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Microbial resource management of one-stage partial nitritation/anammox

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Summary

About 30 full-scale partial nitritation/anammox plants are established, treating mostly sewage sludge reject water, landfill leachate or food processing digestate. Although two-stage and one-stage processes each have their advantages, the one-stage configuration is mostly applied, termed here as oxygen-limited autotrophic nitrification/denitrification (OLAND), and is the focus of this review. The OLAND application domain is gradually expanding, with technical-scale plants on source-separated domestic wastewater, pre-treated manure and sewage, and liquors from organic waste bioenergy plants. A 'microbial resource management' (MRM) OLAND framework was elaborated, showing how the OLAND engineer/operator (1: input) can design/steer the microbial community (2: biocatalyst) to obtain optimal functionality (3: output). In the physicochemical toolbox (1), design guidelines are provided, as well as advantages of different reactor technologies. Particularly the desirable aeration regime, feeding regime and shear forces are not clear yet. The development of OLAND trickling filters, membrane bioreactors and systems with immobilized biomass is awaited. The biocatalyst box (2) considers 'Who': biodiversity and its dynamic patterns, 'What': physiology, and 'Where': architecture creating substrate gradients. Particularly community dynamics and extracellular polymeric substances (EPS) still require insights. Performant OLAND (3) comprises fast start-up (storage possibility; fast growth of anammox bacteria), process stability (endured biomass retention; stress resilience), reasonable overall costs, high nitrogen removal efficiency and a low environmental footprint. Three important OLAND challenges are elaborated in detailed frameworks, demonstrating how to maximize nitrogen removal efficiency, minimize NO and N_2O emissions and obtain through OLAND a plant-wide net energy gain from sewage treatment.

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Introduction

The discovery of anoxic ammonium-oxidizing bacteria (AnAOB) around 15 years ago has led to the development of several partial nitritation/anammox processes for biological nitrogen removal, including the one-stage oxygen-limited autotrophic nitrification/denitrification (OLAND) process (Kuai and Verstraete, 1998; Pynaert *et al.*, 2003; Vlaeminck *et al.*, 2010). In OLAND, the first reaction consists of the aerobic oxidation of about half of the ammonium to nitrite (partial nitritation), performed by aerobic ammonium-oxidizing bacteria (AerAOB). The second reaction, performed by the AnAOB, is the anoxic oxidation of the residual ammonium with nitrite to mainly dinitrogen gas and some nitrate (anammox). Combing the nitritation (Barnes and Bliss, 1983) and anammox (Strous *et al.*, 1998) stoichiometries, yields the overall OLAND stoichiometry (Eq. 1), with the first and second biomass term respectively displaying the growth of AerAOB and AnAOB.

$$\begin{aligned} & NH_4^+ + 0.792\ O_2 + 0.080\ HCO_3^- \rightarrow \\ & \quad 0.435\ N_2 + 0.111\ NO_3^- + 1.029\ H^+ + \\ & \quad 0.052\ CH_{1.4}O_{0.4}N_{0.2} + 0.028\ CH_2O_{0.5}N_{0.15} + 1.460\ H_2O \end{aligned} \tag{1}$$

In comparison, the stoichiometry of the conventional biological process for nitrogen removal, i.e. nitritation and nitratation (nitrification; Barnes and Bliss, 1983) and denitrification, with for instance methanol (Mateju *et al.*, 1992), exhibits the following reaction:

$$\begin{aligned} & NH_4^+ + 1.856\ O_2 + 1.058\ CH_3OH + 0.103\ HCO_3^- \rightarrow \\ & \quad 0.457\ N_2 + 0.907\ H^+ + 0.744\ CO_2 + \\ & \quad 0.421\ CH_{1.4}O_{0.4}N_{0.2} + 2.452\ H_2O \end{aligned} \tag{2}$$

Overall, OLAND consumes 100% less organic carbon, produces about 90% less sludge and consumes almost 60% less oxygen compared with nitrification/denitrification (Mulder, 2003). As such, the treatment of wastewaters with a low biodegradable chemical oxygen demand (bCOD) to N ratio (< 2–3) saves 30–40% of the overall costs (Fux and Siegrist, 2004). Depending on the wastewater characteristics and reactor operation, additional nitrogen conversions can take place, including aerobic nitrite oxidation to nitrate (nitratation) by nitrite-oxidizing bacteria (NOB) and reduction of nitrate or nitrite with organic carbon to nitrogen gas (heterotrophic denitrification). The latter requires at least 4.1 g bCOD g^{-1} NO_3^--N and 2.7 g bCOD g^{-1} NO_2^--N using wastewater organics (Mateju *et al*., 1992). Recent information on the diversity of pathways, enzymes and phylogeny of the mentioned microbial key players can be found in Vlaeminck and colleagues (2011).

