimate of how much of the (produced) biomass washed out.

Total protein washout from SBFR- was 0.10 g between day 556 and 621 (distributed over 6 cycles). In this period, about 4.4 g NO_2^--N was consumed, thus 0.022 g protein washed out per g NO_2^--N consumed. Total protein washout from SFBR+ was 0.18 g between day 551 and 621 (distributed over 11 cycles). In this period about 7.0 g NO_2^--N was consumed, thus 0.026 g protein washed out per g NO_2^--N consumed. The growth yield of *M. oxyfera* is unknown, but assuming it is similar to the growth yield of the anaerobic nitrite consuming Anammox bacteria, viz. 0.054 g protein/g NO_2^-N (Strous et al., 1999), it can be estimated that 41–48 % of the produced biomass washed out from the reactors. This indicates that, even though in this enrichment study a long settling time of 2 h was applied, compared to only 15 min applied by Raghoebarsing et al. (2006) and 1–2 h applied by Ettwig et al. (2009), biomass washout may have significantly decelerated enrichment. In the periods when stagnating or decreasing nitrite consumption rates were observed, biomass washout was not quantified, but it seems likely, these can mainly be attributed to (temporarily higher) biomass washout. After placement of the membrane in SFBR- the nitrite consumption rate stabilized or even increased suggesting the importance of efficient biomass retention. Prolonged reactor operation is required to see the effect on the long term. Biomass that washed out was also examined under the microscope (results not shown). In the effluent from both reactors single cells and small flocs of up to 60 µm were observed, which indicated this biomass had poor settling characteristics.

2.3.4 Microbiological composition

The presence and abundance of *M. oxyfera* bacteria in the reactors was assessed by sequence analysis and FISH. Sequence analysis of 16S rRNA clones obtained from biomass from the reactors and subsequent phylogenetic analyses confirmed the presence of *M. oxyfera* bacteria in both the inoculum and after 5 months of enrichment. The 'NC10' sequences obtained in this study were similar to sequences found in previous studies in which also *M. oxyfera* bacteria were enriched or detected (fig. 2.5) (Hu et al., 2009; Ettwig et al., 2009, 2008; Raghoebarsing et al., 2006). In addition, microscopic analysis using FISH of biomass from the reactors showed an increase of *M. oxyfera* bacteria over the course of the enrichment. The amount of *M. oxyfera* bacteria in the inoculum was too low to be detected by FISH. After 8 months of enrichment, the bacteria were observed in both reactors and after 13 months of enrichment *M. oxyfera* bacteria dominated both reactors (70–80 % of the population;

SFBR+ after 13 months of enrichment is represented in fig. 2.6), confirming the results from section 2.3.2 that denitrification of nitrite coupled to anaerobic methane oxidation was the dominant process in both reactors.

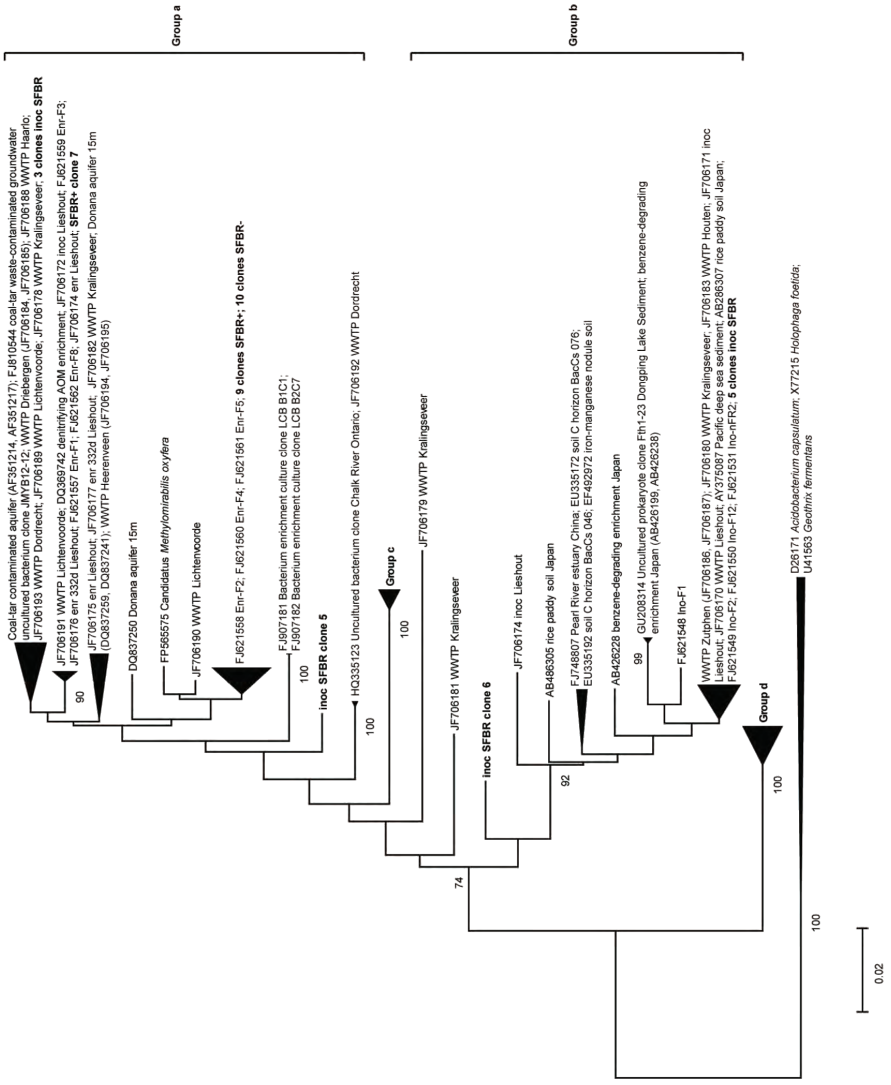

Figure 2.5: Phylogenetic tree of 16S rRNA sequences of the 'NC10' phylum with *Acidobacteria* as the outgroup. Sequences obtained in this study are shown in boldface. The tree was constructed with MEGA4 software using the neighbour-joining method and pairwise deletion of gaps. The tree topology was tested by bootstrap analysis (1000 replicates).

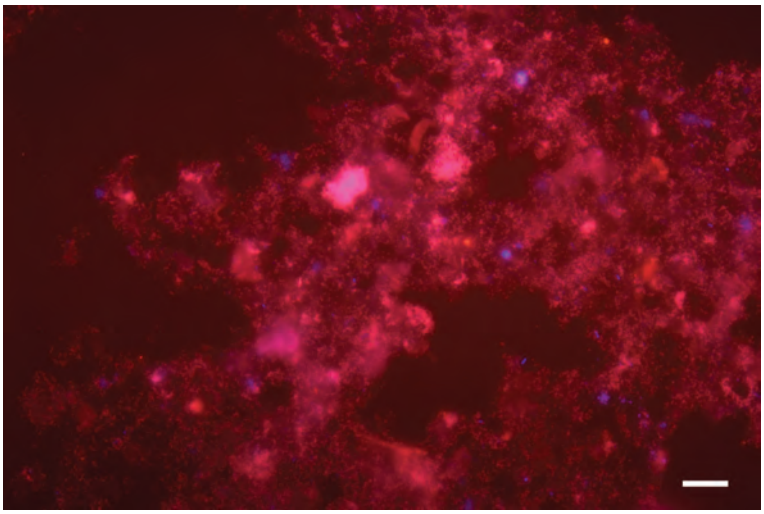

Figure 2.6: Fluorescence in situ hybridization of biomass from SFBR+ after 13 months of enrichment. Fluorescence micrograph after hybridization with probes DBACT1027 (Cy3; red) specific for 'NC10' bacteria; and EUB mix (probes EUB338 I-III; Cy5; dark blue), detecting nearly all eubacteria. Due to co-hybridization with the specific and general probes, the *M. oxyfera* bacteria appear pink. The scale bar indicates 20 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.3.5 Outlook

The coupled removal of nitrogen and dissolved methane makes a process with denitrifying methanotrophic bacteria a promising treatment for effluents from direct low-temperature anaerobic sewage treatment. Emission of dissolved methane present in the effluent would lead to greenhouse gas emissions. Using the dissolved methane for denitrification decreases the potential greenhouse gas emissions from direct low-temperature anaerobic wastewater treatment and the dissolved methane (20 mg NO_2^--N/L d at 10 °C) is enough to remove nearly all nitrogen (47 mg NO_2^--N/L d) in the effluent (typically containing 50 mg NO_2^--N/L), thus avoids the need for an external carbon source for denitrification. Autotrophic nitrogen removal with Anammox could also be applied for treatment of effluent from anaerobic wastewater treatment, but this would still require removal of dissolved methane (Hendrickx et al., 2012b).

The volumetric nitrite consumption rates of both enrichment reactors are low compared to other denitrifying systems. For the treatment of effluent from anaerobic sewage treatment plants, containing 50 mg N/L, the present results would dictate a

long hydraulic retention time of 1.3 (SFBR+)-1.5 d (SFBR-). The volumetric rates have to be increased by an order of magnitude. The low growth rates of denitrifying methanotrophic bacteria necessitate efficient biomass retention. Preliminary results with applying a membrane for complete biomass retention in SFBR- suggested that the decreasing trend in NLR could be stopped or even reversed in a short period of 30 d in which the membrane was applied for effluent collection. Therefore, further research should focus on using systems with better biomass retention, such as membrane bioreactors, reactors with granular sludge or biofilms to increase the volumetric conversion rates to the desired values.

In the proposed concept for sewage treatment at low temperatures, the reactor for denitrification coupled to anaerobic methane oxidation is fed with the effluent from anaerobic sewage treatment, containing ammonium, dissolved CH_4 and residual COD; and with a recycle flow from the nitritation reactor, containing nitrite and traces of dissolved oxygen (fig. 2.1). These conditions could trigger processes other than denitrification coupled to anaerobic methane oxidation such as Anammox and heterotrophic denitrification, competing for nitrite with denitrifying methanotrophic bacteria. Recently, it was shown that under ammonium limitation, but with nitrite and methane supplied in excess, Anammox and *M. oxyfera* bacteria could co-exist (Luesken et al., 2011a). Also traces of oxygen present in the effluent from the nitritation reactor could have an effect on the denitrifying methanotrophs. Luesken et al. (2012) showed that addition of 2 % and 8 % of oxygen to *M. oxyfera* enriched cultures resulted in a direct decrease of nitrite and methane consumption rates and changes in gene expression showed *M. oxyfera* was under oxidative stress. Therefore, in addition to improved biomass retention, further research topics should include the performance of the proposed concept at wastewater temperatures, competition for nitrite and effects of traces of oxygen.

2.4 Conclusions

- Denitrifying methanotrophic bacteria offer a possible solution to treatment of effluent from low-temperature anaerobic sewage treatment plants, such as a UASB-digester system.

- Maximum volumetric consumption rates of enrichment cultures of *M. oxyfera* (70–80 %) were 33.5 mg NO_2^--N/L d (using synthetic medium) and 37.8 mg NO_2^--N/L d (using medium containing effluent from a sewage treatment plant) were achieved. These denitrification rates need to be increased an order of magnitude before the process could be considered for practical applications.

- Biomass washout occurred throughout the enrichment and significantly decelerated enrichment. Therefore, further research should focus on systems with better

biomass retention, such as membrane bioreactors, reactors with granular sludge or biofilms to increase the volumetric consumption rates to the desired values.

3

Enrichment of denitrifying methanotrophic bacteria from municipal wastewater sludge in a membrane bioreactor at 20 °C

Abstract

Simultaneous nitrogen and methane removal by the slow growing denitrifying methanotrophic bacterium '*Candidatus* Methylomirabilis oxyfera' offers opportunities for a new approach to wastewater treatment. However, volumetric nitrite consumption rates should be increased by an order of magnitude before application in wastewater treatment becomes possible. A maximum volumetric nitrite consumption rate of 36 mg NO_2^--N/L d was achieved in a membrane bioreactor inoculated with wastewater sludge and operated at 20 °C. This rate is similar to maximum rates reported in literature, though it was thought that by strict biomass retention using membranes, higher rates would be achieved. In experiments lasting several years, growth was not stable: every experiment showed a decrease in activity after 1–2 years. The cause remains unknown. Rates increased after addition of copper and operating a membrane bioreactor at shorter hydraulic retention times. Further research should focus on long-term effects of copper addition and operation at hydraulic retention times in the order of hours using membrane bioreactors.

A modified version of this chapter has been accepted for publication as Kampman, C., Temmink, H., Hendrickx, T.L.G., Zeeman, G., and Buisman, C.J.N. Enrichment of denitrifying methanotrophic bacteria from municipal wastewater sludge in a membrane bioreactor at 20 °C. *Journal of Hazardous Materials*, 2014. doi: 10.1016/j.jhazmat.2014.04.031.

3.1 Introduction

The concomitant removal of nitrogen and methane by the denitrifiying methanotrophic bacterium '*Candidatus* Methylomirabilis oxyfera' offers opportunities for a new approach to wastewater treatment (Kampman et al., 2012; Luesken et al., 2011b). Kampman et al. (2012) proposed a concept in which *M. oxyfera*-type bacteria are used to treat effluent from direct, low-temperature anaerobic municipal wastewater treatment. For the anaerobic treatment they proposed to use a combination of an upflow anaerobic sludge bed (UASB) reactor and a sludge digester, referred to as UASB-digester (Zhang et al., 2012; Álvarez et al., 2008; Mahmoud, 2008; Mahmoud et al., 2004). In addition, the concept comprised a reactor with denitrifying methanotrophic bacteria and a nitritation reactor to supply the denitrifiers with nitrite. Applying this concept would make it possible to benefit from all advantages associated with anaerobic treatment (see e.g. Lema and Omil, 2001; Lettinga, 1995). At the same time the two major problems, viz. removal of nitrogen, which causes eutrophication, and dissolved methane, a greenhouse gas, from the effluent would be solved (Kampman et al., 2012).

M. oxyfera-type bacteria have successfully been enriched from a mixture of sludge from municipal wastewater treatment and freshwater sediment (Hu et al., 2009) and from sludge from industrial wastewater treatment (Luesken et al., 2011b). Also enrichment from freshwater sediments using medium containing 10 % effluent from an aerobic municipal wastewater treatment plant (MWWT effluent) was successful (Kampman et al., 2012). Enrichment using municipal wastewater sludge as sole inoculum source has not been reported. However, these sludges are widely available inocula, potentially abundant with denitrifying methanotrophic bacteria already adapted to municipal wastewater conditions. By means of 16S rRNA screening, *M. oxyfera*-type bacteria were identified in activated sludge samples of eight out of nine selected municipal wastewater treatment plants (Luesken et al., 2011b).

Before practical application of denitrifying methanotrophic bacteria in municipal wastewater treatment is possible, further research is required. Foremost, higher and more stable volumetric denitrification rates at lower process temperatures should be achieved. Hitherto, maximum rates as high as 38 mg NO_2^--N/L d have been observed at 30 °C (Kampman et al., 2012; Ettwig et al., 2009). After reaching a maximum, rates stabilized (Zhu et al., 2012; Ettwig et al., 2009, 2008), or even decreased (Kampman et al., 2012; Hu et al., 2011). This may have been due to washout of biomass (Kampman et al., 2012), missing growth factors or product inhibition (Ettwig et al., 2008).

M. oxyfera enrichment reactors that were described thus far relied on settling for biomass retention. Biomass washout may have significantly decelerated enrichment, contributing to the observed stabilization and even to the decrease of the denitrification rates. From sequencing fed-batch reactors (SFBRs) enriched in *M. oxyfera*-

type bacteria an estimated 41–48 % of grown biomass washed out during periods of increasing and stabilizing rates (Kampman et al., 2012). The aforementioned decreasing rates might be due to biomass washout exceeding growth. This could be overcome by ensuring complete biomass retention using a membrane. For enrichment of microorganisms that couple anaerobic methane oxidation to sulfate reduction and have comparable low growth rates, membrane bioreactors (MBRs) were successfully applied (Meulepas et al., 2009). Kampman et al. (2012) installed an ultrafiltration membrane for effluent extraction in one of the *M. oxyfera*-type bacteria enriched SFBRs. This seemed to stop or even reverse the decreasing trend in denitrification rates, suggesting a positive effect of complete biomass retention.

To supply potentially missing growth factors Kampman et al. (2012) tested addition of 10 % (v/v) MWWT effluent. Slightly higher volumetric denitrification rates (38 mg NO_2^--N/L d vs. 34 mg NO_2^--N/L d) and shorter doubling times (1.7 months vs. 1.9 months) were observed in a reactor fed with medium containing MWWT effluent than in a reactor fed with synthetic medium. However, also in the MWWT effluent fed reactor, stabilizing and decreasing rates were observed. This indicatedd that, even though by addition of MWWT effluent missing growth factors may have been supplied, additional impediment existed. Potentially limiting growth factors are a nitrogen source for assimilation and copper. As an alternative nitrogen source, Ettwig et al. (2008) added ammonium to the medium of one enrichment culture on one occasion, but no effect was observed. To observe an effect, it might be necessary to feed a reactor with ammonium for a longer period. Copper may be required for methane oxidation. *M. oxyfera* expresses particulate methane monooxygenase (pMMO), but no soluble methane monooxygenase (sMMO) (Ettwig et al., 2010). Aerobic methanotrophs expressing pMMO require higher levels of copper than methanotrophs that also express sMMO (Graham et al., 1993; Collins et al., 1991). Increasing copper levels may result in higher pMMO expression by *M. oxyfera*-type bacteria and thereby higher denitrification rates. Inhibition by unidentified intermediates or products may be prevented by increasing the washout of such compounds using shorter hydraulic retention times (HRT). This could easily be tested in a MBR, in which strict uncoupling of HRT and sludge retention time (SRT) is possible.

In this research enrichment of denitrifying methanotrophic bacteria from municipal wastewater treatment sludge at 20 °C, which is 5–15 °C lower than in most enrichments described, was studied. To achieve increased volumetric denitrification rates, enrichment was performed in MBRs, ensuring complete biomass retention. The operation of the two reactors described by Kampman et al. (2012) was continued and performance was compared with MBR performance. Also, several additives (100 % effluent from aerobic municipal wastewater treatment, ammonium, increased copper concentration, autoclaved sludge and filtrate from autoclaved sludge) and operational strategies (shorter HRT) were tested with the aim of increasing denitrification rates.

After successful enrichment one of the reactors was spiked with concentrated effluent from the SFBRs. The effluent contained *M. oxyfera*-type bacteria enriched from freshwater sediment (Kampman et al., 2012), but possibly also limiting growth factors. Finally, the potential to apply a process with denitrifying methanotrophic bacteria for treatment of effluent from direct low-temperature anaerobic municipal wastewater treatment is discussed.

3.2 Materials and methods

3.2.1 Setup of enrichment reactors

Two enrichments were performed in two anoxically operated submerged membrane bioreactors (MBRs; fig. 3.1) with total volumes of 7.3 L (MBR1) and 7.2 L (MBR2), and each with a working volume of 4.6 L. Operation of two previously described enrichment reactors (Kampman et al., 2012) was continued. Originally both were operated as sequencing fed-batch reactors (SFBRs) but after 623 days of operation one of the reactors was equipped with a membrane, and operated as MBR (now referred to as MBR3). Both reactors had a total volume of 10 L, with a working volume of 5.3–6.7 L when operated as SFBR and a working volume of 6.7 L when operated as MBR.

All four reactors were continuously fed with mineral medium and, to provide substrate and maintain anoxic conditions, CH_4/CO_2 (5.0–10 mL/min, 93.6–95.0 % CH_4, 5.0–6.4 % CO_2). To provide mixing and sufficient gas transfer, gas was recirculated. The gas was added from the bottom of the reactor, through a glass diffuser producing small bubbles. MBR1 and MBR2 were operated at 21 resp. 20 ± 1 °C; MBR3 and the SFBR were operated at 30 ± 1 °C. The MBRs were equipped with an ultrafiltration membrane (VFU-250, Memos Membranes Modules Systems GmbH) to retain all biomass. Reactor operation was controlled and data (pH, temperature) were acquired using FieldPoint modules and LabVIEW 7.0 (National Instruments). Extraction of effluent was controlled using level switches and transmembrane pressure (TMP). Effluent was extracted when the level switch was in contact with the liquid, i.e. at a liquid volume > 4.6 L. At a TMP below 200 mbar, effluent was extracted from the reactor through the membrane. On the few occasions the TMP increased to more than 200 mbar effluent was extracted from the reactor directly and stored at 4 °C. This effluent, containing suspended biomass, was returned to the reactor after decreasing the TMP by backwashing the membrane. To prevent an increase of TMP above 200 mbar, effluent was extracted for a maximum of 8 min, followed by 2 min relaxation. Furthermore, the membrane was backwashed with permeate at least once per month and a high surface shear on the membrane was maintained by placing the membrane in a riser through which the recirculated gas was sparged. The setup of the SFBRs was described by Kampman et al. (2012).

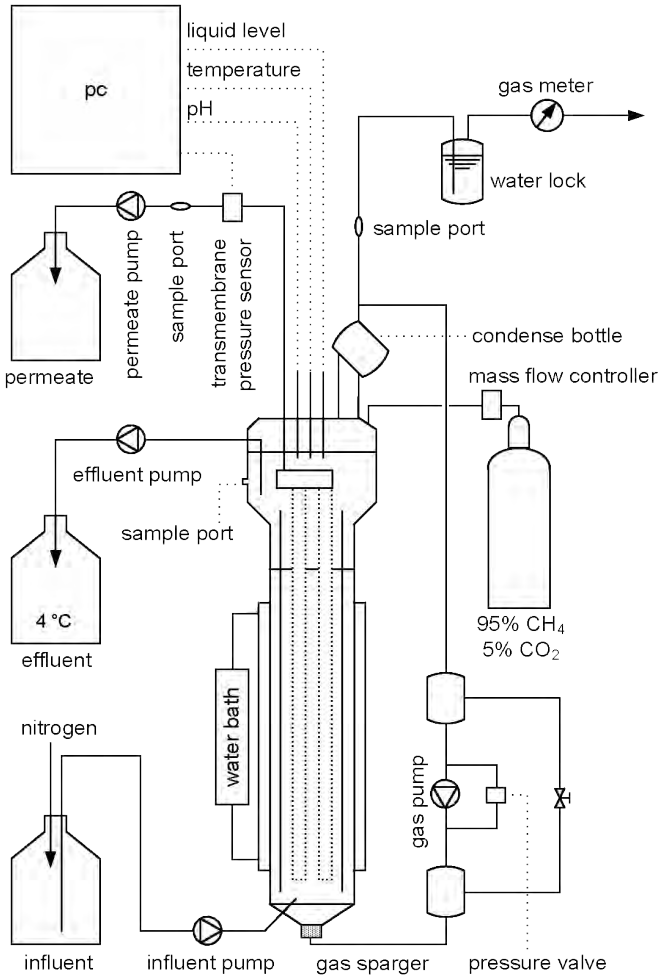

Figure 3.1: Setup of the submerged membrane bioreactors applied for the 20 °C enrichment of denitrifying methanotrophic bacteria from a mixture of municipal wastewater treatment sludge.

3.2.2 Inoculum

MBR1 and MBR2 were inoculated with 1.0 g volatile solids (VS)/L (0.37 g protein/L) of a mixture of municipal wastewater sludge. The mixture consisted of equal amounts of digested primary sludge, secondary sludge and digested secondary sludge from the

municipal wastewater treatment plant (WWTP) in Ede, The Netherlands. At this plant, wastewater with a low biochemical oxygen demand (BOD) : N ratio of 3.6 was treated at a long SRT of 23 d (secondary sludge).
Primary and secondary sludge were anaerobically digested at SRTs of 17 d and 22 d respectively. These values for BOD : N ratio and SRT were similar to the values characterizing sludge samples in which Luesken et al. (2011b) detected *M. oxyfera*-type bacteria. Prior to inoculation the sludge was washed to remove dissolved COD, which could otherwise serve as substrate for heterotrophic denitrification. The sludge was centrifuged (digested primary sludge and fresh secondary sludge 5 min, digested secondary sludge 10 min, at 2500 g) and the pellets were resuspended in water; this was repeated four times. Hereafter the sludge was centrifuged one more time, the pellets were resuspended in a small amount of water and added to the reactors. The two SFBRs, one of which was continued as MBR3, were inoculated with sediment from ditches in Ooijpolder, The Netherlands, as described by Kampman et al. (2012).

3.2.3 Mineral medium

The reactors were fed with a mineral medium (Kampman et al., 2012). The nitrite concentration in the medium ranged from 0.014 to 0.980 g NO_2^--N/L and was increased as denitrification rates increased. The concentration of nitrate in the medium ranged from 0.12 to 0.056 g NO_3^--N/L and was decreased as heterotrophic denitrification rates decreased. To control the pH at 6.5–8, 5.0–6.4 % CO_2 was added in the gas phase and 0.05–1.5 g $KHCO_3$/L was added to the medium. In addition, to provide potential growth factors, the medium fed to MBR1, MBR2 and SFBR contained 10 % (v/v) 0.2 µm filtered effluent from municipal WWTP Bennekom, The Netherlands. On average the effluent contained 1.3 mg BOD/L, 24 mg chemical oxygen demand (COD)/L, 2.1 mg Kjeldahl-N/L and 3.8 mg NO_2^-+NO_3^-/L. These concentrations were too low to cause enrichment of heterotrophic denitrifying bacteria. During reactor operation medium composition was adjusted in an attempt to increase volumetric nitrite consumption rates (see section 3.2.4).

3.2.4 Operation of enrichment reactors

During the first 7–10 months of reactor operation nitrite consumption rates were low. Hereafter, consumption rates increased. Frequently the nitrite loading rates (calculated as the daily nitrite addition per maximum working volume) were adjusted. The nitrite loading rate was changed by changing HRT (5–67 d) or medium concentration (0.014–0.980 g NO_2^--N/L), to approach the nitrite consumption rate. Therefore 3–7 times per week the reactor nitrite concentration was estimated. On some occasions nitrite was limiting. To prevent or resolve limitation, at nitrite concentrations < 3 mg NO_2^--N/L the nitrite loading rate was increased. On some other

occasions nitrite accumulation occurred. To prevent too high nitrite accumulation, at concentrations > 30 mg NO_2^--N/L the medium supply was stopped and restarted at a concentration < 15 mg NO_2^--N/L, at a lower nitrite loading rate. To increase volumetric denitrification rates several adjustments were made to medium composition and reactor operations. An overview of the adjustments is presented in table 3.1 and the adjustments are explained in section 3.3.2. Due to the low growth rate of the bacteria it was expected that it would take several months before an increase in denitrification rates due to adjusted operations would be perceptible. Therefore multiple, carefully selected, strategies were applied to the different reactors at the same time rather than testing only a few strategies in duplicate.

3.2.5 Sampling and analytical methods

3–7 times per week 1–2 mL liquid samples were withdrawn from the reactors. Test strips (Merckoquant, Merck chemicals) were used to estimate nitrite and nitrate concentrations in these samples. Every 7–20 days and when changes were made to reactor operation, nitrite and nitrate concentrations and gas composition were measured. Ion chromatography was used to measure nitrite and nitrate (Kampman et al., 2012). Once per week 50 µL gas samples were analyzed. Gas chromatography was used to measure methane, nitrogen, carbon dioxide and oxygen pressure (Kampman et al., 2012). VS was analyzed according to APHA Standard method 2540 (APHA et al., 1998). Protein samples were hydrolyzed (Kampman et al., 2012) and analyzed according to the modified Hartree-Lowry method (Hartree, 1972).

3.3 Results

3.3.1 Enrichment of denitrifying methanotrophic bacteria

The presence of *M. oxyfera*-type bacteria in the inoculum of the MBRs and after 4 and 12 months of MBR operation was confirmed using molecular tools (sequence analysis and Fluorescence in situ hybridization; data not shown). Fluorescence in situ hybridization showed that after 12 months of reactor operation, and also in periods of decreasing volumetric nitrite consumption rates, *M. oxyfera*-type bacteria dominated both MBRs (60–70 %). MBR3 and SFBR were also dominated by *M. oxyfera*-type bacteria (70–80 %; Kampman et al., 2012). In MBR1, MBR2 and the SFBR these bacteria also remained dominant at decreasing volumetric nitrite consumption rates; in MBR3 microbial composition was not analyzed at decreasing rates. In all reactors *M. oxyfera*-type bacteria were present both in suspension and as a biofilm on the reactor walls.

3.3.2 Nitrite consumption rates

MBR1

After 10 months of low volumetric nitrite consumption rate in MBR1 (period I, partly shown in fig. 2a), the rate started to increase and reached 12 mg NO_2^--N/L d on day 421 (period II). In fig. 2 the nitrite consumption rate was represented by the nitrite loading rate. This is an accurate representation because in general nearly all nitrite added was removed. To further elevate the consumption rate, more biomass enriched in *M. oxyfera*-type bacteria was added (0.46 g protein). Biomass was collected from the effluent of the SFBRs during months 11–15 of the enrichment and might have contained growth factors as well. In the SFBRs at that time consumption rates were increasing. After biomass addition the rate in MBR1 continued to increase, reaching a maximum of 36 mg NO_2^--N/L d at day 457 (period III; see also table 1.1). Assuming exponential growth, a rate of 30–43 mg NO_2^--N/L d was expected on day 469 (calculated assuming a nitrite consumption rates ranging from 3.4 nmol NO_2^--N/mi mg protein (Ettwig et al., 2009) to 8.6 nmol NO_2^--N/min mg protein (Luesken et al., 2011b), 70 % of protein being from *M. oxyfera*-type bacteria, and a doubling time of 1.7–1.9 months (Kampman et al., 2012)). This indicated that the increase in rate was probably due to the addition of biomass and not due to potential growth factors added. After reaching a maximum in MBR1, the rate stabilized at a slightly lower value of 34 mg NO_2^--N/L d. After a second addition of biomass from the effluent of MBR3 and SFBR collected during periods of stabilizing and decreasing rates (1.0 g protein; collected during months 15–21 of the enrichment) on day 623, only a slight growth in rate to 37 mg NO_2^--N/L d (day 655; period IV) was observed. This is much lower than expected from the amount of biomass added. Hereafter, despite the complete biomass retention, the nitrite consumption rate went down. The rate could not be increased by adjusting the medium composition at day 707 (period V; section 3.3.2). The rate increased again when, after 934 days, the copper concentration of the medium was changed from 3 to 6 µM. More copper was added, because possibly *M. oxyfera*-type bacteria require copper for the expression of pMMO. As a result, the rate increased from 13 to 16 mg NO_2^--N/L d (period VI). The rate was still going up when medium was prepared without addition of MWWT effluent and the reactor contents of MBR2 and SFBR were transferred to MBR1 (period VII). All biomass from these reactors was retained in MBR1; the excess liquid was extracted via the membrane. This transfer of biomass resulted in an increase of the nitrite consumption rate from 16 to 34 mg NO_2^--N/L d, which corresponded to the sum of the nitrite consumption rates of all three reactors.

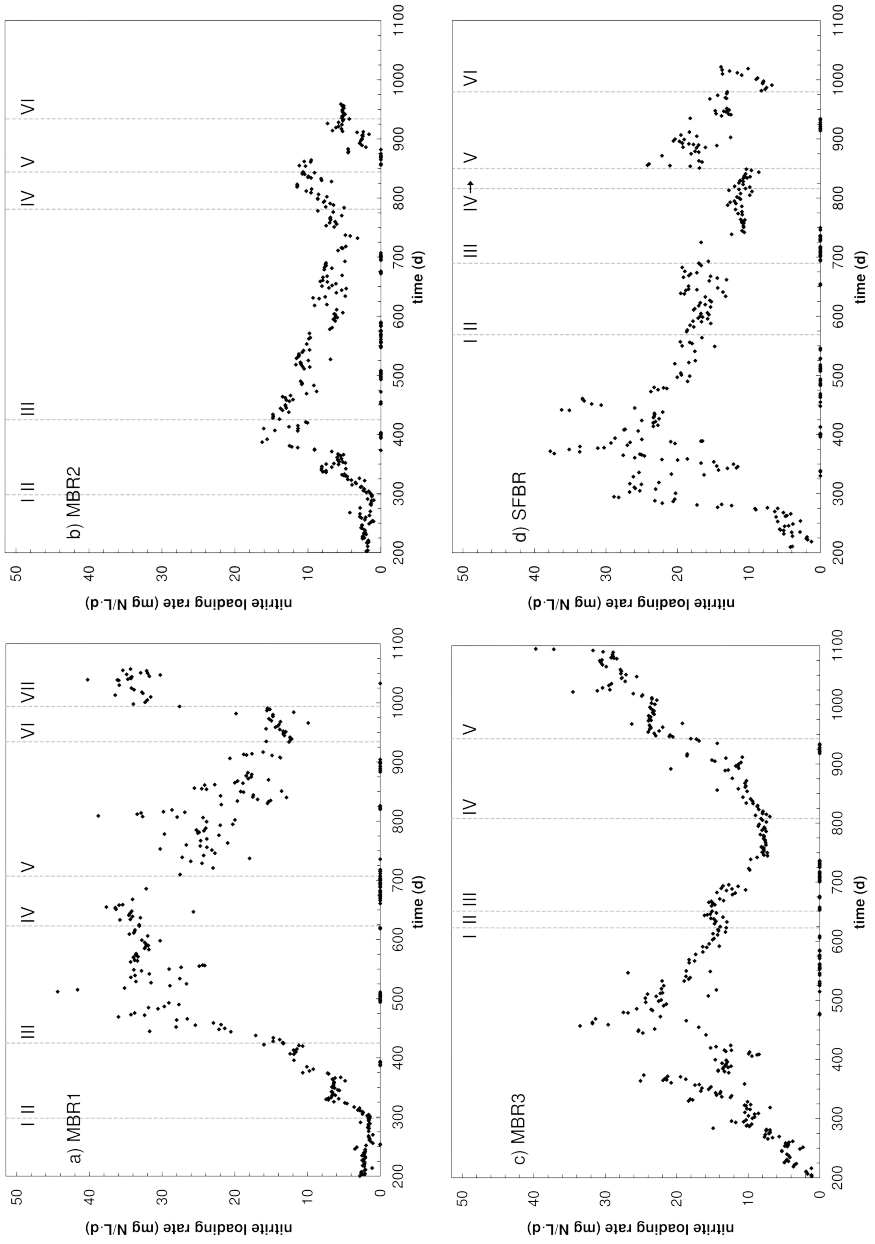

Figure 3.2: Nitrite loading rate applied to (a) MBR1, (b) MBR2, (c) MBR3 and (d) SFBR. Periods indicated by Roman numerals are described in section 3.3.2. Period I of fig. 3.2(c) and (d) were described by Kampman et al. (2012).

MBR2

Until day 421 MBR1 and MBR2 were duplicate reactors and showed a similar course of enrichment. However, as a control, MBR2 was not spiked with effluent biomass from two SFBRs enriched in *M. oxyfera*-type bacteria. Nitrite consumption rates increased to a maximum rate of 16 mg NO_2^--N/L d on day 387 (period II). Hereafter, the rates slowly decreased to 6 mg NO_2^--N/L d (period III). The rates increased again after 781 days (period IV; table 1.1). However, this was most likely due to heterotrophic denitrification (section 3.3.2).

MBR3

As described by Kampman et al. (2012), after placing a membrane in a SFBR, now referred to as MBR3, the decreasing trend in volumetric nitrite consumption rates could be stopped or even reversed (period II in fig. 2c). However, after a month the rate went down again and eventually stabilized around 8 mg NO_2^--N/L d. On day 808 the HRT was decreased from 61 to 19 days to add trace elements and to remove potentially inhibiting intermediates or products at a higher rate. While decreasing the HRT, the nitrite loading rate remained unchanged by decreasing the nitrite concentration of the medium. In response, the volumetric nitrite consumption rate gradually increased to 15 mg NO_2^--N/L d. To increase the nitrite loading rate, the HRT was gradually decreased from 19 to 9 days (period III). On day 943 the HRT was further decreased from 9 to 2.3 days, again while maintaining a constant nitrite loading rate. The nitrite consumption rates subsequently increased to a new maximum of 31 mg NO_2^--N/L d at an HRT of 1.3 days (period IV; table 1.1).

SFBR

Kampman et al. (2012) observed decreasing nitrite consumption rates (period I, fig. 2d). Consumption rates in SFBR continued to go down during prolonged reactor operation (period II). Adding more biomass concentrated from the effluent on day 816 did not result in increased rates (section 3.3.2).

Adjustments that did not affect nitrite consumption rates

To alleviate possible growth factor limitation, MBR1 was fed with an adjusted medium. The fraction of MWWT effluent in the medium was changed from 10 % to 100 % and NH4Cl was added (4 mg N/L) as a potential nitrogen source for assimilation. Also the trace element concentration was tripled to increase the availability of (potential) trace elements. No effect was observed; the decrease in rate that had started before adjusting the medium composition continued (period V in fig. 3.2a). Autoclaved sludge from municipal wastewater treatment (0.92 g VS, 0.92 g

Table 3.1: Effect of different additives, to medium and reactors, and shorter hydraulic retention times on volumetric nitrite consumption rates.

Adjustment to reactor operation	**Time (d) and period of adjustments**				**Effect**
	MBR1	**MBR2**	**MBR3**	**SFBR**	
Addition of biomass enriched in M. oxyfera-type bacteria					
Addition of 0.46 g protein	421(III)				Nitrite consumption rate increased from 12 to 36 mg NO_2^--N/L d
Addition of 1.0 g protein	623(IV)				Nitrite consumption rate increased from 34 to 37 mg NO_2^--N/L d
Addition of reactor contents MBR2 and SFBR	994(VII)				Nitrite consumption rate increased from 16 to 34 mg NO_2^--N/L d
Addition of 0.46 g protein/L				816(III)	No effect observed
Adjustments to medium composition					
Increased the fraction MWWT effluent from 10 to 100 %; increased the nutrient concentration by a factor 3; addition of 4 mg NH_4Cl-N/L	707(VI)				Decrease in nitrite consumption rates that started at day 655 continued
Increased the copper concentration from 3 to 6 μM	934(VII)				Nitrite consumption rate increased from 13 to 16 mg NO_2^--N/L d; still increasing at the end of the period
Addition of autoclaved sludge		781(IV)			Increased heterotrophic denitrification, before an effect on denitrifying methanotrophic activity could be observed operational problems occurred
Shorter HRT					
Decreased the HRT from 61 to 19 d			808(IV)		Nitrite consumption rate increased from 8 to 15 mg NO_2^--N/L d and still increasing at the end of the reported period; in this period HRT was gradually decreased from 19 to 9 d
Decreased the HRT from 9 to 2.4 d			943(V)		Nitrite consumption rate increased from 15 to 36 mg NO_2^--N/L d and still increasing at the end of the reported period; in this period HRT was gradually decreased from 2.4 d to 1.3 d

COD) was added to MBR2. Sludge was added to provide potential trace elements for denitrifying methanotrophic bacteria or substrate for other microorganisms present, potentially producing growth factors or removing inhibitory compounds, were supplied. The addition resulted in an elevated nitrite consumption rate (period IV in fig. 3.2b), probably due to increased heterotrophic denitrification. Unfortunately, operational problems occurred on day 833, before an effect of COD addition on denitrifying methanotrophic activity could be observed.

Addition of biomass collected from the effluent of the SFBRs to MBR1 resulted in increased rates (section 3.3.2). However, returning biomass collected from the effluent of the SFBR (0.66 g protein; collected during months 21–26 of the enrichment when rates were decreasing) on day 816 did not result in an increase in nitrite consumption rate (period III).