Currently about 30 partial nitritation-anammox applications are operating at full scale. In four of these, partial nitritation and anammox are spatially separated (van der Star *et al*., 2007; Desloover *et al*., 2011; Tokutomi *et al*., 2011), while in all others, a one-stage process is executed for sewage sludge reject water treatment at 17 locations (Beier and Schneider, 2008; Joss *et al*., 2009; Wett *et al*., 2010a; T. Hülsen, oral comm.), for landfill leachate treatment at five locations (Hippen *et al*., 2001; Denecke *et al*., 2007; Rekers *et al*., 2008) and for industrial wastewaters at four locations (Abma *et al*., 2010; T. Hülsen, oral comm.). Some debate exists on the preference between one-stage versus two-stage configuration. Distinct advantages of the two-stage process include: (i) the partial nitritation and the anammox step can be optimized individually, including nitratation suppression in the first stage, (ii) the risk is lower for AnAOB to be overgrown by denitrifiers in case of higher bCOD/N ratio in the influent (Lackner *et al*., 2008), since most bCOD will be degraded in the preceding stage, (iii) smaller quantities of AnAOB-enriched inoculum are required for a fast start-up (Jaroszynski and Oleszkiewicz, 2011), and (iv) the risk of oxygen inhibition for AnAOB is lower, provided no oxygen enters the anammox stage (Jaroszynski and Oleszkiewicz, 2011). Distinct advantages of the one-stage process include: (i) investment costs are significantly lower, (ii) the process control is less complex, (iii) the risk of inhibiting AnAOB with nitrite is lower, and (iv) data so far indicate that the N_2O emissions from the one-stage process are 0.4–1.3% of the nitrogen load (Joss *et al*., 2009; Kampschreur *et al*., 2009b; Weissenbacher *et al*., 2010), whereas two-stage emissions amount to 2.3–6.6% (Kampschreur *et al*., 2008; Desloover *et al*., 2011). Given the prevalence of full-scale realizations of the one-stage process, this is the focus of this review.

Despite of many operational full-scale one-stage plants, several OLAND aspects are still unknown. Therefore, a novel conceptual framework is presented in this review providing insights into the key points for successful OLAND operation and revealing challenges for further research and development. Human resource management (HRM) engages a high-performing employee for a particular dedicated job, and ensures his/her continued performance by offering an attractive package of rewards and conditions. Analogous to HRM, Verstraete and colleagues (2007) proposed to apply microbial resource management (MRM) in environmental biotechnology, in order to reveal strategies to obtain and maintain a highly performant microbial community. To properly manage complex microbial systems, the engineer needs well-documented concepts, reliable tools and a set of default values.

MRM framework for OLAND

The OLAND engineering question is to remove nitrogen from wastewater which has a low bCOD/N ratio ($\leq$ 2–3) and which displays a temporal variability in physicochemical composition and flow rate. Given the abovementioned cost-efficiency of OLAND compared with conventional nitrification/denitrification, its application to a **broad range of wastewater types** can entail overall benefits with regard to cost and energy savings (Fig. 1, box 0). Besides the established plants on sewage sludge digestate, landfill leachate and specific industrial wastewaters, technical-scale installations were established for the treatment of source-separated domestic wastewater, i.e. black water digestate (Meulman *et al*., 2010; Verstraete and Vlaeminck, 2011). Further, at lab scale, OLAND treatment has been demonstrated for digested manure (Villegas *et al*., 2011), urine (Udert *et al*., 2008) and sewage-like nitrogen concentrations (De Clippeleir *et al*., 2011a). Additionally, the feed-in tariffs for renewable energy from biomass are appreciable, and for instance up to 0.30 EUR kWh^{-1} of electricity is recovered in the European Union (Europe's Energy Portal, 2011). This provides an incentive to build dedicated anaerobic (co-)digestion plants recovering energy from various organic waste streams (Holm-Nielsen *et al*., 2009). OLAND development in this area is expected since its application can further maximize energy recovery by a post-digestion stage while minimizing energy consumption for nitrogen treatment.