3.3.3 Biomass growth and decay

The amount of biomass in the reactors could not be measured because it was present in suspension as well as in a biofilm on the reactor wall. Instead, a maximum amount of biomass present in the reactors was estimated from the maximum volumetric nitrite consumption rates, using the assumptions from section 3.3.2. At the maximum rates, the total amount of denitrifying methanotrophic biomass in the reactors was estimated to be 1.4–3.5 g for MBR1, 0.6–1.5 g for MBR2, 2.0–5.0 g for MBR3 and 2.1–5.3 g for SFBR. Net doubling times were estimated from the increase in volumetric nitrite consumption rate (period II and III in fig. 3.2a and b). The observed doubling time in MBR1 was 1.3 months, increasing to 2 months after addition of biomass from the SFBRs. The doubling time might have become longer due to limited availability of growth factors or the presence of inhibiting compounds. The observed doubling time in MBR2 was 0.9 months. At the extremely long SRTs applied in this research, the decay constant may have had a large impact on the observed doubling time.

3.4 Discussion

Using molecular tools, *M. oxyfera*-type bacteria have been identified in samples from municipal WWTPs (Luesken et al., 2011b). In this research for the first time *M. oxyfera*-type bacteria were enriched from municipal wastewater sludge only. Moreover a low temperature (20 °C) was applied. Molecular tools and nitrite and methane consumption rates showed that denitrification of nitrite coupled to anaerobic methane oxidation was the dominant process in the enrichment reactors. At a low temperature of 20 °C a maximum volumetric nitrite consumption rate of 36 mg NO_2^--N/L d was achieved using a MBR system. This maximum rate is similar to maximum rates reported in literature, achieved in sequencing (fed-)batch reactors at 30 °C (Kampman et al., 2012; Ettwig et al., 2009). After installation of a membrane in a SFBR, a

maximum rate of 31 mg NO_2^--N/L d was achieved; the rates in the other MBR and in the SFBR did not rise above 16 and 24 mg NO_2^--N/L d respectively. The maximum rates need to be increased before the process can be considered for practical applications. Although it was expected that enrichment rate and maximum volumetric nitrite consumption rates could be enhanced by applying a MBR with complete biomass retention, this research shows that complete biomass retention alone does not result in higher volumetric nitrite consumption rates. The decreasing trend in nitrite consumption rate that was stopped or even reversed after Kampman et al. (2012) placed a membrane in a SFBR was only temporary; after a month rates decreased again. In this study, the effect of different additives and operational conditions on the rate was investigated (table 1.1), indicating that operating a MBR at a shorter HRT and feeding a reactor with an increased influent copper concentration resulted in higher rates. However, maximum rates were similar to maximum rates in literature and should be increased by an order of magnitude before practical application in wastewater treatment. The effect of different operational conditions and additives are in more detail discussed below.

3.4.1 Biomass washout

In SFBRs an increase of volumetric nitrite consumption rates was observed after 7–8 months (Kampman et al., 2012). In MBRs the rates started to increase only after 10 months of low volumetric nitrite consumption rates. This was probably because the inoculum of the MBRs contained a lower amount of *M. oxyfera*-type bacteria (data not shown). However, in the MBRs the observed doubling times were considerably shorter (0.9–1.3 months) than in the SFBRs (1.7–1.9 months; (Kampman et al., 2012)). This indicated that MBRs do facilitate faster enrichment rates. Shorter doubling times may be achieved when the SRT is controlled at a lower value. Also the decay rate of *M. oxyfera*-type bacteria was low. Stabilizing (Zhu et al., 2012; Ettwig et al., 2009, 2008) and slowly decreasing rates (Kampman et al., 2012; Hu et al., 2011) were observed in several studies, independent of inoculum, reactor type, enrichment temperature and medium composition. In the MBRs applied in this study biomass retention was complete, thus decreasing rates could not have been caused by biomass washout. Possible explanations are substrate limitation, growth factor limitation or inhibition by intermediates or products.

3.4.2 Substrate limitation

Methane and nitrite were supplied in excess at all times and therefore substrate limitation can also be excluded as an explanation for the decreasing rates.

3.4.3 Growth or inhibiting factors

Possibly *M. oxyfera*-type bacteria require growth factors or other, heterotrophic, organisms to produce growth factors or to remove inhibitory intermediates or products. During the enrichment, organic substrates present in the inoculum got depleted and growth of heterotrophic organisms became dependent on the consumption of decay products. At a low concentration of decay products, buildup of inhibitory compounds and decay of *M. oxyfera*-type bacteria may have occurred. After addition of biomass enriched in *M. oxyfera*-type bacteria, collected from the effluent of the SFBRs during periods of increasing nitrite consumption rates, to one of the MBRs the nitrite consumption rate increased. The rate decreased in the other MBR that was inoculated with wastewater sludge only. In contrast, when biomass collected during periods of decreasing rates was added, the rate did not increase or only slightly increased. These results indicated that biomass collected during periods with increasing rates contained not just *M. oxyfera*-type bacteria, but also growth factors or substrate for heterotrophic microorganisms that provided growth factors or removed inhibitory compounds. Biomass collected during periods of decreasing rates did not provide such compounds and addition did therefore not result in higher consumption rates. Eventually, in all reactors nitrite consumption rates went down, indicating growth factors or substrate for heterotrophs became limiting. When biomass from three reactors was combined in one single reactor, the nitrite consumption rate increased, matching the sum of the consumption rates in all three reactors, viz. 34 mg NO_2^--N/L d. It remains to be investigated if the consumption rate will increase to a new maximum or will stabilize or decrease again. The results, however, suggested that to achieve higher consumption rates, not only biomass concentration should be increased, but also growth factors or substrate for heterotrophic bacteria are required.

3.4.4 Copper addition

To supply potential growth factors, MBR1 was fed with medium with increased copper concentrations. As a result nitrite consumption rates increased. This indicated that higher copper concentrations facilitate higher consumption rates. An explanantion would be that copper is required for expression of pMMO by *M. oxyfera*-type bacteria and that higher copper concentrations result in higher pMMO expression.

3.4.5 HRT

The results of this paper clearly show that decreasing the HRT immediately increased the consumption rate, changing from 8 mg NO_2^--N/L d at an HRT of 61 days to a new maximum of 31 mg NO_2^--N/L d at an HRT of 1.4 days. When decreasing the HRT, the influent nitrite concentration was decreased accordingly while trace element concentrations remained constant. Consequently the rate at which these trace elements were

supplied was increased, possibly alleviating a nutrient shortage. Also, at a shorter HRT, the rate at which potentially inhibitory intermediates or products are washed out was increased. Further research at even shorter HRTs in the order of hours, which are also required for practical application, is necessary to evaluate if decreasing nitrite consumption rates can be avoided and if a rate will be achieved that is suitable for a practical application in wastewater treatment.

3.5 Conclusions

- *M. oxyfera*-type bacteria can be enriched from a mixture of sludge from municipal wastewater treatment in a membrane bioreactor at 20 °C, fed with medium containing 10–100 % effluent from municipal wastewater treatment.
- Despite the lower enrichment temperature (20 °C vs. 30 °C), the maximum denitrification rate of 36 mg NO_2^--N/L d achieved in the present enrichment matches the highest rate reported in literature.
- The observed stagnation and subsequent decrease in nitrite consumption rate observed in the enrichment reactors is not due to limited biomass retention; the cause remains unknown.
- Nitrite consumption rates were increased by increasing the influent copper concentration and operating a MBR at shorter HRTs.

4

Effect of temperature on denitrifying methanotrophic activity of '*Candidatus* Methylomirabilis oxyfera'

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. Previous studies showed that similar maximum volumetric denitrification rates could be achieved at 20 °C and 30 °C. Moreover, shorter doubling times were achieved in membrane bioreactors at 20 °C than in sequencing fed-batch reactors at 30 °C. This study showed that biomass enriched at 20 °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.

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

4.1 Introduction

In response to climate change and fossil fuel depletion, there is a trend towards energy self-sufficient municipal wastewater treatment. Wastewater treatment plants (WWTPs) can become (close to) self-sufficient by a combination of energy savings and energy recovery. To achieve this, in temperate zones the main focus is on 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 e.g. a combination of an upflow anaerobic sludge bed reactor and a sludge digester (UASB-digester) (Zhang et al., 2013; Álvarez et al., 2004; Mahmoud et al., 2004). The main disadvantages of this system are that nutrients are not removed and that methane is dissolved in the effluent. In order to overcome these disadvantages, Kampman et al. (2012) 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 nitritation reactor. By applying this concept energy can be saved, because of lower aeration requirements than conventional activated sludge treatment. Also 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. 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 and Lant, 2006). In the proposed concept, nitrite-nitrogen and methane are concomitantly removed (eq. 4.1) (Raghoebarsing et al., 2006), through the activity of denitrifying methanotrophic bacteria (Kampman et al., 2012).

$$3\,CH_4 + 8\,NO_2^- + 8\,H^+ \longrightarrow 3\,CO_2 + 4\,N_2 + 10\,H_2O \tag{4.1}$$

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, e.g. in summer conditions or in (sub)tropical regions. This, however, may require addition of biogas to provide sufficient methane. In these cases also a UASB-digester combined with the anammox process could be used (Hendrickx et al., 2012b).

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) achieved 80 % nitritation at an average nitrogen concentration of 43 mg N/L and a temperature of 18–25 °C, Yang et al. (2007) achieved > 95 % nitritation at an average nitrogen concentration of 60 mg N/L and a temperature of 11.9–26.5 °C.

16S rRNA sequences similar to that of the denitrifying methanotrophic bacterium '*Candidatus* Methylomirabilis oxyfera' have been detected in sediments and wastewater sludge from temperate zones. From these inocula *M. oxyfera*-type bacteria have been enriched (Kampman et al., 2012; Zhu et al., 2012; Luesken et al., 2011b; Raghoebarsing et al., 2006). The bacteria have been enriched at temperatures ranging from 20–35 °C (see table 4.1). Similar maximum volumetric denitrification rates of 34–38 mg NO_2^--N/L d were achieved at 20 °C (chapter **??**) and 30 °C (Kampman et al., 2012; Ettwig et al., 2009) and doubling times were shorter in membrane bioreactors (MBR) at 20 °C than in sequencing fed-batch reactors (SFBR) at 30 °C (0.9 and 1.3 months in the MBRs vs. 1.7 and 1.9 months in the SFBRs) (chapter **??**). Activity and growth of denitrifying methanotrophic bacteria at temperatures < 20 °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., 2011b) with a doubling time in the order of weeks, lower optimum temperatures were observed for organisms enriched at or adapted to lower temperatures. Hendrickx et al. (2014) observed a temperature optimum of 20–30 °C for anammox enriched at 10 °C. Hu et al. (2013) reported similar maximum activities of a 30 °C anammox culture and a 12 °C adapted anammox culture. However, anammox bacteria enriched at 12 °C had 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 an application in low-temperature anaerobic wastewater treatment where temperature fluctuates between 10–20 °C, less biomass (retention) is required if biomass with a lower temperature optimum, yet similar maximum rate, is enriched.

In this chaper 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 20 °C and at 30 °C. Nitrite and methane consumption rates and nitrogen gas production 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.

Table 4.1: Overview of denitrifying methanotrophic enrichment reactors, enrichment temperatures and maximum activity. The abbreviation MBR is used for a membrane bioreactor, SBR is a sequencing batch reactor and SFBR is a sequencing fed-batch reactor.

Inoculum	**Enrichment temp. (°C)**	**Maximum activity (mg N/L d)**			**Enrichment reactor**	**Reference**
		NO_2^-	NO_3^-	NO_2^-+NO_3^-		
Mixture of WWTP sludge, The Netherlands	20	16			MBR	Kampman et al., 2014
Mixture of WWTP sludge and biomass from enrichments (Kampman et al., 2012)	20	37			MBR	Kampman et al., 2014
Mixture of WWTP sludge and fresh water sediment, Australia	22		0.91		SBR	Hu et al., 2009
Sludge industrial wastewater treatment, The Netherlands	20 - 23	15			SFBR	Luesken et al., 2011b
Minerotrophic peatland, The Netherlands	25	14			SFBR	Zhu et al., 2012
Freshwater sediment, The Netherlands	25			12	SFBR	Raghoebarsing et al., 2006
Freshwater sediment, The Netherlands; synthetic medium	30	34			SFBR	Kampman et al., 2012
Freshwater sediment, The Netherlands; medium with 10 % wastewater	30	38			SFBR	Kampman et al., 2012
Freshwater sediment, The Netherlands	30	36			SFBR	Ettwig et al., 2009
Biomass from enrichment (Raghoebarsing et al., 2006)	30	11			CSTR	Ettwig et al., 2008
Mixture of WWTP sludge and fresh water sediment, Australia	35	24[a]	28; 6.4 [a]		SBR	Hu et al., 2009
Biomass from 35 °C enrichment (Hu et al., 2009); nitrite fed	35	17			SBR, pulse fed with nitrite	Hu et al., 2011
Biomass from 35 °C enrichment (Hu et al., 2009); nitrate fed	35		0.54		SBR	Hu et al., 2011

[a] In a batch test the reactor was fed with both nitrite and nitrate, nitrate consumption was lower than during normal reactor operation, when the reactor was fed with nitrate only.

4.2 Materials and methods

4.2.1 Setup of activity tests

Activity tests were performed on two anoxically operated reactors enriched in *M. oxyfera*-type bacteria. Reactor R30, a sequencing fed-batch reactor (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 (chapter 3; Kampman et al., 2012). Reactor R20, a membrane bioreactor (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 (1.00 ± 0.32 g protein) of MBR operation (3). 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 % CH4, 5.0–6.4 % CO2; in reactors in excess at all times) (chapter 3;Kampman et al., 2012). 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.

4.2.2 Procedure of 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), 20.4 °C (day 2.3–4.3), 15.9 °C (day 7.0–8.3), 11.4 °C (day 9.0–11.3) and 24.9 °C (day 12.9–15.2) subsequently. R30 was operated at 29.9 °C (day 0.0–2.3), 20.4 °C (day 2.3–4.3), 15.8 °C (day 7.0–8.3), 11.1 °C (day 9.0–11.3) and 25.1 °C (day 12.9–15.2) subsequently. Nitrite concentration and methane and nitrogen gas pressures 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. In between the activity tests at different temperatures nitrite was added or the influent was started and the reactor was fed with 5.0 mL/min CH_4/CO_2 (95/5%).

4.2.3 Analyses

Nitrite and nitrate were measured by ion chromatography. Methane, nitrogen gas, carbon dioxide and oxygen were 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). After setting a new experimental temperature, a minimum equilibration time of 1.5 h was applied to allow redistribution of gasses between gas and liquid phase before analyses were started.

4.2.4 Calculations

Zero-order nitrite and methane consumption rates and nitrogen gas production 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 activities of the reactors could not be directly compared. For determining 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 (4.2), 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).

$$k = A \times e^{\frac{-E_a}{R \times T}} \tag{4.2}$$

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

4.3 Results and discussion

4.3.1 Conversion rates and temperature optimum

At all temperatures tested methane oxidation and nitrite denitrification with concomitant nitrogen gas production occurred in both R20 and R30 (as in fig. 4.1). The results indicated 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 (fig. 4.2). Methane consumption rate and nitrogen gas production rate were highest at 20 °C, viz. 15 ± 2 mg/L d and 38 ± 3 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

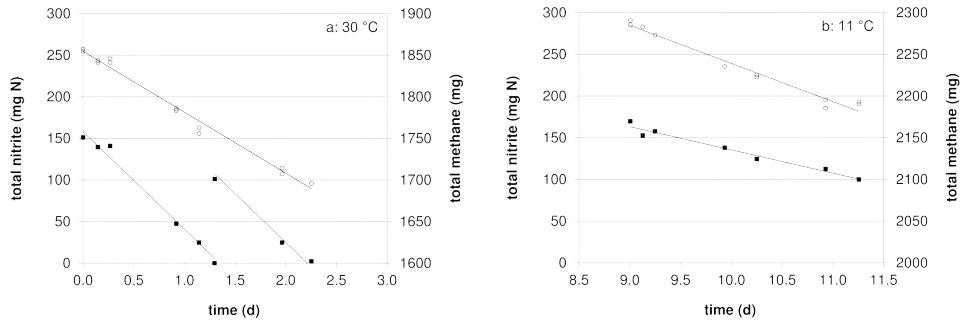

Figure 4.1: Coupling of methane (∘) oxidation and nitrite (□) denitrification in R30 during whole culture batch tests at temperatures of (a) 30 °C, more nitrite was added after 1.3 d, and (b) 11 °C.

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 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 ± 7.0 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 (fig. 4.2). Also methane consumption rates and nitrogen gas production rates were highest at 25 °C 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. Nitrogen gas was produced at a rate of 24.1 ± 0.1 mg/L d at both 25 °C and 30 °C. In the temperature range of 11–25 °C the conversion rates increased by a factor 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 20 °C. Whilst the activity of the biomass in R30 was highest at 25–30 °C , the activity of the biomass enriched at 20 °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 (chapter 3). This indicated long term (> 3 months) activity of the biomass at lower temperatures is possible and biomass might have changed due to the lower temperature after spiking. However, enrichment of denitrifying methanotrophic bacteria at 30 °C is not interesting for low temperature applications. Since at 20 °C similar maximum denitrification rates, shorter doubling times (chapter 3) and a lower temperature optimum were observed, for temperate zones biomass should be enriched at ≤ 20 °C.

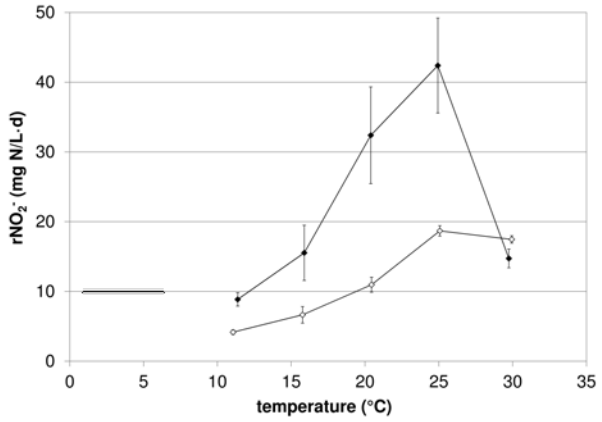

Figure 4.2: Volumetric nitrite consumption rates of R20 (◆) and R30 (◇) at different temperatures. The error bars indicate the standard error in the estimate of the slope. Methane consumption and nitrogen gas production showed similar patterns.

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 20 °C, respectively) was higher than during continuous operation (14 mg NO_2^--N/L d vs. 17.5 ± 0.5 mg NO_2^--N/L d at 30 °C in R30 and 18 mg NO_2^--N/L d vs. 41 ± 7 mg NO_2^--N/L d at 20 °C in R20). After the tests continuous operation was resumed and nitrite was consumed at a rate of 14 mg NO_2^--N/L d in both R20 and R30, indicating changes in temperature did not or only hardly influence the stability of the process. Nitrite was not limiting during continuous operation, thus could not have resulted in apparent low nitrite consumption rates. What could have caused the difference in rate between activity tests and continuous operation is unknown.

4.3.2 Nitrite affinity

In most activity tests the nitrite consumption rates did not decrease at decreasing nitrite concentrations, even if nitrite was consumed to < 0.015 mg NO_2^--N/L (R30 at 30 °C fig. 4.1a). This indicateds that the nitrite affinity of the denitrifying methanotrophic culture was high, which is in good agreement with the high affinity of 1 µg NO_2^--N/L reported by Ettwig et al. (2008). The affinity was higher than the affinity reported by He et al. (2013).

Table 4.2: CH4, NO2- and N2 molar ratios during activity tests at temperatures of 11–30 °C in R20 and R30.

Reactor	**Temperature (°C)**	**CH_4 :**	**NO_2^- :**	**N_2**
R20	11	6.0	8	7.6
	16	3.8	8	5.1
	20	3.3	8	4.7
	25	2.2	8	2.5
	30	3.6	8	6.1
R30	11	12.0	8	12.3
	16	6.5	8	8.7
	20	3.3	8	6.6
	25	4.0	8	5.2
	30	4.4	8	5.5
Theoretical ratio		3	8	4

4.3.3 Conversion ratios

The molar consumption or production ratios $CH_4 : NO_2^- : N_2$ were in good agreement with the theoretical ratio of $3\,CH_4 : 8\,NO_2^- : 4\,N_2$ (table 4.2) at temperatures of 16–30 °C in R20 and 20–30 °C in R30 (table 4.2). At lower temperatures methane consumption rates and nitrogen gas production 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 significant nitrate consumption occurred. Thus, the nitrogen gas production that could not be explained by nitrite-denitrification could not be explained by nitrate-denitrification either. Possibly at lower temperatures a changed metabolism occurred.

4.3.4 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 20 °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, that also perform nitrite-denitrification and have a similar low growth rate (Hendrickx et al., 2014; Hu et al., 2013). Considering the long doubling times of denitrifying methanotrophic bacteria, efficient biomass retention is required, e.g. as flocs, granules or biofilms, is required. 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 to 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 < 20 °C. However, the duration of the activity tests was too short to observe any growth (doubling time denitrifying methanotrophic bacteria 1–2 months). Long term reactor operation or enrichment at < 20 °C is required to verify this assumption.

Temperature does not only affect biological activity, it also influences e.g. 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 dissolved methane concentration is about 20 mg/L. This is enough to sustain nitrite-dependent anaerobic methane oxidation (Kampman et al., 2012). In summer, at a temperature of 20 °C the dissolved methane concentration is 16 mg/L (assuming Henry's law, atmospheric pressure, and 70 % methane in the biogas). Possibly, to sustain denitrification at 20 °C biogas has to be added. However, this is only a small fraction of the biogas produced, viz. 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 has confirmed that the nitrite affinity of denitrifying methanotrophic bacteria is high, as was already reported by Ettwig et al. (2008); nitrite consumption rates did not decrease as nitrite concentrations decreased to < 0.015 mg NO_2^--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).

4.4 Conclusions

- For the first time activity of denitrifying methanotrophic bacteria at temperatures < 20 °C was shown.
- Activity of denitrifying methanotrophic biomass enriched at 20 °C was highest at 20–25 °C. At a temperature of 30 °C the activity dropped.
- Denitrifying methanotrophic biomass enriched at 30 °C showed a maximum activity at 25–30 °C.
- Denitrifying methanotrophic bacteria have a high affinity for nitrite; consumption rates did not decrease as nitrite concentrations decreased to < 0.015 mg NO_2^--N/L. This means that if denitrifying methanotrophic bacteria are applied in wastewater treatment low nitrogen effluent concentrations are feasible.

5

Effect of low concentrations of dissolved oxygen on the activity of denitrifying methanotrophic bacteria

Abstract

Through anaerobic treatment, chemical energy can be recovered from municipal wastewater as biogas. Effluent from direct anaerobic wastewater treatment at low temperatures, however, still contains nitrogenous compounds (mainly ammonium) and considerable amounts of dissolved methane. After nitritation, methane can be used as electron donor for denitrification by the anaerobic bacterium '*Candidatus* Methylomirabilis oxyfera'. It was shown that in presence of 0.7 % O_2 denitrifying methanotrophic activity slightly increased and returned to its original level after oxygen had been removed. Therefore, traces of oxygen that bacteria are likely to be exposed to in wastewater treatment are not expected to affect the denitrification process. At 1.1 % O_2, methane consumption increased with 118 %, nitrite consumption rate increased with 58 %. After removal of oxygen, methane consumption rate fully recovered, nitrite consumption rate returned to 88 %. 2.0 % O_2 inhibited denitrifying methanotrophic activity. Nitrite consumption rate decreased with 60 % and did not recover after removal of oxygen. Further studies should evaluate if intermittent addition of oxygen results in increased growth rates.

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

5.1 Introduction

Municipal wastewater is generally treated in aerobic activated sludge processes. In these processes chemical energy (chemical oxygen demand, COD) is converted to carbon dioxide, which is emitted to the atmosphere. Through anaerobic treatment, the chemical energy can be recovered as biogas instead. Effluent from direct anaerobic wastewater treatment at low temperatures, however, still contains nitrogenous compounds (mainly ammonium) and considerable amounts of dissolved methane. Nitrogen should be removed from the effluent because it can cause eutrophication. Methane is a gas with a high global warming potential and should be removed to prevent it from escaping to the atmosphere (Kampman et al., 2012). Using methane for nitrogen removal would be a promosing technological solution for both problems.

Conventionally, nitrogen is removed from wastewater using sequential nitrification and heterotrophic denitrification. However, for the latter process an electron donor is required. The effluent from low-temperature anaerobic treatment systems for municipal wastewater contains insufficient carbon sources to sustain heterotrophic denitrification. Addition of an external carbon source is expensive and not sustainable (Sun et al., 2010; Modin et al., 2007; Thalasso et al., 1997). Methane has been considered a cheap and readily available electron donor (Modin et al., 2007; Houbron et al., 1999; Thalasso et al., 1997). At lower temperatures anaerobically pretreated water contains a relatively high concentration of dissolved methane that could be used for denitrification. The dissolved methane concentration is 15–20 mg/L assuming Henry's law (calculated for atmospheric pressure, 20 resp. 10 °C and 70 % methane in the biogas). 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 and Lant, 2006).

In an association between aerobic methane oxidizing bacteria and heterotrophic denitrifying bacteria, methane can serve as the sole carbon source. Methane oxidizing bacteria convert methane to carbon dioxide. They also excrete soluble organic compounds such as methanol. These compounds are subsequently used as electron donors by the denitrifying bacteria (reviewed by Modin et al., 2007). The theoretical ratio of methane to nitrate consumption for this process is 1.27 (Modin et al., 2007). However, due to the presence of oxygen, in practice ratios of more than 2.2 were observed (Modin et al., 2008). This means that to remove 50 mg NO_2^--N/L, a nitrogen concentration typical for municipal wastewater, more than1.3 mol CH_4/mol NO_2^-, or more than 75 mg CH_4/L, would be required (assuming a ratio of more than 2.2 mol methane/mol nitrate and oxygen equivalents of 2.86 g O_2/g NO_3^--N and 1.71 g O_2/g NO_2^--N). Consequently, the methane dissolved in the effluent might not be enough to sustain denitrification. Additional methane from the anaerobic treatment step could be added to obtain more denitrification. However, if this suffices, it would still significantly reduce energy recovery: the total methane production from

the anaerobic step is approximately 91 mg CH_4/L municipal wastewater, assuming a concentration of 0.6 g COD/L and 66 % COD removal (Mahmoud et al., 2004).

Recently, microorganisms have been discovered that couple denitrification to anaerobic methane oxidation (Haroon et al., 2013; Raghoebarsing et al., 2006). The denitrifying methanotrophic archaea '*Candidatus* Methanoperedens nitroreducens' reduce nitrate to nitrite while performing reverse methanogenesis (Haroon et al., 2013). Denitrifying methanotrophic bacteria related to '*Candidatus* Methylomirabilis oxyfera' '*Candidatus* Methylomirabilis oxyfera' reduce nitrite to nitrogen gas while oxidizing methane (Kampman et al., 2012; Luesken et al., 2011b; Hu et al., 2011). After nitritation of the effluent from anaerobic treatment, these bacteria can be applied to remove both nitrite and methane from the effluent (Kampman et al., 2012; Luesken et al., 2011b). *M. oxyfera* produces oxygen internally, similar to (per)chlorate reducing bacteria. The latter convert (per)chlorate to chlorite, which is subsequently split into oxygen and chloride by a chlorite dismutase (Van Ginkel et al., 1996). *M. oxyfera* converts nitrite to nitric oxide, which is converted to nitrogen gas and oxygen by an unidentified nitric oxide dismutase (Ettwig et al., 2010). Even though these bacteria exist under anoxic conditions, they produce and consume their own oxygen (Ettwig et al., 2010). Part of this oxygen (3/4) is used for aerobic methane oxidation while the remainder (1/4) may be used for e.g. respiration by a terminal oxidase (Wu et al., 2011a). Contrary to denitrifying methanotrophic archaea, denitrifying methanotrophic bacteria conduct complete denitrification to nitrogen gas. These bacteria consume methane and nitrite in a ratio close to the theoretical ratio of 0.375 (Kampman et al., 2012; Luesken et al., 2011b; Hu et al., 2011). At this ratio only 21 mg CH_4/L is required to remove 50 mg NO_2-N/L, viz. only $1/4^{th}$ of the methane produced during anaerobic treatment of municipal wastewater is required for denitrification. Thus, the dissolved methane in the effluent from direct anaerobic wastewater treatment at low temperatures suffices to drive complete denitrification (Kampman et al., 2012). If not all dissolved methane is utilized, the remaining methane could be removed aerobically in the nitritation process.

Despite the highly exergonic reaction ($\Delta G^{0'}$ −928 kJ/mol CH4, Raghoebarsing et al., 2006) performed by denitrifying methanotrophic bacteria, they grow slowly. *M. oxyfera*-like bacteria have a doubling time of 1–2 months (chapter 2;Kampman et al., 2012). The doubling time of bacteria performing aerobic methane oxidation ($\Delta G^{0'}$ −818 kJ/mol CH4, Thauer and Shima, 2008), employing the same methane oxidation pathway, such as *Methylomonas capsulatus* can be as short as 13 h (Foster and Davis, 1966). The doubling time of heterotrophic denitrifiers typically is 5 h (Tchobanoglous et al., 2003). Rates of oxygen dependent reactions in *M. oxyfera*-like bacteria, such as methane oxidation and energy conserving reactions catalyzed by terminal oxygen reductases, might be increased by addition of oxygen (Luesken et al., 2012). Short term exposure to air did not result in reduced activity of *M.*

oxyfera-like bacteria (Ettwig et al., 2009). In contrast, Luesken et al. (2012) showed irreversible inhibition of denitrifying methanotrophic bacteria upon oxygen exposure. Exposure to 2 % (initial concentration) of oxygen resulted in a decrease of methane and nitrite consumption rates of 25 and 57 %, respectively. Exposure to 8 % of oxygen, depleted within 42 hours, resulted in a 72 % (methane) and 81 % (nitrite) decrease in consumption rates. Within the first 8 hours after oxygen exposure was stopped, no full recovery of activity was observed. The methane consumption rate was 61 % lower than before oxygen exposure. It was also shown that expression of the genes involved in nitrite and nitrate reduction decreased as a result of exposure to 8 % oxygen. Possibly, oxygen enhances or does not affect denitrifying methanotrophic activity at low concentrations. However, at increased levels, resulting from oxygen production at high denitrifying methanotrophic activity or from external oxygen addition, the bacteria might be inhibited. This might explain the reduced activity observed in enrichment reactors after reaching a maximum denitrification rate (chapter 3;Kampman et al., 2012; Hu et al., 2011) and thus a maximum oxygen production rate. In this case other organisms may be required to remove excess oxygen and prevent or reduce oxygen inhibition.

It remains to be investigated if denitrifying methanotrophic bacteria are affected by oxygen partial pressures below 2 % (Kampman et al., 2012; Shen et al., 2012). Possibly, lower concentrations would not inhibit, but even enhance the growth rate of denitrifying methanotrophic bacteria. In addition, in wastewater treatment denitrifying methanotrophic bacteria might be exposed to traces of oxygen, remaining from the nitritation process required to produce nitrite (Kampman et al., 2012). Therefore, in the present study the effect of 0.7 % (0.3 mg dissolved O_2/L), 1.1 % (0.5 mg dissolved O_2/L), and 2.0 % (1.0 mg dissolved O_2/L), on denitrifying activity was tested. The results and consequences for enhancing enrichment rate of denitrifying methanotrophic bacteria and their application in wastewater treatment are discussed.

5.2 Materials and methods

5.2.1 Reactor activity test

Three membrane bioreactors (MBRs) and one sequencing fed-batch reactors (SFBRs) were operated for more than 2 years. Operational details and results of these reactors were previously described in detail in chapter 3 and by Kampman et al. (2012). The original inocula of the reactors originated from environments with fluctuating oxygen concentrations. MBR1 was inoculated with a mixture of sludge from municipal wastewater treatment. MBR2 was inoculated with a mixture of sludge and effluent enriched in *M. oxyfera* from two SFBRs inoculated with freshwater sediment. MBR3 and the SFBR were inoculated with freshwater sediment. In wastewater treatment

bacteria were exposed to alternating oxic and anoxic conditions. In sediments, denitrifying methanotrophic bacteria have been found close to the oxic/anoxic interface (Zhu et al., 2012; Raghoebarsing et al., 2006) where they were exposed to fluctuating concentrations of oxygen for short periods of time (Luesken et al., 2012).

All reactors were equipped with on line sensors for oxidation-reduction potential, pH and temperature.

For a period of 11 days the four reactors enriched in *M. oxyfera*-like bacteria were exposed to oxygen. Oxygen was supplied in a mixture with methane (92 % CH_4, 8.0 % O_2) at a flow rate of 5.1 mL/min. After these 11 days, the reactors were flushed for 3 days with CH_4/CO_2 (95/5 %) at an increased flow rate of 20 mL/min to rapidly remove remaining oxygen. After these 3 days the CH_4/CO_2 gas flow rate was set back to 5 mL/min. The effect of this period of oxygen exposure was evaluated from long-term reactor monitoring. To assess the effect of oxygen on the microbial composition immediately after exposure to oxygen the reactors were sampled for Fluorescence In Situ Hybridization (FISH; described in section 5.2.3).

5.2.2 Experimental setup batch tests

The effect of oxygen partial pressures of 0.7 % (0.3 mg dissolved O_2/L), 1.1 % (0.5 mg/L) and 2.0 % (1.0 mg/L) on denitrifying methanotrophic activity was tested in 0.6 L bottles. All oxygen concentrations and also controls without oxygen were tested in duplicate (referred to as A and B). Biomass from a sequencing fed-batch reactor (SFBR) enriched in *M. oxyfera*-like bacteria that was inoculated with freshwater sediment (Kampman et al., 2012) was first siphoned to 1 L bottles under constant flushing with nitrogen gas. Then, in an anaerobic chamber the test bottles were filled with 250 mL reactor sample, 7.5 mL 209 g 3-(N-morpholino)propanesulfonic acid (MOPS)/L (pH 7.0, final concentration 6.28 g MOPS/L) and 0.6 mL 14 g NO_2^--N/L (final concentration of 32 mg NO_2^--N/L). Hereafter, the bottles were flushed with methane for 10 min and after 3 h equilibration time nitrite and methane consumption rates were determined. After 2–3 days pure oxygen (3.0 mL, 5.0 mL and 10 mL to achieve final concentrations of 0.7 %, 1.0 % and 2.0 % respectively) was added. The bottles were shaken vigorously after which activity measurements were resumed immediately. After 22 h oxygen was removed: the head space of the bottles was replaced with nitrogen (five cycles of vacuum and purge) and flushed with CH_4. After 3 h equilibration time, activity was measured for 3–4 more days to establish recovery of the denitrification activity.

During the whole test, the bottles were incubated at 30 °C and mixed by magnetic stirring (100 rpm). Oxygen concentration in the bottles was measured on line every minute (PSt3 non-invasive oxygen sensors and Oxy-4 trace oxygen meter, PreSens). Biogas composition and nitrite concentration were measured 2–3 times per day (section 5.2.3). Nitrite was added to the bottles at a concentration of 28 mg NO_2^--N/L

every time its concentration was close to zero. For calculations and data representation the concentration at the moment of addition was added to the measured concentration just before addition.

5.2.3 Analyses

Gas composition (O_2, N_2, CH4 and CO_2) was measured by gas chromatography, using 0.5 mL samples. Nitrite and nitrate concentrations were estimated 3–7 times per week using test strips (Merckoquant, Merck chemicals) to monitor reactor performance. Nitrite and nitrate concentrations were measured by ion chromatography. These methods were described in detail by Kampman et al. (2012).

FISH was performed as described by Ettwig et al. (2008), except that samples were stored at −18 °C and a hybridization buffer with 20 % formamide was used. To target bacteria affiliated with the 'NC10' phylum probes S-*-DBACT-0193-a-A-18 (DBACT193) and S-*-DBACT-1027-a-A-18 (DBACT1027) (Raghoebarsing et al., 2006) were used. S-Sc-aProt-0968-a-A-18 (ALF968; Neef, 1997) and L-C-gProt-1027-a-A-17 (GAM42a; Manz et al., 1992) were applied to detect resp. *Alpha-* and *Gammaproteobacteria*. EUB mix was used to target almost all bacteria (Daims et al., 1999) and DAPI was used to stain all DNA.

5.3 Results and discussion

5.3.1 Reactors fed with 8 % oxygen

Effect of oxygen on oxidation-reduction potential, pH and nitrite consumption rates

For a period of 11 days all reactors were fed with 92 % CH_4 and 8 % O_2 (5.1 mL min^{-1}), instead of a mixture of 95 % CH_4 and 5 % CO_2. The presence of oxygen was observed when after 11 days a regular check was performed to evaluate if sufficient methane and carbon dioxide were present. Methane was present in excess, carbon dioxide was not detected. Instead of carbon dioxide, 0.6 % (MBR2 and SFBR), 0.9 % (MBR1) and 1.2 % (MBR3) O_2 was measured. The fact was not discovered before, because no direct effects on reactor performance were observed: the oxidation-reduction potential did not increase upon oxygen addition, the pH did not increase in the absence of carbon dioxide and nitrite consumption rates did not drop. In section 5.3.2 it is shown that at low oxygen concentrations the nitrite consumption rates in batch experiments could double. If this also happened in the reactors remained unnoticed. The procedure was to gradually increase the nitrite loading rate (to prevent too high nitrite accumulation) at the moment that nitrite in the reactors was close to 0 mg NO_2^--N/L to match the nitrite consumption rates. The period of

oxygen exposure in the reactors was too short to observe a significant increase in nitrite consumption rates.

After reestablishing anoxic conditions, tests were performed to check if denitrification still occurred. Significant denitrifying methanotrophic activity was still present, showing that oxygen had not irreversibly inhibited the bacteria. Hereafter reactor operation was continued.

Effect of oxygen on nitrite consumption rates during prolonged reactor operation

Long-term behavior of each reactor after the accidental exposure to oxygen was different. Biomass in MBR2 seemed negatively affected by exposure to oxygen (fig. 5.1a): the same nitrite loading rate of 11 mg NO_2^--N/L d was applied before and during oxygen exposure. However, at the same loading rate, 3 weeks after oxygen exposure rapid nitrite accumulation occurred. The nitrite loading rate in MBR2 therefore had to be decreased from 11 to 3 mg NO_2^--N/L d to match the consumption rate. Hereafter slow recovery took place and 2 months after the oxygen exposure a volumetric nitrite consumption rate fluctuating between 5–6 mg NO_2^--N/L d was reached at which it remained for another two months. In MBR1 the decrease (data not shown) and in MBR3 (fig. 5.1b) the increase in volumetric nitrite consumption rates that had already started before exposure to oxygen continued. The rate in SFBR was not affected and consumption rate remained around 19 mg NO_2^--N/L d (data not shown).

Oxygen sensitivity of denitrifying methanotrophs and the role of the side population in oxygen removal

Although the reactors were continuously fed with 8 % O_2, when it was noticed, only 0.6 % O_2–1.2 % O_2 was measured in the reactors. The lower concentrations in the reactor than in the feed, show that oxygen was consumed in the reactors. All enrichment cultures of denitrifying methanotrophic bacteria described in literature, contained more than 20 % unidentified bacteria. Luesken et al. (2012) showed an increased expression of genes involved in methane oxidation of several aerobic methanotrophic bacteria upon oxygen exposure. These bacteria belonged to the classes of *Alpha-* and *Gammaproteobacteria*. In the present study samples taken before (available for MBR1 and SFBR) and immediately after oxygen exposure (available for all four reactors) were analyzed for the presence of bacteria belonging to these classes. Both before and after oxygen exposure *Alpha-* and *Gammaproteobacteria* were present. In the SFBR an increased amount of *Gammaproteobacteria* was observed (visually using FISH) after the period of oxygen exposure; in MBR1 no increase was observed. From the observed oxygen removal, the presence of proteobacteria in all reactors and the observed increase of proteobacteria in the SFBR it is hypothesized that the presence of a side population, consisting of e.g. aerobic methanotrophic bacteria and aerobic

heterotrophic organisms, able to consume oxygen, may be important for reducing the effect of oxygen on denitrifying methanotrophic bacteria. To investigate the effect of oxygen in more detail additional batch tests were performed.