The developed MRM framework links the OLAND process input (1), its biocatalyst (2) and its output (3). The physicochemical **toolbox (1: Input)** of the OLAND engineer consists of a number of operational choices (Fig. 1, box 1). Most of these parameters are relatively fixed and have to be considered while designing the reactor and its control possibilities. Other parameters represent a degree of freedom and can be decided during start-up or at steady-state operation. Each of the engineering tools can have an impact on the **OLAND microbial community (II: biocatalyst)**. The complex multitude of microbial community characteristics can comprehensively be subdivided in three areas, which are strongly interlinked (Fig. 1, box 2): who is there, what are they doing and where are they doing it. With the physicochemical toolbox, the OLAND engineer tries to achieve the best biocatalyst properties,

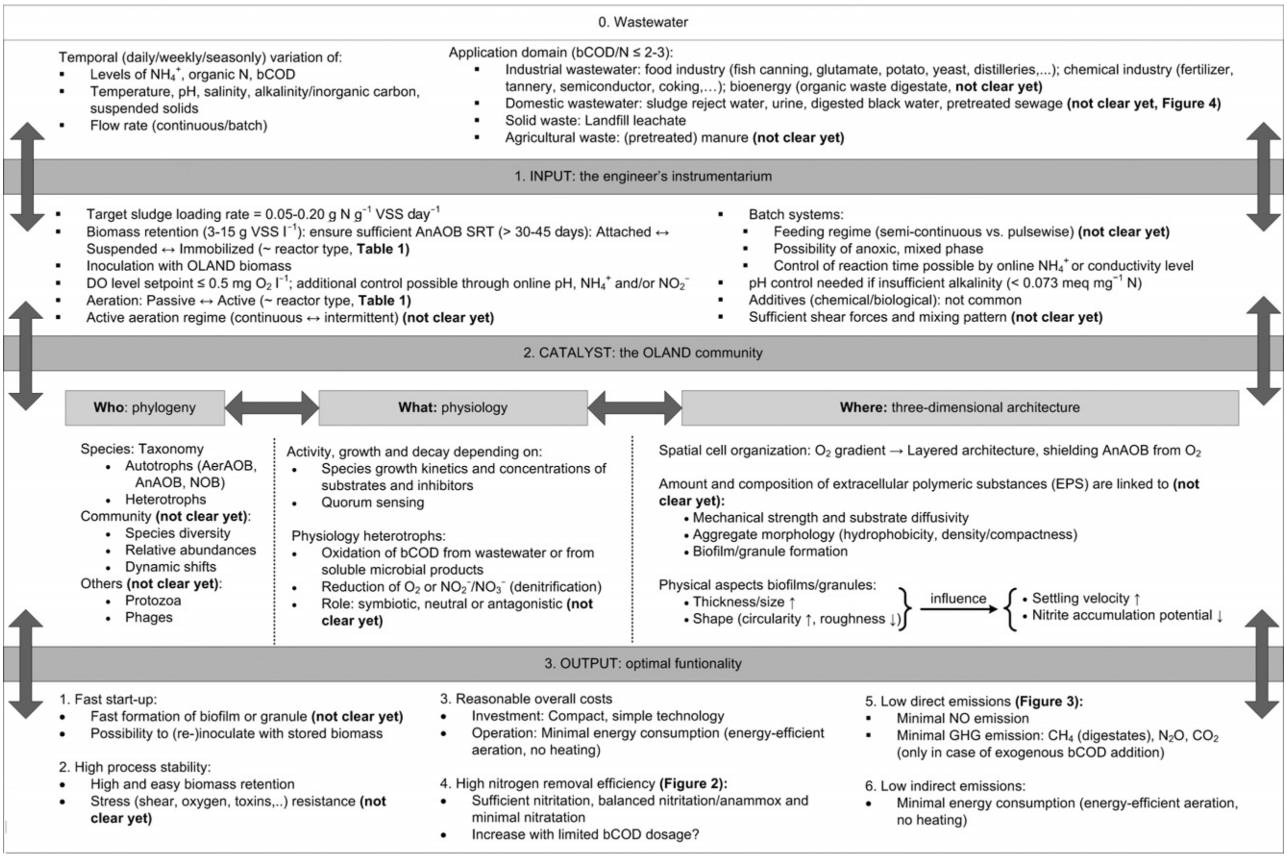

Fig. 1. Microbial resource management view on the OLAND process. AerAOB and AnAOB, aerobic and anoxic ammonium-oxidizing bacteria; NOB, nitrite-oxidizing bacteria; GHG, greenhouse gas; bCOD, biodegradable chemical oxygen demand; GHG, greenhouse gas; DO, dissolved oxygen; VSS, volatile suspended solids.

in order to obtain **optimal functionality (III: Output)** of the OLAND process (Fig. 1, box 3). For this aspect, six process objectives can be distinguished: fast start-up, high process stability, reasonable overall costs, high nitrogen removal efficiency, low NO and N_2O emissions, and a low energy balance.