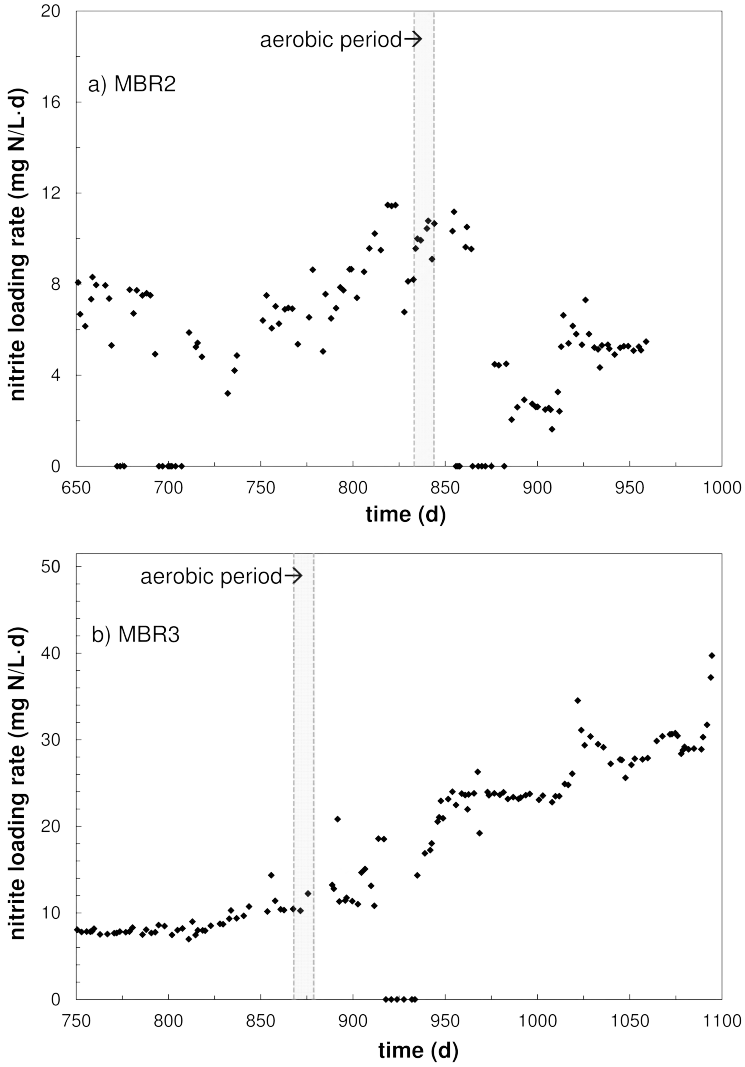

Figure 5.1: Volumetric nitrite loading rate in (a) MBR2 (at 20 °C) and (b) MBR3 (at 30 °C) in time. The grey bar indicates an 11 d period of oxygen exposure. 8 % O_2 was fed to the reactors, at the end of the aerobic period 0.6 % (MBR2) and 1.2 % (MBR3) were measured in the reactors. A loading rate of 0 mg NO_2^--N/L d was set in the event of too high nitrite accumulation. In all other cases nitrite consumption rates were well represented by the nitrite loading rate.

5.3.2 Effect of 0.7, 1.1 and 2.0 % oxygen on denitrifying methanotrophic activity

All the batch tests, the results of which will be described in more detail below, demonstrated denitrifying methanotrophic activity before, during and after exposure to 0.7 % (0.3 mg/L), 1.1 % (0.5 mg/L), as illustrated by fig 5.2, and 2.0 % (1.0 mg/L) O_2. The results indicated that the highest consumption rates of methane and nitrite along with higher production rates of nitrogen gas could be achieved in presence of 1.1 %. 0.7 % O_2 hardly affected activity; in presence of 2.0 % O_2 the activity decreased and did not recover the first three days after oxygen was removed. In the controls without oxygen, a constant denitrification rate of 27 mg NO_2^--N/L d was observed for the duration of the tests.

Comparison of initial activity in test bottles and SFBR

Biomass from the SFBR was used for the activity tests. At the start of the tests, the volumetric nitrite consumption rate in the bottles was 22–27 mg NO_2^--N/L d. These rates were somewhat higher than the 19 mg NO_2^--N/L d measured in the reactor at the time the sludge was sampled. This was probably due to the biomass sampling method, which led to different biomass concentrations in the bottles than in the reactor and because of different mixing conditions in the batch tests compared to the reactors. In the batch tests constant, concentration independent conversion rates were observed. Therefore, conducting the tests at different substrate or product concentrations than in the reactor could not have caused differences in rates. Differences in rates between the bottles were probably caused by different biomass concentrations. The average conversion ratios of $CH_4 : NO_2^- : N_2$ were close to the stoichiometrical ratio of $3 : 8 : 4$ (table 5.1).

Development of oxygen concentration in the test bottles

Biomass in the batch tests was exposed to oxygen for 22 h. (Facultative) aerobic bacteria were present in the enrichment culture: in all batch tests, after a lag time of 7 h, oxygen consumption started (fig. 5.2). Possibly oxygen was consumed by proteobacteria (section 5.2.1). Oxygen may have been consumed by nitrifying bacteria as well, although no nitrate production was observed. Nitrate could have been removed by denitrification without accumulating in the system. Oxygen remained present throughout all tests. After 22 h oxygen was still present at an average of 0.15 % (0.08 mg/L) in the tests started with 0.7 % O_2, 0.29 % (0.14 mg/L) in the tests started with 1.1 % O_2 and 0.96 % (0.47 mg/L) in the tests started with 2.0 % O_2. These results indicated that higher initial oxygen concentrations resulted in higher oxygen consumption rates (table 5.1).

0.7 % **Oxygen**

Oxygen was added at initial concentrations of 0.7, 1.1 and 2.0 %. In presence of 0.7 % O_2 the average methane, nitrite and nitrogen gas conversion rates increased to resp. 113 %, 107 % and 111 % of the rates in the anoxic period (fig. 5.3, table 5.1). After oxygen was removed, the average methane, nitrite and nitrogen gas conversion rates rate were 104 %, 99 % and 95 % of the rate before oxygen addition, respectively. Both in presence of and after removal of oxygen, conversion ratios were close to the theoretical ratio of $3\,CH_4 : 8\,NO_2^- : 4\,N_2$. Thus, only a minor increase of conversion rates was observed in presence of 0.7 % O_2 and when oxygen was removed, rates returned to equal rates as before oxygen exposure.

1.1 % **Oxygen**

Addition of 1.1 % O_2 increased all conversion rates (fig. 5.2, fig. 5.3, table 5.1). The average methane consumption rate increased to 218 % of the rate before oxygen addition; nitrite consumption rate increased to 158 % and nitrogen gas production rate increased to 125 %. Since especially the methane consumption rate increased, the conversion ratio deviated from the theoretical $CH_4 : NO_2^- : N_2$ ratio and was $3 : 6.7 : 2.9$ on average. Possibly the increased methane consumption was due to aerobic methane oxidation. Based on a stoichiometric ratio for $O_2 : CH_4$ of $2 : 1$, aerobic methane oxidizers alone could, however, only have been responsible for a maximum of 7.3 mg CH_4/L d. Methane consumption rates increased with 11 mg /L d. This means that, in addition to aerobic methanotrophic activity, also the activity of denitrifying methanotrophic bacteria must have increased. This is supported by the observed increase in nitrite consumption rates and nitrogen gas production rates. The average $CH_4 : NO_2^- : N_2$ ratio was $3 : 6.7 : 2.9$. The stoichiometry shifted towards methane. Nitrite may have partially been removed by aerobic methane oxidation coupled to denitrification. However, according to above stoichiometry, not all nitrite consumed was converted to nitrogen gas. It is unclear to what compounds, besides nitrogen gas, the nitrite was converted. After oxygen was removed, the methane consumption rate returned to the rate in the first anoxic period. On average, nitrite consumption rate returned to 88 % (in one bottle 71 %, in the other bottle 105 %) and nitrogen gas production rate returned to 81 % of the rate in the first anoxic period. The average $CH_4 : NO_2^- : N_2$ ratio was $3 : 7.8 : 4.1$, indicating that denitrification coupled to anaerobic methane oxidation was the dominant process. The different response in the duplicates with respect to the nitrite consumption rate could not be explained.

2.0 % **Oxygen**

Although low oxygen concentrations enhance denitrifying methanotrophic activity, a concentration of 2.0 % O_2 resulted in a decrease in most conversion rates (fig. 5.3,

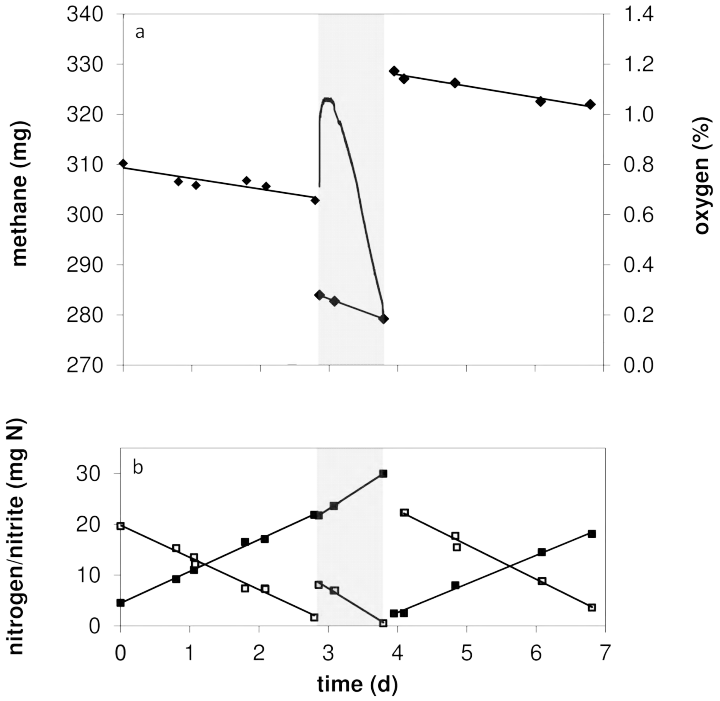

Figure 5.2: Results from an activity test with 1.1 % O_2 and at 30 °C. Figure (a) shows the total amount of methane (◆) and oxygen (—), figure (b) shows the total amount of nitrogen gas (■) and nitrite (□) in time. The grey blocks indicate the aerobic period; 1.1 % O_2 was added at the start of this period. Methane and nitrite consumption rate and nitrogen gas production rate slightly increased in presence of oxygen.

table 5.1). Methane consumption rate was 101 % of the rate before exposure. However, denitrifying methanotrophic activity decreased: nitrite consumption rate and nitrogen gas production rate were decreased by 60 % and 42 % respectively. Luesken et al. (2012) observed a 25 % lower methane consumption rate and a 57 % lower nitrite consumption rate at the same oxygen concentration, which is in good agreement with our results. Similar to the tests at 1.1 % O_2 and as observed by Luesken et al. (2012), at 2.0 % O_2 the stoichiometry shifted towards aerobic methane oxidation. This may be due to inhibition of the denitrification process in presence of oxygen. Moreover, denitrifying methanotrophic bacteria may prefer oxygen as electron acceptor (Luesken et al., 2012). Possibly also activity of aerobic methanotrophs, if present, increased due to lower oxygen limitation at increased oxygen concentrations. The $CH_4 : NO_2^- : N_2$ ratio was $3 : 4.7 : 2.9$. The discrepancy between nitrite consumption and nitrogen gas production could not be explained. Possibly

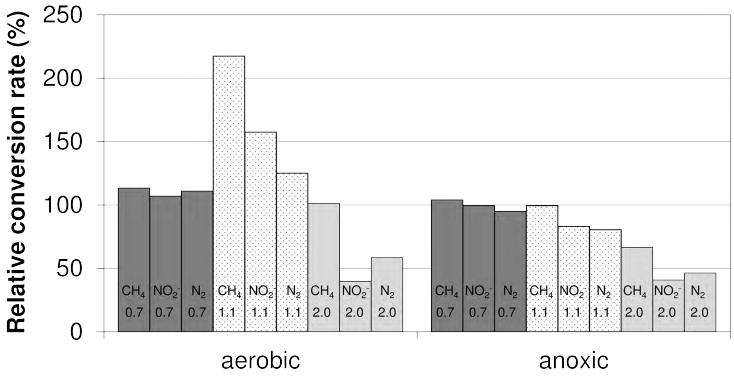

Figure 5.3: Relative conversion rates in activity tests with 0.7, 1.1 and 2.0 % O_2. Conversion rates were calculated relative to the conversion rate in the first anoxic period (100 %), which is not shown in the graphs.

other nitrogenous compounds, such as nitrogen incorporated in cell debris or ammonium added with the influent (4 mg NH_4^+-N/L) were converted to nitrogen gas by nitrification-denitrification. After removing oxygen the methane consumption rate in bottle A was only 38 % of the initial rate, in bottle B this was 95 % of the initial rate. Nitrite consumption and nitrogen gas production amounted to 36–46 % (resp. bottle A and B) and 29–64 % (resp. bottle A and B) of the initial activity, respectively. Since consumption of methane and nitrite and production of nitrogen gas were coupled, it is unclear how methane consumption could have recovered in bottle B, while consumption of nitrite and nitrogen gas have not. In bottle B, similar to the experiments at 1.1 % O_2 the stoichiometry shifted towards methane oxidation: the ratio $CH_4 : NO_2^- : N_2$ was $3 : 5.7 : 3.1$; in bottle A the ratio was $3 : 12 : 4.2$. The difference between the bottles cannot be explained.

Contribution of aerobic microorganisms to increased conversion rates

If aerobic methanotrophs would have been responsible for the increased methane consumption rate, at 2.0 % O_2 rates similar to or higher than at 0.7 or 1.1 % O_2 would be expected. However, whereas at and 0.7 and 1.1 % O_2 the consumption rates increased, at 2.0 % O_2 the methane consumption rate was similar to the rate before oxygen addition (fig. 3). This supports the hypothesis that denitrifying methanotrophic bacteria exhibit increased activity in presence of ≤ 1.1 % O_2. After exposure to 2.0 % O_2 and remarkably also after exposure to 1.1 % O_2 denitrifying methanotrophic activity did not completely recover. Irreversible inhibition by 2 % oxygen was also reported by Luesken et al. (2012).

5.3.3 Final discussion

Increased oxygen concentrations, resulting either from increased denitrifying methanotrophic activity or from an external oxygen source could both stimulate or inhibit denitrifying methanotrophic bacteria, depending on the prevailing concentrations. At the here observed increased methane and nitrite consumption rates at oxygen concentrations $\leq 1.1\,\%$ O_2, it is likely that also the biomass growth rates increased. Therefore addition of low concentrations of oxygen offers opportunities to accelerate the enrichment of the slow growing denitrifying methanotrophic bacteria. Long term oxygen addition to continuous reactors with denitrifying methanotrophic activity is needed to confirm this hypothesis.

Low growth rates do not have to be a problem for full-scale application. Anaerobic ammonium oxidation for example is successfully applied now. However, first enough sludge is required to start a plant (Shen et al., 2012). If growth rates can be increased, e.g. by applying a system with intermittent micro-aerobic and anaerobic periods, startup times of reactors with denitrifying methanotrophic bacteria may be reduced. In this manner increased biomass concentrations could be achieved, resulting in higher volumetric denitrification rates and therefore lower volume requirements.

The denitrifying methanotrophic activity in enrichment reactors showed cyclic behavior (chapter 3). It is hypothesized that this behavior was caused by oxygen. Oxygen may either be inhibiting or limiting. At increasing denitrification rates, *M. oxyfera* produced oxygen at a higher rate and as a result oxygen, may have accumulated. This may have resulted in inhibition of denitrifying methanotrophic bacteria. On the contrary, oxygen consumption by aerobic methanotrophs and nitrifying bacteria oxygen may have taken away oxygen for denitrifying methanotrophic bacteria, resulting in decreased denitrifying methanotrophic rates. Moreover, intermediates of methane oxidation (e.g. methanol or formaldehyde) may have accumulated, due to different oxygen affinity of the different enzymes involved in methane oxidation (Costa et al., 2001), thereby inhibiting denitrifying methanotrophic bacteria. Once oxygen levels were reduced, the amount of denitrifying methanotrophic bacteria increased again and the above described cycle was repeated. How to break through this cyclic behaviour should be investigated. Inhibiting oxygen levels may be prevented by addition of a carbon source for aerobic heterotrophic organisms, removing oxygen. Intermittent microaerobic and anaerobic conditions may be applied to increase the growth rate of denitrifying methanotrophic bacteria if oxygen is limiting.

The observed positive effect of low concentrations of oxygen are promising when considering full scale application in wastewater treatment. Addition of a low amount of oxygen may enhance the growth rate of denitrifying methanotrophic bacteria and thereby enable higher enrichment rates. Moreover, in wastewater treatment denitrifying methanotrophic bacteria are likely to be exposed to traces of oxygen (Kampman et al., 2012). The present study shows that denitrifying methanotrophic bacteria are

not inhibited by 0.7 % O_2 (0.3 mg O_2/L). Therefore, no effect of short term exposure to traces of oxygen is expected, i.e. in case of calamities or exposure to oxygen remaining from the nitritation process are not anticipated to result in a collapse of a system with denitrifying methanotrophic bacteria.

5.4 Conclusions

- Contrary to previous findings, oxygen did not necessarily inhibit denitrifying methanotrophic activity. Low concentrations of oxygen may even be beneficial although this effect is not well understood.
- After exposure to oxygen for 11 d (8.0 % in the feed, after 11 d 0.6 to 1.2 % in the reactors) three out of four denitrifying methanotrophic cultures do not seem influenced by the presence of oxygen. In another culture denitrification rates decreased with 75 %.
- Addition of 0.7 % O_2 (0.3 mg O_2/L) resulted in a slight increase in denitrifying methanotrophic activity and activities recovered after oxygen removal. Therefore, traces of oxygen that bacteria are likely to be exposed to in wastewater treatment are not expected to affect the denitrification process.
- At 1.1 % O_2 (0.5 mg O_2/L) denitrifying methanotrophic activity increased: methane consumption increased to 158 %, nitrite consumption rate increased to 125 %. After removal of oxygen, methane consumption rate returned to the level of before oxygen exposure, nitrite consumption rate only reached 88 %.
- 2.0 % O_2 (1.0 mg O_2/L) inhibited denitrifying methanotrophic activity. Nitrite consumption rate decreased with 40 %. After removal of oxygen rates did not recover.
- Further studies should evaluate if intermittent addition of oxygen results in increased growth rates.

Table 5.1: Methane, nitrite and oxygen consumption rates and nitrogen gas production rates in controls; tests with 0.7, 1.0 and 2.0 % O_2 in the headspace in the first anoxic period, before oxygen exposure; during oxygen exposure; and in the second anoxic period, after oxygen exposure. A and B indicate the duplicates, pO_2 indicates the oxygen concentration at the end of the aerobic aerobic period.

	first anoxic period			**aerobic period**					**second anoxic period**		
	CH_4 mg/L d	NO_2^- mg N/L d	N_2 mg N/L d	CH_4 mg/L d	NO_2^- mg N/L d	N2 mg N/L d	O_2 mg/L d	pO_2 %	CH_4 mg/L d	NO_2^- mg N/L d	N_2 mg N/L d
control											
average	13	27	31								
A	12	27	31								
B	14	27	32								
0.7 % O_2											
average	11	26	24	12	29	27	25	0.15	12	28	24
A	11	27	22	14	28	28	28	0.13	11	27	23
B	11	26	26	10	29	26	22	0.17	12	29	25
1.1 % O_2											
average	9	24	26	20	38	33	29	0.29	9	21	22
A	9.6	23	28	19	40	31	27	0.37	9.3	15	21
B	8.3	25	25	20	37	35	31	0.21	9.3	28	23
2.0 % O_2											
average	7	24	20	7	10	12	42	1.0	5	10	10
A	6.1	22	19	5.3	7.8	7.8	39	0.89	2.4	8.2	5.9
B	7.6	26	21	9.2	12	16	45	1.1	7.5	12	14

6

General discussion

6.1 Introduction

In response to fossil fuel depletion and climate change, new technologies to recover energy and resources from wastewater are developed. Denitrifying methanotrophic bacteria can contribute to this development, by removing nitrogen (as nitrite), which causes eutrophication, and dissolved methane, a greenhouse gas, from the effluent of anaerobic municipal wastewater treatment at low temperatures. Application of a denitrifying methanotrophic process would simultaneously solve these two problems associated with anaerobic treatment. In addition, there no longer is a need to add an external carbon source to accomplish heterotrophic denitrification.

In this final chapter, the status of the research and further steps required to achieve application of a denitrifying methanotrophic process for treatment of effluent of anaerobic wastewater treatment at low temperatures will be discussed.

6.2 Comparison of denitrification coupled to anaerobic methane removal with other technologies for nitrogen removal

Denitrification coupled to anaerobic methane oxidation (DAMO) was compared to conventional nitrification-denitrification, nitrification-denitrification via the nitrite route, anaerobic ammonium oxidation (anammox), combinations of DAMO and anammox, and denitrification coupled to aerobic methane oxidation. These processes were compared based on energy consumption and energy recovery, sludge production, methane removal, consumption of (external) carbon sources and their research status. In this section the comparison made in chapter 1 is summarized, to explain why DAMO is the preferred process.

(Conventional) nitrification-denitrification is not feasible for the treatment of effluent of anaerobic wastewater treatment because the effluent does not contain enough organic carbon sources to accomplish sufficient heterotrophic denitrification. The effluent does contain dissolved methane (15–86.5 mg/L). Since methane has a high global warming potential (25 times the potential of CO_2, IPCC 2007), the dissolved

methane should be removed from the effluent to prevent it from escaping to the atmosphere. DAMO can simultaneously remove 50 mg N/L (a nitrogen concentration typical for municipal wastewater) and 21 mg CH_4/L. If not enough dissolved methane is present, a small amount of methane may be added, although this slightly reduces the energy production. Denitrification coupled to aerobic methane oxidation cannot be applied, since this process may require more methane for denitrification than is produced during anaerobic wastewater treatment. anammox bacteria do not consume methane. Although less oxygen is required for nitritation than when DAMO is applied, because not all, but only 55–60 % of ammonium has to be converted to nitrite, extra oxygen would be required to aerobically oxidize the dissolved methane. As a result, the energy consumption of the anammox process to treat effluent of anaerobic treatment of municipal wastewater at low temperatures, is slightly higher than of the DAMO process (fig. 6.1, Hendrickx et al. 2012a). A process combining anammox and DAMO bacteria is particularly interesting at low dissolved methane concentrations: since DAMO and anammox bacteria both consume part of the nitrogen, less methane will be used for denitrification. As a result, in the case of low-temperature anaerobic municipal wastewater treatment dissolved methane will remain in the effluent and additional methane removal by e.g. an aerobic process will be required. A process with DAMO bacteria, DAMO archaea and anammox bacteria consuming nitrate, ammonium and methane consumes nitrogen (nitrate + ammonium) and methane in the same ratio as DAMO. The oxygen or energy consumption of this process was not reported. Since the process is more complex than DAMO, the latter process is preferred.

6.3 DAMO knowledge development

DAMO is the preferred process for removal of methane and nitrogen from the effluent of low-temperature anaerobic municipal wastewater treatment for environmental and energy reasons. However, it is a new process. Therefore in this study the DAMO process was investigated in more detail. Denitrifying methanotrophic bacteria were enriched from two different inocula. The enrichment was performed in sequencing fed-batch reactors and membrane bioreactors to determine the effect of biomass retention. Also, the effect of several additives, hydraulic retention time (HRT), temperature and oxygen on the nitrite removal rate was determined. The results of this research and the implications thereof are addressed.

6.3.1 Enrichment of denitrifying methanotrophic bacteria and the effect of biomass retention

In this thesis the successful enrichment of denitrifying methanotrophic bacteria similar to '*Candidatus* Methylomirabilis oxyfera' was described. The bacteria were enriched

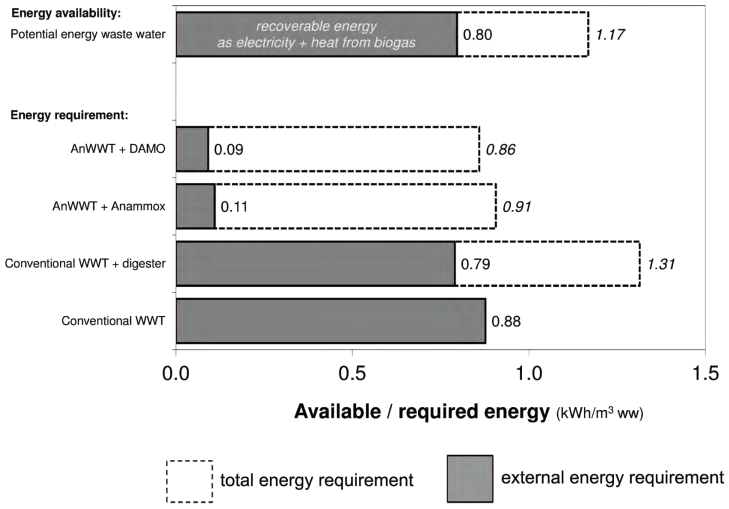

Figure 6.1: Calculated energy requirements for municipal wastewater treatment at 15 °C in different processes. The top bar represents the chemical energy contained in the wastewater (dotted bar) and how much of this can be recovered (grey bar). The dotted bars for the treatment processes represent the total energy requirement; the grey bars indicated the external energy requirement (total energy requirement corrected for on-site energy production from biogas). Anaerobic wastewater treatment (AnWWT) combined with DAMO or anammox, is compared to conventional activated sludge treatment with conventional nitrification-denitrification using an external carbon source (adopted from Hendrickx et al. 2012a).

in two sequencing fed-batch reactors (SFBRs; chapter 2) and two membrane bioreactors (MBRs; chapter 3). The SFBRs were inoculated with freshwater sediment and operated at 30 °C (chapter 2). Only after 6.5 months an increase in denitrification rates was observed. The observed doubling time was long, viz. 1.7–1.9 months. Eventually, maximum volumetric denitrification rates of 34–38 mg NO_2^--N/L d were achieved in a reactor fed with synthetic medium and a reactor fed with medium prepared with 10 % (v/v) effluent from aerobic municipal wastewater treatment, respectively. Although biomass washout was not considered in previous research with similar reactor systems (Luesken et al., 2011b; Ettwig et al., 2009; Hu et al., 2009; Raghoebarsing et al., 2006), analyses of the effluent of the SFBRs confirmed that biomass washout may have significantly hampered enrichment rates. An estimated 41 %–48 % of produced biomass washed out during periods with increasing denitrification rates. Washout may have exceeded growth in periods of decreasing rates, as occurred after 1.5–2 years. It is likely that biomass washout also delayed enrichment rates in previously described reactors.

To facilitate complete biomass retention, and thereby increase enrichment rates and volumetric denitrification rates, two additional enrichments were performed in membrane bioreactors (MBRs; chapter 3). These reactors were inoculated with sludge from municipal wastewater treatment, operated at 20 °C and fed with medium containing 10 % (v/v) effluent from aerobic municipal wastewater treatment. After 10 months of operation, the denitrification rates started to increase and doubling times of 0.9–1.3 months were observed. In one of the MBRs, after spiking with biomass from the effluent of the two SFBRs, a maximum rate of 36 mg NO_2^--N/L d was achieved. In the other reactor a maximum rate of 16 mg NO_2^--N/L d was achieved. The shorter doubling times compared to the SFBRs indicated that by complete biomass retention, the enrichment of denitrifying methanotrophic bacteria can be accelerated. However, biomass retention alone did not result in denitrification rates which were sufficiently high for a practical application. In addition, similar to the SFBRs, also in the MBRs the denitrification rates decreased after 1–1.5 years.

6.3.2 Potential growth factors and inhibition

As stated above, in all enrichments the denitrification rates decreased after 1–2 years (chapters 2 and 3). This could not be explained by biomass washout as it not only occurred in the SFBRs but also in the MBRs. Although it was possible to enhance the denitrification rate in one of the MBRs by addition of biomass from the effluent of the SFBRs, a second addition had little impact. The first addition of biomass from the effluent of the SFBRs may have introduced growth factors for the denitrifying methanotrophic bacteria as it was sampled during periods of increasing denitrification rates in the SFBRs. In addition, it may have contained substrate for (heterotrophic) supporting organisms, viz. organisms that excreted growth factors for denitrifying methanotrophs, or that removed inhibitory intermediates and/or products. The second addition consisted of biomass collected in periods of decreasing denitrification rates and may not have contained such compounds. No effect was observed when a reactor was fed, to supply potential growth factors, with 100 % effluent from an aerobic municipal wastewater treatment plant. Also no effect was observed when trace element concentrations in the influent were increased or when, to provide a potential nitrogen source for assimilation, 4 mg NH_4^+-N/L was added to the medium. Higher rates were observed after addition of increased amounts of copper (381 µg/L instead of 64 µg/L), a cofactor for particulate methane monooxygenase. Possibly by addition of copper, more particulate methane monooxygenase was produced, which may have resulted in increased methane and nitrite removal rates. The denitrification rates also increased when an SFBR in which a membrane was installed for effluent extraction, was operated at a shorter (HRT). The HRT was decreased from 61 d to 1.3 d, after which the denitrification rate rates increased from 8 to 36 mg NO_2^--N/L d. To be able to operate the reactor at a shorter HRT, while applying the same nitrite

loading rate, a lower influent nitrite concentration was applied. However, the trace element addition was not changed. As a result the trace element load was higher and potential inhibiting intermediates and/or products that may have accumulated at the longer HRT were washed out at a higher rate at the shorter HRT. It is not clear if the higher denitrification rates were due to the shorter HRT or increased trace element load.

6.3.3 Effect of temperature on denitrifying methanotrophic activity

Although denitrifying methanotrophs were enriched before, this was mostly carried out at temperature of 25 °C or above; only one enrichment was performed at 20–23 °C (Luesken et al., 2011b). For a practical application to treat the effluent from e.g. a cold upflow anaerobic sludge bed (UASB)-digester system, these microorganisms also need to be able to cope with lower temperatures. Therefore, in this study an enrichment reactor was operated at 20 °C, which is 5–15 °C lower than most reported enrichments. Maximum denitrification rates of 20 and 30 °C cultures, both started with low-temperature inocula, were similar. However, the doubling times in MBRs operated at 20 °C were significantly shorter. If the latter was due to the lower enrichment temperature, or were caused by complete biomass retention remained unknown. The activity of denitrifying methanotrophic bacteria at 11–30 °C was assessed in short-term experiments (chapter 4). These experiments showed that the biomass enriched at 20 °C had an optimum temperature of 20–25 °C. The activity dropped when the temperature was increased to 30 °C. Biomass enriched at 30 °C had an optimum temperature of 25–30 °C.

In winter, wastewater temperatures can be as low as 5–10 °C (Lettinga et al., 2001). The results of the short-term tests indicated that denitrifying methanotrophic bacteria are still active at such low temperatures. However, long term operation at such low temperatures is required. For this purpose, denitrifying methanotrophic bacteria should either be enriched at temperatures below 20 °C, or adaptation of biomass enriched at higher temperatures to lower temperatures should be further investigated.

6.3.4 Effect of oxygen on denitrifying methanotrophic bacteria

Though denitrifying methanotrophic bacteria live under anoxic conditions, they produce oxygen during denitrification and use this oxygen to oxidize methane. It was hypothesized that by supplying oxygen, methane oxidation would proceed faster, and as a result, denitrification and enrichment rates could be enhanced.

In this research, the effect of initial concentrations of 0.7, 1.1 and 2.0 % oxygen (measured in the test bottles) was tested (chapter 5). Denitrifying methanotrophic

activity slightly increased in the presence of 0.7 % O_2 and after oxygen removal the activity returned to its original level. At 1.1 % O_2, methane consumption increased with 118 % and the nitrite removal rate increased with 58 %. After removal of oxygen, methane consumption rate returned to its original level, while nitrite consumption rate only reached 88 % of the level before oxygen addition. 2.0 % O_2 inhibited denitrifying methanotrophic activity. The nitrite removal rate decreased with 60 % and did not recover during the three days following removal of oxygen.

When feeding the continuously operated reactors with 8 % O_2 for 11 d (0.6–1.2 % in the reactors) three out of four denitrifying methanotrophic cultures did not seem to be affected by the presence of oxygen. In another culture, the denitrification rate decreased with 75 %, which may have been caused by oxygen inhibition. Since the biomass contained more than 20 % unidentified bacteria, it is hypothesized that these unidentified bacteria removed most of the surplus oxygen that was not required for methane oxidation by denitrifying methanotrophic bacteria. The presence of a diverse side population able to consume oxygen, limited by the availability of organic substrate, may have been important for reducing a negative effect of oxygen on the denitrifying methanotrophic bacteria. Traces of oxygen that bacteria are likely to be exposed to in wastewater treatment, are therefore not expected to affect the denitrification process.

It is hypothesized that the cyclic behavior observed in the enrichment reactors, viz. an increase of denitrification rates, followed by a decrease after 1–2 years, and eventually increasing rates, was induced by oxygen. Oxygen may have either been inhibiting or limiting. Inhibition could be the result of the increased oxygen production rates, resulting in oxygen accumulation, accompanying increased denitrification rates. On the other hand, the side-population may have competed with denitrifying methanotrophic bacteria for the produced oxygen and thereby have limited the DAMO process. Moreover, depending on the oxygen concentration, intermediates of methane oxidation (e.g. methanol or formaldehyde) may have accumulated (Costa et al., 2001). As a result of accumulation or limitation, denitrification rates decreased. As rates decreased, inhibiting compounds may have been slowly removed by more resistant organisms or washed out, or oxygen consuming organisms decreased in numbers due to oxygen limitation. Hereafter, the denitrification rates could increase again. Further studies should identify an optimal oxygen concentration for denitrifying methanotrophic bacteria. Inhibiting oxygen concentrations may be prevented by addition of an oxygen-reducing compound or a substrate, e.g. a carbon source, for aerobic organisms. If oxygen is limiting, intermittent microaerobic and anaerobic conditions may be applied to increase the denitrifying methanotrophic rates.

6.4 Prospects and recommendations for further research

The progress in research on denitrifying methanotrophic bacteria is limited due to the low growth rate and the limited number of enrichment cultures that is available (Hu et al., 2009)). However, gradually more information becomes available that may lead to application of denitrifying methanotrophic processes in (municipal) wastewater treatment.

6.4.1 Growth factor requirements and inhibition

After 1–2 years, in all four enrichment reactor a decrease in denitrification activity was observed . The reason for this is still not found. The present research indicated that growth factor limitation, accumulation of a toxic intermediate or product, or the lack of substrate for a heterotrophic organisms excreting growth factors or removing inhibiting compounds may have resulted in decreased activity.

The results indicated that copper, a cofactor for methane monooxygenase catalyzing methane oxidation, is a limiting growth factor. When the copper concentration in the influent was increased, the denitrification rate increased. Neither copper speciation nor its bioavailability were determined, however, these should be taken into account in further research. Long-term tests are required to evaluate if copper addition can stimulate the denitrification rate and to determine the optimum copper concentration to achieve this. No other potential growth factors have been identified. Addition of effluent from municipal wastewater treatment which may contain growth factors, at this stage, did not result in higher rates.

The denitrification rate also increased when the HRT was decreased. It should be tested if this was due to an increased supply of trace elements, or due to washout of inhibiting compounds. To test if the increase was due to increased trace element supply rates, a higher trace element load can be applied, at a long HRT. To determine if the increased rates were due to washout of inhibiting compounds, trace element load should not be increased at a decreased HRT. After decreasing the HRT, the rates increased to a rate similar to the maximum volumetric rate that was observed before. Prolonged reactor operation at a short HRT is required to see if the rates increase beyond the previously observed maximum of 36 mg NO_2^--N/L d.

6.4.2 Operational temperature

The maximum denitrification rates in the reactors operated at 20 and 30 °C were similar. In short-term batch tests at a temperature of 11 °C denitrification activity was still observed, albeit 73 resp. 76 % lower than in the reactors at 20 and 30 °C. This is promising for a practical application, because the temperature of municipal wastewater in temperate zones can be as low as 5–10 °C (Lettinga et al., 2001). However,

this is only possible if sufficiently high biomass concentrations can be maintained, i.e. if a high biomass retention is possible at low temperatures (section 6.4.4). In the short-term tests a strong decrease in activity took place when the temperature of the 20 °C enrichment was increased to 30 °C. The effect of a more gradual change of temperature, more representative of changes in wastewater temperatures during the year, should be tested. Moreover, enrichments should be performed at temperatures below 20 °C and the temperature of existing enrichments should be decreased, to determine the best startup strategy for municipal wastewater treatment in temperate zones.

6.4.3 The role of oxygen in denitrifying methanotrophic bacteria

The denitrification rate increased when biomass from an enrichment reactor was exposed to 1.1 % O_2. To study if it is possible to achieve higher denitrification rates, oxygen should be added to an enrichment culture intermittently, undesired growth of aerobic methanotrophs and nitrite oxidizing bacteria should be avoided.

6.4.4 Achieving improved biomass retention

The enrichment of slow growing bacteria, such as denitrifying methanotrophic bacteria, requires efficient biomass retention. Experience with the anammox process has taught that, as long as biomass is retained efficiently, low growth rates do not have to be a problem. Moreover, slow growth rates can even be beneficial since less sludge has to be disposed (Shen et al., 2012). Even though at this stage other factors seem to limit the denitrification rate, further research should consider growth of denitrifying methanotrophic bacteria in flocs, granules or biofilms. Using retention-based systems, biomass washout is minimized and high biomass concentrations can be obtained, as a result of which high volumetric denitrification rates can be achieved. In this research, complete biomass retention was achieved using MBRs. However, even though MBRs are applied for treatment of municipal wastewater (Liao et al., 2006), investment and operational costs are high (Melin et al., 2006) and therefore application of an MBR is not a preferred option. Instead, growth of denitrifying methanotrophic bacteria in a biofilm or as granules would be preferred.

In the enrichment reactors considerable biofilm formation on the reactor walls was observed. Shi et al. (2013) operated a membrane biofilm reactor, with, amongst other organisms, denitrifying methanotrophic bacteria. Such a system, in which gaseous methane is supplied by a membrane, is not applicable for treatment of effluent of low-temperature anaerobic municipal wastewater treatment because dissolved methane needs to be removed. It should be studied if biomass can be grown on suspended or fixed carrier material, e.g. in a moving bed biofilm reactor or a rotating biological contactor. Both have successfully been applied for anammox (Christensson et al.,

2013; Van Hulle et al., 2010). Alternatively, the possibility to grow the denitrifying methanotrophs in granules should be studied.

There are many theories that explain the formation of granules (Hulshoff Pol et al., 2004). One common theory is that to grow biomass in granules a selection pressure, viz. a high upflow velocity and/or gas loading rate, has to be applied. As an example, in a UASB reactor for removal of organic substrate from municipal wastewater, successful granulation was obtained at an upflow velocity of 0.5–2 m/s (Francese et al., 1998). At such high rates, light sludge flocs will washout, while only heavier particles, such as sludge granules, can be retained. Other theories include that granules are formed upon colonization of suspended solids from the influent or an inert carrier that is supplied to the reactor. Biofilm formation follows the same principles (Hulshoff Pol et al., 2004). Small granules and thin biofilms are required for diffusion of methane to the denitrifying methanotrophic bacteria.

6.4.5 Development of a denitrifying methanotrophic reactor

The present research, results reported by Luesken et al. (2011b) and by Hu et al. (2009), have shown that denitrifying methanotrophic bacteria can be enriched from (municipal) wastewater treatment sludge, which therefore can be used as an inoculum for starting a DAMO reactor. Assuming a typical nitrogen concentration of 50 mg N/L, 21 mg CH_4/L is required for denitrification. This means that the effluent of a low temperature anaerobic municipal wastewater treatment reactor contains (almost) enough (viz. 15–86.5 mg/L) dissolved methane to drive denitrification. The concentration of methane in the effluent of anaerobic treatment should not just be calculated, based on Henry's law because this could underestimate the concentration of dissolved methane. Instead, concentrations should be determined from mass balances or, preferably, should be measured. This is important because this concentration determines if biogas should be added (during the summer period) to obtain sufficient denitrification or if (during the winter) additional removal of dissolved methane is required. At the maximum denitrification rate of 38 mg NO_2^--N/L d, an HRT of 1.3 d would be required. Conventional denitrification typically has an HRT of 3–4 h. Thus, for a practical application of denitrification coupled to anaerobic methane oxidation for sewage treatment, denitrification rate needs to be increased by an order of magnitude.

To supply DAMO bacteria with nitrite, a nitritation step is required. At low temperatures this can be accomplished either at low oxygen concentrations or by aeration duration control. A one-stage nitritation-DAMO process in which nitritation, DAMO, and possibly aerobic oxidation of excess dissolved methane are combined, would be preferred over a two-stage process. In such a system, the denitrifying methanotrophic bacteria should be located in the anoxic interior of a granule or biofilm. The ammonium oxidizing bacteria are located on the oxic outside of the granule or

biofilm. However, methane should be transferred from the oxic bulk liquid to the anoxic interior of the granule or biofilm. This implies that aerobic methane oxidation probably will take place and insufficent methane may be available for methanotrophic denitrification. A two-stage process. in which nitrite is recirculated from a nitritation reactor seems to be a better alternative although relatively high recirculation rates need to be applied to obtain sufficient nitrogen removal. This effluent recirculation requires energy and with the effluent traces of oxygen are fed to the DAMO process. Although short-term exposure to $\leq 1.1\,\%$ O_2 oxygen or lower did not negatively affect denitrifying methanotrophic bacteria, this should be verified in long-term reactor operation. Moreover, the effect of ammonium, entering the DAMO reactor with the anaerobic effluent, on a DAMO process should be determined. In presence of ammonium and nitrite, anammox bacteria are expected to grow in a DAMO reactor. If a significant part of nitrogen is removed by anammox, dissolved methane may remain in the effluent. An advantage of the two-stage system is that excess methane (in winter time) will be removed in the nitritation reactor by aerobic methanotrophs.

In conclusion, before a demonstration scale DAMO reactor can be designed and applied, many research questions remain to be answered. However, similar problems have successfully been overcome for slow-growing anammox bacteria and currently more than 30 full-scale anammox plants are in operation (Vlaeminck et al., 2012). Once these research questions have been answered, a treatment concept consisting of a UASB-sludge digester, a DAMO reactor for denitrification coupled to anaerobic methane oxidation and a nitritation step, either separate or integrated with the denitrification, offers opportunities for low temperature energy-neutral or producing municipal wastewater treatment.

Summary

In response to climate change and fossil fuel depletion, there is a trend towards energy self-sufficient municipal wastewater treatment. This can be achieved by anaerobic treatment, with, for example, an upflow anaerobic sludge bed (UASB) reactor and a sludge digester. However, anaerobic treatment does not remove nitrogen. Moreover, when applying anaerobic municipal wastewater treatment at low temperatures, the effluent contains a high concentration of dissolved methane. Both nitrogen and methane have to be removed from the effluent, since nitrogen causes eutrophication and methane has a high global warming potential. A denitrifying methanotrophic process would simultaneously solve these two problems associated with anaerobic treatment. Such a process could be applied in a new concept for low-temperature anaerobic municipal wastewater treatment, consisting of a UASB-digester system, a reactor for denitrification coupled to anaerobic methane oxidation (DAMO), and a nitritation reactor to supply the denitrifiers with nitrite.

In this research denitrifying methanotrophic bacteria similar to '*Candidatus* Methylomirabilis oxyfera' were enriched from freshwater sediment, using sequencing fed-batch reactors (SFBRs) operated at 30 °C. Maximum denitrification rates of 33.5–37.8 mg NO_2^--N/L d were achieved after 12–15 months of enrichment. These rates should be increased by an order of magnitude before application in wastewater treatment becomes possible. However, after reaching these maximum rates, denitrifcation rates decreased. Results indicated that biomass washout may have significantly decelerated enrichment. To obtain higher volumetric consumption rates, further research focussed on systems with complete biomass retention.

Membrane bioreactors (MBRs) with complete biomass retention were inoculated with wastewater sludge and operated at 20 °C. Shorter doubling times were achieved in these MBRs than in the SFBRs. After 14–15 months of enrichment, maximum denitrification rates of 16–36 mg NO_2^--N/L d were reached. The highest rate of 36 mg NO_2^--N/L d was observed in a MBR to which biomass from the effluent of the SFBRs was added. This biomass sample may have contained growth factors for denitrifying methanotrophic bacteria, which were missing in the other reactor. The maximum rates were similar to the maximum rates observed for the SFBRs and the maximum rates reported in literature. Thus, higher denitrification rates could not be achieved by strict biomass retention using membranes alone. Moreover, after reaching a maximum, also in the MBRs a decrease in activity was observed. The cause remains unknown. Rates increased after addition of copper and operating a membrane bioreactor at shorter hydraulic retention times. To achieve higher dentirification rates, further research should focus on long-term effects of copper addition and operation at hydraulic retention times in the order of hours using membrane bioreactors.

In short-term batch tests the activity of denitrifying methanotrophic bacteria at 11–30 °C was tested. This study showed that biomass enriched at 20 °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. At a temperature of 11 °C denitrification activity was still observed, albeit 73 resp. 76 % lower than in the reactors at 20 and 30 °C. The effect of a more gradual change of temperature, more representative of changes in wastewater temperatures during the year, should be studied. 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.

The slow growing *M. oxyfera*-like bacteria produce oxygen from nitrite. Rates of oxygen dependent reactions in these bacteria may be increased by addition of oxygen. As a result, enrichment rates may be increased. Moreover, in wastewater treatment denitrifying methanotrophic bacteria are likely to be exposed to traces of oxygen. When reactors were fed with 8.0 % O_2, reactor concentrations decreased to 0.6 to 1.2 % O_2. Enrichments contained maximally 80 % denitrifying methanotrophic bacteria, the side-population may have consumed the oxygen. Three out of four denitrifying methanotrophic cultures did not seem influenced by the presence of oxygen. In another culture denitrification rates decreased with 75 %. In addition, the effect of 0.7, 1.0 and 2.0 % O_2 was tested. In presence of 0.7 % O_2 denitrifying methanotrophic activity slightly increased and returned to its original level after oxygen had been removed. Therefore, traces of oxygen that bacteria are likely to be exposed to in wastewater treatment are not expected to affect the denitrification process. At 1.1 % O_2, methane consumption increased with 118 %, nitrite consumption rate increased with 58 %. After removal of oxygen, methane consumption rate fully recovered, nitrite consumption rate returned to 88 %. 2.0 % O_2 inhibited denitrifying methanotrophic activity. Nitrite consumption rate decreased with 60 % and did not recover after removal of oxygen. It was hypothesized that the cyclic trend in denitrication rates observed in all enrichment reactors may be induced by oxygen. Further research should study the optimal oxygen concentrations for denitrifying methanotrophic bacteria and will determine if oxygen removal should be facilitated, or if oxygen should be added, to increase denitrifying methanotrophic rates.

Although, before a demonstration scale DAMO reactor can be designed and applied, many research questions remain to be answered, a treatment concept consisting of a UASB-sludge digester, a DAMO reactor for denitrification coupled to anaerobic methane oxidation and a nitritation step, either separate or integrated with the denitrification, offers opportunities for low temperature energy-neutral or producing municipal wastewater treatment.

Samenvatting

Er is, in reactie op klimaatverandering en uitputting van de voorraad fossiele brandstoffen, een trend naar energie-zelfvoorzienende zuivering van huishoudelijk afvalwater. Dit kan bereikt worden met gebruik van, bijvoorbeeld, een upflow anaerobic sludge bed (UASB) reactor en een slibvergister. Anaerobe afvalwaterzuivering verwijdert echter geen stikstof. Wanneer anaerobe zuivering van huishoudelijk afvalwater wordt toegepast bij lage temperaturen bevat het effluent naast stikstof een hoge concentratie opgelost methaan. Zowel stikstof als methaan moeten uit het effluent verwijderd worden, aangezien stikstof eutroficatie veroorzaakt en methaan een sterk broeikasgas is. Een denitrificerend methanotroof proces zou deze twee problemen gerelateerd aan anaerobe behandeling tegelijkertijd kunnen oplossen. Een dergelijk proces zou toegepast kunnen worden in een nieuw concept voor anaerobe zuivering van huishoudelijk afvalwater bij lage temperaturen, bestaande uit een UASB-vergister, een reactor voor denitrificatie gekoppeld aan anaerobe methaanoxidatie (DAMO) en een nitritatiereactor om de denitrificeerders van nitriet te voorzien.

In dit onderzoek zijn denitrificerende methanotrofe bacteriën gelijkend op '*Candidatus* Methylomirabilis oxyfera' verrijkt uit zoetwatersediment, in sequencing fed-batch reactoren (SFBRs) bedreven bij 30 °C. Maximale denitrificatiesnelheden van 33.5–37.8 mg NO_2^--N/L d werden bereikt na 12–15 maanden verrijking. Deze snelheden moeten met een ordegrootte verhoogd worden voordat praktische toepassing in afvalwaterzuivering haalbaar is. Na het bereiken van deze maximumsnelheden namen de denitrificatiesnelheden echter af. De resultaten duidden erop dat biomassauitspoeling de verrijking significant kan hebben vertraagd. Om hogere volumetrische consumptiesnelheden te bereiken zijn in het vervolgonderzoek systemen met complete biomassaretentie bestudeerd.

Membraanbioreactoren (MBRs) met complete biomassaretentie zijn geënt met afvalwaterzuiveringsslib en bedreven bij 20 °C. In deze MBRs werden kortere verdubbelingstijden bereikt dan in de SFBRs. Na 14–15 maanden verrijking werden maximale denitrificatiesnelheden van 16–36 mg NO_2^--N/L d bereikt. De hoogste waarde van 36 mg NO_2^--N/L d werd gemeten in een MBR die was aangeënt met biomassa uit het effluent van de SFBRs. Deze biomassa zou groeifactoren voor denitrificerende methanotrofe bacteriën bevat kunnen hebben, welke ontbraken in de andere reactor. De maximumsnelheden waren vergelijkbaar met de maximumsnelheden waargenomen in de SFBRs en de maximumsnelheden gerapporteerd in de literatuur. Dus, hogere denitrificatiesnelheden konden niet bereikt worden door enkel strikte biomassaretentie door middel van membranen. Daarnaast, nadat een maximum bereikt werd, nam ook in de MBRs de activiteit af. De oorzaak hiervan is onbekend. De snelheden namen weer toe na toediening van koper aan het influent en bij het bedrijven van een reactor met kortere hydraulische verblijftijden. Om hogere denitrificatiesnelheden te bereiken dient toekomstig onderzoek zich te richten op lange termijn effecten van

kopertoediening en kortere hydraulische retentietijd in de orde van enekle uren met gebruik van MBRs.

In batchtests is gedurende enkele dagen de activiteit van denitrificerende methanotrofe bacteriën bij 11–30 °C gemeten. Dit onderzoek liet zijn dat biomassa verrijkt bij 20 °C een optimumtemperatuur van 20–25 °C had en dat de activiteit sterk afnam bij het verhogen van de temperatuur tot 30 °C. Biomassa verrijkt bij 30 °C had een optimum temperatuur van 25–30 °C. Bij een temperatuur van 11 °C werd nog denitrificatie waargenomen, de snelheid was echter 73 resp. 76 % lager dan in tests met een temperatuur van 20 and 30 °C. Het effect van een geleidelijker temperatuurverandering, representatiever voor de schommelingen in afvalwatertemperatuur gedurende het jaar, dient onderzocht te worden. Deze resultaten duiden aan dat biomassa verrijkt uit entmateriaal afkomstig van lage temperaturen zich heeft aangepast aan de verrijkingstemperatuur en dat daarom lage temperatuurverrijking het meest geschikt is voor toepassingen in afvalwaterzuivering bij lage temperatuur.

De langzaam groeiende *M. oxyfera* bacteriën produceren zuurstof uit nitriet. Snelheden van zuurstofafhankelijke reacties in deze bacteriën zouden mogelijk verhoogd kunnen worden door de toediening van zuurstof. Als gevolg hiervan zouden verrijkingssnelheden verhoogd kunnen worden. Daarnaast is het waarschijnlijk dat denitrificerende methanotrofe bacteriën tijdens afvalwaterzuivering worden blootgesteld aan sporen lage zuurstofconcentraties. Wanneer 8.0 % O_2 werd toegediend aan reactoren namen reactorconcentraties af tot 0.6 to 1.2 % O_2. De verrijkingsculturen bevatten maximaal 80 % denitrificerende methanotrofe bacteriën, de rest van de populatie zou de zuurstof geconsumeerd kunnen hebben. Drie van de vier denitriferende methanotrofe verrijkingen schenen niet beïnvloed te zijn door de aanwezigheid van zuurstof. In een andere verrijking nam de activiteit af met 75 %. Daarnaast werd het effect van 0.7, 1.0 and 2.0 % O_2 getest. In aanwezigheid van 0.7 % O_2 nam de denitriferende methanotrofe activiteit licht toe en keerde na de verwijdering van zuurstof terug naar het niveau van voor de zuurstofblootstelling. Sporen van zuurstof aan welke de bacteriën gedurende afvalwaterzuivering kunnen worden blootgesteld hebben naar verwachting geen effect op het denitrificatieproces. Bij 1.1 % O_2, nam de methaanconsumptiesnelheid toe met 118 %, de nitrietconsumptiesnelheid nam toe met 58 %. Na zuurstofverwijdering keerde de methaanconsumptie terug tot het niveau van voor zuurstofblootstelling, nitrietconsumptiesnelheden herstelden tot 88 %. 2.0 % O_2 remde
denitrificerende methanotrofe activiteit. De nitrietconsumptiesnelheid nam af met 60 % en herstelde niet volledig na zuurstofverwijdering. De hypothese is dat de cyclische trend
waargenomen in denitrificatiesnelheden in alle verrijkingsreactoren veroorzaakt werd door zuurstof. In toekomstig onderzoek dient de optimale zuurstofconcentratie voor denitrificerende methanotrofe bacteriën bepaald te worden en moet aanwijzingen

geven of zuurstofverwijdering of zuurstoftoediening nodig is om denitriferende methanotrofe activiteit te verhogen.

Voordat een demonstratieschaal DAMO reactor ontworpen en toegepast kan worden dienen nog veel onderzoeksvragen beantwoord te worden,toch biedt een zuiveringsconcept bestaande uit een UASB-sludge vergister, een DAMO-reactor voor denitrificatie gekoppeld aan anaeorbe methaanoxidatie en een nitritatiestap, gescheiden of geïntegreerd met de denitrificatie kansen voor energie-neutrale of energie-producerende zuivering van huishoudelijk afvalwater bij lage temperaturen.

Bibliography

Ahn, Y.H. Sustainable nitrogen elimination biotechnologies: A review. *Process Biochemistry*, 41(8):1709–1721, 2006.

Akanyeti, I., Temmink, H., Remy, M., and Zwijnenburg, A. Feasibility of bioflocculation in a high-loaded membrane bioreactor for improved energy recovery from sewage. *Water Science and Technology*, 61(6):1433–1439, 2010.

Álvarez, J.A., Armstrong, E., Presas, J., Gómez, M., and Soto, M. Performance of a UASB-digester system treating domestic wastewater. *Environmental Technology*, 25(10):1189–1199, 2004.

Álvarez, J.A., Armstrong, E., Gómez, M., and Soto, M. Anaerobic treatment of low-strength municipal wastewater by a two-stage pilot plant under psychrophilic conditions. *Bioresource Technology*, 99(15):7051–7062, 2008.

Angenent, L.T., Karim, K., Al-Dahhan, M.H., and Dom'iguez-Espinosa, R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends in Biotechnology*, 22(9):477–485, 2004.

APHA, AWWA, and WEF. *Standard Methods for the Examination of Water and Wastewater*. 20^{th} edition, 1998.

Blackburne, R., Yuan, Z., and Keller, J. Demonstration of nitrogen removal via nitrite in a sequencing batch reactor treating domestic wastewater. *Water Research*, 42 (8-9):2166–2176, 2008.

CBS. Centraal Bureau voor de Statistiek, Statline, www.cbs.nl.

Chernicaro, C.A.L. Post-treatment options for the anaerobic treatment of domestic wastewater. *Reviews in Environmental Science and Bio/Technology*, 5:73–92, 2006.

Christensson, M., Ekstrom, S., Chan, A. A., Le Vaillant, E., and Lemaire, R. Experience from start-ups of the first ANITA Mox plants. *Water Science and Technology*, 67(12):2677–2684, 2013.

Collins, M.L.P., Buchholz, L.A., and Remsen, C.C. Effect of copper on *Methylomonas albus BG8*. *Applied and Environmental Microbiology*, 57(4):1261–1264, 1991.

Cookney, J., McAdam, E.J., Cartmell, E., and Jefferson, B. Recovery of methane from anaerobic process effluent using poly-di-methyl-siloxane membrane contactors. *12^{th} World Congress on Anaerobic Digestion*, 2010.

Costa, C., Vecherskaya, M., Dijkema, C., and Stams, A.J.M. The effect of oxygen on methanol oxidation by an obligate methanotrophic bacterium studied by in vivo

c-13 nuclear magnetic resonance spectroscopy. *Journal of Industrial Microbiology & Biotechnology*, 26(1-2), 2001.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., and Wagner, M. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*, 22(3):434–444, 1999.

Elmitwalli, T.A., Oahn, K.L.T., Zeeman, G., and Lettinga, G. Treatment of domestic sewage in a two-step anaerobic filter/anaerobic hybrid system at low temperature. *Water Research*, 36(9):2225–2232, 2002.

Ettwig, K.F., Shima, S., Pas-Schoonen, K.T. Van de, Kahnt, J., Medema, M.H., Op den Camp, H.J.M., Jetten, M.S.M., and Strous, M. Denitrifying bacteria anaerobically oxidize methane in the absence of *Archaea*. *Environmental Microbiology*, 10(11):3164–3173, 2008.

Ettwig, K.F., Van Alen, T., Pas-Schoonen, K.T. Van de, Jetten, M.S.M., and Strous, M. Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. *Applied and Environmental Microbiology*, 75(11):3656–3662, 2009.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., Schreiber, F., Dutilh-B.E., Zedelius, J., De Beer, D., Gloerich, J., Wessels, H.J.C.T., Van Alen, T., Luesken, F., Wu, M.L., Pas-Schoonen, K.T. Van de, Op den Camp, H.J.M., Janssen-Megens, E.M., Francoijs, K.-J., Stunnenberg, H., Weissenbach, J., Jetten, M.S.M., and Strous, M. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature*, 464(7288):543–548, 2010.

Foster, J.W. and Davis, R.H. A methane-dependent coccus with notes of classification and nomenclature of obligate methane-utilizing bacteria. *Journal of Bacteriology*, 91(5):1924–1931, 1966.

Francese, A., Cordoba, P., Duran, J., and Sineriz, F. High upflow velocity and organic loading rate improve granulation in upflow anaerobic sludge blanket reactors. *World Journal of Microbiology & Biotechnology*, 14(3):337–341, 1998.

Ginige, M.P., Bowyer, J.C., Foley, L., Keller, J., and Yuan, Z. A comparative study of methanol as a supplementary carbon source for enhancing denitrification in primary and secondary anoxic zones. *Biodegradation*, 20(2):221–234, 2009.

Graham, D.W., Chaudhary, J.A., Hanson, R.S., and Arnold, R.G. Factors affecting competition between Type-I and Type-II methanotrophs in 2-organism, continuous-flow reactors. *Microbial Ecology*, 25(1):1–17, 1993.

Hanaki, K., Wantawin, C., and Ohgaki, S. Nitrification at low-levels of dissolved-oxygen with and without organic loading in a suspended-growth reactor. *Water Research*, 24(3):297–302, 1990.

Hanson, R.S. and Hanson, T.E. Methanotrophic bacteria. *Microbiological Reviews*, 60(2):439–471, 1996.

Haroon, M.F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z., and Tyson, G.W. Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature*, 500(7464):567–570, 2013.

Hartley, K. and Lant, P. Eliminating non-renewable CO_2 emissions from sewage treatment: An anaerobic migrating bed reactor pilot plant study. *Biotechnology and Bioengineering*, 95(3):384–398, 2006.

Hartree, E.F. Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Analytical Biochemistry*, 48(2):422–427, 1972.

He, Z., Cai, C., Geng, S., Lou, L., Xu, X., Zheng, P., and Hu, B. Modeling a nitrite-dependent anaerobic methane oxidation process: Parameters identification and model evaluation. *Bioresource Technology*, 147:315–320, 2013.

Hellinga, C., Schellen, A.A.J.C., Mulder, J.W., Van Loosdrecht, M.C.M., and Heijnen, J.J. The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water. *Water Science and Technology*, 37(9):135–142, 1998.

Hendrickx, T.L.G., Kampman, C., Zhang, L., Zeeman, G., Temmink, H., and Buisman, C.J.N. Energy balance for direct anaerobic wastewater treatment. 2012a.

Hendrickx, T.L.G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., and Buisman, C.J.N. Autotrophic nitrogen removal from low strength waste water at low temperature. *Water Research*, 46(7):2187–2193, 2012b.

Hendrickx, T.L.G., Kampman, C., Zeeman, G., Temmink, H., Hu, Z., Kartal, B., and Buisman, C.J.N. Enrichment of fresh water anammox at 10 °C. *Bioresource Technology*, accepted, 2014. doi: 10.1016/j.biortech.2014.04.025.

Houbron, E., Torrijos, M., and Capdeville, B. An alternative use of biogas applied at the water denitrification. *Water Science and Technology*, 40(8):115–122, 1999.

Hu, S., Zeng, R.J., Burow, L.C., Lant, P., Keller, J., and Yuan, Z. Enrichment of denitrifying anaerobic methane oxidizing microorganisms. *Environmental Microbiology Reports*, 1(5):377–384, 2009.

Hu, S., Zeng, R.J., Keller, J., Lant, P. A., and Yuan, Z. Effect of nitrate and nitrite on the selection of microorganisms in the denitrifying anaerobic methane oxidation process. *Environmental Microbiology Reports*, 3(3):315–319, 2011.

Hu, Z., Lotti, T., De Kreuk, M., Kleerebezem, R., Van Loosdrecht, M., Kruit, J., Jetten, M.S.M., and Kartal, B. Nitrogen removal by a nitritation-anammox bioreactor at low temperature. *Applied and Environmental Microbiology*, 79(8):2807–2812, 2013.

Hulshoff Pol, L.W., Castro Lopes, S.I. de, Lettinga, G., and Lens, P.N.L. Anaerobic sludge granulation. *Water Research*, 38(6):1376–1389, 2004.

IPCC. *Climate change 2007: the physical science basis. Contribution of Working Group I to the fourth assessment report of the Intergovernmental Panel on Climate Change*. 2007.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.-P., and Wagner, M. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Applied and Environmental Microbiology*, 64(8):3042–3051, 1998.

Kampman, C., Hendrickx, T.L.G., Luesken, F.A., Van Alen, T.A., Op den Camp, H.J.M., Jetten, M.S.M., Zeeman, G., Buisman, C.J.N., and Temmink, H. Enrichment of denitrifying methanotrophic bacteria for application after direct low-temperature anaerobic sewage treatment. *Journal of Hazardous Materials*, 227: 164–171, 2012.

Kampman, C., Temmink, H., Hendrickx, T.L.G., Zeeman, G., and Buisman, C.J.N. Enrichment of denitrifying methanotrophic bacteria from municipal wastewater sludge in a membrane bioreactor at 20 °C. *Journal of Hazardous Materials*, 2014. doi: 10.1016/j.jhazmat.2014.04.031.

Kartal, B., Kuenen, J.G., and Loosdrecht, M.C.M. van. Sewage treatment with anammox. *Science*, 328(5979):702–703, 2010.

Kim, D.J., Chang, J.S., Lee, D.I., Han, D.W., Yoo, I.K., and Cha, G.C. Nitrification of high strength ammonia wastewater and nitrite accumulation characteristics. *Water Science and Technology*, 47(11):45–51, 2003.

Kornaros, M., Marazioti, C., and Lyberatos, G. A pilot scale study of a sequencing batch reactor treating municipal wastewater operated via the UP-PND process. *Water Science and Technology*, 58(2):435–438, 2008.

Kuntke, P., Smiech, K.M., Bruning, H., Zeeman, G., Saakes, M., Sleutels, T.H.J.A., Hamelers, H.V.M., and Buisrnan, C.J. N. Ammonium recovery and energy production from urine by a microbial fuel cell. *Water Research*, 46(8):2627–2636, 2012.

Lema, J.M. and Omil, F. Anaerobic treatment: A key technology for a sustainable management of wastes in Europe. *Water Science and Technology*, 44(8):133–140, 2001.

Lettinga, G. Anaerobic digestion and wastewater treatment systems. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 67(1): 3–28, 1995.

Lettinga, G. and Hulshoff-Pol, L.W. UASB-process design for various types of wastewaters. *Water Science and Technology*, 24(8):87–107, 1991.

Lettinga, G., Rebac, S., and Zeeman, G. Challenge of psychrophilic anaerobic wastewater treatment. *Trends in Biotechnology*, 19(9):363–370, 2001.

Li, C. and Fang, H.H.P. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Critical Reviews in Environmental Science and Technology*, 37(1):1–39, 2007.

Liao, B., Kraemer, J.T., and Bagley, D.M. Anaerobic membrane bioreactors: Applications and research directions. *Critical Reviews in Environmental Science and Technology*, 36(6):489–530, 2006.

Lofrano, G. and Brown, J. Wastewater management through the ages: A history of mankind. *Science of the Total Environment*, 408(22):5254–5264, 2010.

Luesken, F.A., Sáchez, J., Alen, T.A. van, Sanabria, J., Op den Camp, H.J.M., Jetten, M.S.M., and Kartal, B. Simultaneous nitrite-dependent anaerobic methane and ammonium oxidation processes. *Applied and Environmental Microbiology*, 77 (19):6802–6807, 2011a.

Luesken, F.A., Van Alen, T.A., Biezen, E. Van der, Frijters, C., Toonen, G., Kampman, C., Hendrickx, T.L.G., Zeeman, G., Temmink, H., Strous, M., Op den Camp, H.J.M., and Jetten, M.S.M. Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. *Applied Microbiology and Biotechnology*, 92(4):845–854, 2011b.

Luesken, F.A., Wu, M.L., Op den Camp, H.J.M., Keltjens, J.T., Stunnenberg, H., Francoijs, K.-J., Strous, M., and Jetten, M.S.M. Effect of oxygen on the anaerobic methanotroph '*Candidatus* Methylomirabilis oxyfera': Kinetic and transcriptional analysis. *Environmental Microbiology*, 14(4):1024–1034, 2012.

Mahmoud, N. High strength sewage treatment in a UASB reactor and an integrated UASB-digester system. *Bioresource Technology*, 99(16):7531–7538, 2008.

Mahmoud, N., Zeeman, G., Gijzen, H., and Lettinga, G. Anaerobic sewage treatment in a one-stage UASB reactor and a combined UASB-digester system. *Water Research*, 38(9):2347–2357, 2004.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.H. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria - Problems and solutions. *Systematic and Applied Microbiology*, 15(4), 1992.

Melin, T., Jefferson, B., Bixio, D., Thoeye, C., De Wilde, W., De Koning, J., Graaf, J. Van der, and Wintgens, T. Membrane bioreactor technology for wastewater treatment and reuse. *Desalination*, 187(1-3):271–282, 2006.

Meulepas, R.J.W., Jagersma, C.G., Gieteling, J., Buisman, C.J.N., Stams, A.J.M., and Lens, P.N.L. Enrichment of anaerobic methanotrophs in sulfate-reducing membrane bioreactors. *Biotechnology and Bioengineering*, 104(3):458–470, 2009.

Modin, O., Fukushi, K., and Yamamoto, K. Denitrification with methane as external carbon source. *Water Research*, 41(12):2726–2738, 2007.

Modin, O., Fukushi, K., Nakajima, F., and Yamamoto, K. A membrane biofilm reactor achieves aerobic methane oxidation coupled to denitrification (AME-D) with high efficiency. *Water Science and Technology*, 58(1):83–87, 2008.

Mulder, A. The quest for sustainable nitrogen removal technologies. *Water Science and Technology*, 48(1):67–75, 2003.

Murrell, J.C. and Jetten, M.S.M. The microbial methane cycle. *Environmental Microbiology Reports*, 1(5):279–284, 2009.

Neef, A. Anwendung der In-situ-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen, 1997.

Peng, Y. and Zhu, G. Biological nitrogen removal with nitrification and denitrification via nitrite pathway. *Applied Microbiology and Biotechnology*, 73(1):15–26, 2006.

Rabaey, K. and Verstraete, W. Microbial fuel cells: novel biotechnology for energy generation. *Trends in Biotechnology*, 23(6):291–298, 2005.

Raghoebarsing, A.A., Pol, A., Pas-Schoonen, K.T. Van de, Smolders, A.J.P., Ettwig, K.F., Rijpstra, W.I.C., Schouten, S., Sinninghe Damsté, J.S., Camp, H.J.M. Op den, Jetten, M.S.M., and Strous, M. A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature*, 440(7086):918–921, 2006.

Rozendal, R.A., Hamelers, H.V.M., Euverink, G.J.W., Metz, S.J., and Buisman, C.J.N. Principle and perspectives of hydrogen production through biocatalyzed electrolysis. *International Journal of Hydrogen Energy*, 31(12):1632–1640, 2006.

Salehizadeh, H. and Van Loosdrecht, M.C.M. Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. *Biotechnology Advances*, 22(3):261–279, 2004.

Seghezzo, L., Zeeman, G., Van Lier, J.B., Hamelers, H.V.M., and Lettinga, G. A review: The anaerobic treatment of sewage in UASB and EGSB reactors. *Bioresource Technology*, 65(3):175–190, 1998.

Shen, L., He, Z., Zhu, Q., Chen, D., Lou, L., Xu, X., Zheng, P., and Hu, B. Microbiology, ecology and application of the nitrite-dependent anaerobic methane oxidation process. *Frontiers in Microbiology*, 3(269):1–5, 2012.

Shen, L., Liu, S., Zhu, Q., Li, X., Cai, C., Cheng, D., Lou, L., Xu, X., Zheng, P., and Hu, B. Distribution and diversity of nitrite-dependent anaerobic methane-oxidising bacteria in the sediments of the Qiantang River. *Microbial Ecology*, pages 1–9, 2013.

Shi, Y., Hu, S., Lou, J., Lu, P., Keller, J., and Yuan, Z. Nitrogen removal from wastewater by coupling anammox and methane-dependent denitrification in a membrane biofilm reactor. *Environmental Science & Technology*, 47(20):11577–11583, 2013.

Siddiquee, M.N. and Rohani, S. Lipid extraction and biodiesel production from municipal sewage sludges: A review. *Renewable & Sustainable Energy Reviews*, 15 (2):1067–1072, 2011.

Strous, M., Kuenen, J.G., and Jetten, M.S.M. Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology*, 65(7):3248–3250, 1999.

Sun, S., Nàcher, C. Pellicer i, Merkey, B., Zhou, Q., Xia, S., Yang, D., Sun, J., and Smets, B.F. Effective biological nitrogen removal treatment processes for domestic wastewaters with low C/N ratios: A review. *Environmental Engineering Science*, 27(2):111–126, 2010.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24(8):1596–1599, 2007.

Tchobanoglous, G., Burton, F.L., and Stensel, H.D. *Wastewater engineering: treatment and reuse*. McGraw-Hill, Boston, USA, 4$^{\text{th}}$ edition, 2003.

Ter Heijne, A., Liu, F., Van Rijnsoever, L.S., Saakes, M., Hamelers, H.V.M., and Buisman, C.J.N. Performance of a scaled-up Microbial Fuel Cell with iron reduction as the cathode reaction. *Journal of Power Sources*, 196(18, SI):7572–7577, 2011.

Thalasso, F., Vallecillo, A., García-Encina, P., and Fdz-Polanco, F. The use of methane as a sole carbon source for wastewater denitrification. *Water Research*, 31(1): 55–60, 1997.

Thauer, R.K. and Shima, S. Methane as fuel for anaerobic microorganisms. In Wiegel, J., Maier, R.J., and Adams, M.W.W., editors, *Incredible anaerobes: from physiology to genomics to fuels*, volume 1125 of *Annals of the New York Academy of Sciences*, pages 158–170. 2008.

Tian-Yuan, Z., Yin-Hu, W., Shu-Feng, Z., Feng-Min, L., and Hong-Ying, H. Isolation and heterotrophic cultivation of mixotrophic microalgae strains for domestic wastewater treatment and lipid production under dark condition. *Bioresource Technology*, 149:586–589, 2013.

Toh, S.K., Webb, R.I., and Ashbolt, N.J. Enrichment of autotrophic anaerobic ammonium-oxidizing consortia from various wastewaters. *Microbial Ecology*, 43 (1):154–167, 2002.

Uemura, S. and Harada, H. Treatment of sewage by a UASB reactor under moderate to low temperature conditions. *Bioresource Technology*, 72(3):275–282, 2000.

Van Ginkel, C.G., Rikken, G.B., Kroon, A.G.M., and Kengen, S.W.M. Purification and characterization of chlorite dismutase: A novel oxygen-generating enzyme. *Archives of Microbiology*, 166(5):321–326, 1996.

Van Hulle, S. W. H., Vandeweyer, H. J. P., Meesschaert, B. D., Vanrolleghem, P. A., and Dejans, P. Dumoulin, A. Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams. *Chemical Engineering Journal*, 162(1):1–20, 2010.

Vlaeminck, S.E., De Clippeleir, H., and Verstraete, W. Microbial resource management of one-stage partial nitritation/anammox. *Microbial Biotechnology*, 5(3, SI): 433–448, 2012.

Wang, K. Integrated anaerobic and aerobic treatment of sewage, PhD-thesis, Wageningen University, The Netherlands, 1994.

Wu, M.L., De Vries, S., Van Alen, T.A., Butler, M.K., Op den Camp, H.J.M., Keltjens, J.T., Jetten, M.S.M., and Strous, M. Physiological role of the respiratory quinol oxidase in the anaerobic nitrite-reducing methanotroph '*Candidatus* Methylomirabilis oxyfera'. *Microbiology*, 157(3):890–898, 2011a.

Wu, M.L., Ettwig, K.F., Jetten, M.S.M., Strous, M., Keltjens, J.T., and Van Niftrik, L. A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium '*Candidatus* Methylomirabilis oxyfera'. *Biochemical Society Transactions*, 39(1):243–248, 2011b.

Yang, Q., Peng, Y., Liu, X., Zeng, W., Mino, T., and Satoh, H. Nitrogen removal via nitrite from municipal wastewater at low temperatures using real-time control to optimize nitrifying communities. *Environmental Science & Technology*, 41(23): 8159–8164, 2007.

Zeeman, G. and Lettinga, G. The role of anaerobic digestion of domestic sewage in closing the water and nutrient cycle at community level. *Water Science and Technology*, 39(5):187–194, 1999.

Zhang, L., Hendrickx, T.L.G., Kampman, C., Zeeman, G., Temmink, H., Li, W., and Buisman, C.J.N. The effect of sludge recirculation rate on a UASB-digester treating domestic sewage at 15 °C. *Water Science and Technology*, 66(12):2597–2603, 2012.

Zhang, L., Hendrickx, T.L.G., Kampman, C., Temmink, H., and Zeeman, G. Co-digestion to support low temperature anaerobic pretreatment of municipal sewage in a UASB-digester. *Bioresource Technology*, 148:560–566, 2013.

Zhu, B., Van Dijk, G., Fritz, C., Smolders, A.J.P., Pol, A., Jetten, M.S.M., and Ettwig, K.F. Anaerobic oxidization of methane in a minerotrophic peatland: Enrichment of nitrite-dependent methane-oxidizing bacteria. *Applied and Environmental Microbiology*, 78(24):8657–8665, 2012.

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List of publications

Articles in peer-reviewed journals

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Hendrickx, T.L.G., Kampman, C., Zeeman, G., Temmink, H., Hu, Z., Kartal, B., and Buisman, C.J.N. Enrichment of fresh water anammox at 10 °C. *Bioresource Technology*, accepted, 2014, DOI 10.1016/j.biortech.2014.04.025.

Zhang, L., Hendrickx, T.L.G., Kampman, C., Temmink, H. and, Zeeman, G. Co-digestion to support low temperature anaerobic pretreatment of municipal sewage in a UASB-digester, *Bioresource Technology*, 148: 560-566, 2013.

Kampman, C., Hendrickx, T.L.G., Luesken, F.A., Van Alen, T.A., Op den Camp, H.J.M., Jetten, M.S.M., Zeeman, G., Buisman, C.J.N. and, Temmink, H. Enrichment of denitrifying methanotrophic bacteria for application after direct low-temperature anaerobic sewage treatment. *Journal of Hazardous Materials*, 227: 164-171, 2012.

Hendrickx, T.L.G., Wang, Y., Kampman, C., Zeeman, G., Temmink, H., and Buisman, C.J.N. Autotrophic nitrogen removal from low strength waste water at low temperature. *Water Research*, 46(7):2187-2193, 2012.

Zhang, L., Hendrickx, T.L.G., Kampman, C., Zeeman, G., Temmink, H., Li, W. and, Buisman, C.J.N. The effect of sludge recirculation rate on a UASB-digester treating domestic sewage at 15 °C. *Water Science and Technology*, 66(12):2597-2603, 2012.

Luesken, F.A., Van Alen, T.A., Van der Biezen, E., Frijters, C., Toonen, G., Kampman, C., Hendrickx, T.L.G., Zeeman, G., Temmink, H., Strous, M., Op den Camp, H.J.M. and Jetten, M.S.M. Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. *Applied Microbiology and Biotechnology*, 92(4):845-854, 2011.

Steinbusch, K.J.J., Hamelers, H.V.M., Schaap, J.D., Kampman, C., and Buisman, C.J.N. Bioelectrochemical Ethanol Production through Mediated Acetate Reduction by Mixed Cultures. *Environmental Science & Technology*, 44(1):513-517, 2010.

Articles in professional journals

Hendrickx, T.L.G., Kampman, C., Luesken, F.A., and Temmink, H. Denitrificatie met opgelost methaan uit anaerobe vergisting: nieuwe mogelijkheid voor afvalwaterbehandeling (Denitrification with dissolved methane from anaerobic digestion: novel opportunity for sewage treatment). *H2O*, 14/15, 34-36, 2010.

Conference presentations

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Zhang, L., Hendrickx, T.L.G., Kampman, C., Zeeman, G., Temmink, H., Li, W. and, Buisman, C.J.N. The effect of sludge recirculation rate on a UASB-digester treating domestic sewage at 15 Â°C, X Latin American Workshop and Symposium on Anaerobic Digestion, 23-27 October 2011, Minas Gerais, Brazil.

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Christel Kampman was born on 7 May 1984, in Leiderdorp. After graduating from secondary school in Apeldoorn in 2002 she studied Environmental Sciences at Wageningen University. During both her BSc (graduated in 2005) and MSc (graduated in 2007, with honors) she specialized in environmental technology. Her MSc thesis research was conducted at the Sub-department of Environmental Technology of Wageningen University, studying biological ethanol production from wastewater. She did an internship at AnoxKaldnes AB, Lund, Sweden, focussing on biological treatment of industrial wastewater. In September 2007 she started her PhD research at the Sub-department of Environmental Technology of Wageningen University. Hereafter, she worked as a technologist/project manager at Triqua International B.V. Since February 2014, she is working as a postdoctoral researcher at the Industrial Biotechnology group, part of the Chemical and Biological Engineering Department at Chalmers University of Technology, Gothenburg, Sweden.

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