Input

Many fixed parameters depend on the choice of **reactor type**. The reactor type first determines the way biomass is retained in the system. Indeed, since the doubling time of AnAOB is in the order of 1–2 weeks (Strous *et al*., 1998), the sludge retention time has to exceed this high value. This can be achieved in reactor types relying on attached biomass, i.e. biofilms, suspended biomass such as flocs and/or granules, or immobilized biomass in a gel matrix (e.g. polyethylene glycol, polyvinyl alcohol, alginate, . . .). Table 1 gives the advantages and challenges for each reactor technology in a qualitative way, which should always be interpreted according to case-specific requirements. It should be noted that trickling filters (Schmid *et al*., 2000), immobilized biomass (Yan and Hu, 2009; Zhu *et al*., 2009) and membrane bioreactors (Wyffels *et al*., 2004) were used for separate partial nitritation and anammox, but not yet in a one-stage autotrophic removal process. Immobilization for instance creates diffusion limitations for oxygen, providing anoxic zones for AnAOB, and might be an excellent start-up strategy ensuring biomass retention and activity. Encapsulation of OLAND biofilm in alginate was shown to decrease the AnAOB activity by 60%, yet after 30 days of biomass growth of alginate granules in a sequencing batch reactor (SBR), the original activity was restored.

Biomass retention is most delicate for suspended growth systems (Table 1), and depends on the settleability of the biomass. In a settler (CSTR, continuous stirred-tank reactor) or settling phase (SBR, sequencing batch reactor), sludge settling is a separate step, permitting some optimization. For SBRs, only occasionally, sludge settling problems have been reported due to small N_2 bubbles not detaching from the flocs (Joss *et al*., 2009). Adjustments of the settling phase and occasional addition of flocculant as needed could solve this problem. In general, larger and thus heavier sludge aggregates have a lower nitritation and a higher anammox activity

Table 1. Qualitative comparison of OLAND reactor configurations (advantages indicated in bold).

Biomass growth	Attached (biofilm)			Immobilized	Suspended (flocs and/or granules)			
Reactor configuration	Trickling filter	RBC[a] Fixed/moving	Bed reactor Fixed/moving	Upflow/SBR	MBR	Gas-lift or upflow	SBR	CSTR with settler[b]
Overall costs	**Low**	**Low**	Medium	Medium	High	Medium	Medium	Medium
Area requirement	Medium	High	Medium	**Low**/Medium	Medium	**Low**	Medium	High
Aeration	**Passive**	**Passive**	Active	Active	Active	Active	Active	Active
Ease of DO control	Low	Medium[c]	Medium/**High**	**High**	**High**	**High**	**High**	**High**
Sludge content	Medium	Medium	Medium	Medium	**High**	**High**	Low	Low
Ease of biomass retention	Medium	Medium	Medium	Medium	**High**	Low	Low	Low
Inoculation feasibility[d]	Medium	Low/Medium	Medium/**High**	**High**	**High**	**High**	**High**	**High**
Low HRT feasibility[e]	**Yes**	**Yes**	**Yes**	**Yes**	No	**Yes**	No	No
Risk for mechanical failure	Medium	High	**Low**	**Low**	Medium	**Low**	**Low**	**Low**
Risk for clogging	High	**Low**	High/**Low**	**Low**	High	**Low**	**Low**	**Low**
Operational flexibility	Low	Low	Low/Medium	Medium/**High**	Medium	Medium	**High**[f]	Medium
Operational complexity	**Low**	**Low**	Medium	Medium/High	High	Medium	High	Medium

a. Biofilm can grow on rotating discs (fixed), or on carrier material brought in rotating porous cages (moving).
b. Similar configuration as conventionally used for activated sludge.
c. The bulk DO level can be controlled by the rotation speed (Meulman *et al.*, 2010) and by the immersion level of the discs (Courtens *et al.*, 2011).
d. Assuming sufficient availability of enriched inoculum, attached to carrier material if applicable.
e. Important for wastewaters with low nitrogen level. For SBR and CSTR, this largely increases required settling time or settler volume, whereas for MBR this largely increases the amount of membranes required.
f. Cycle duration can be adjusted to meet effluent requirements (Siegrist *et al.*, 2008), allowing to respond to changes in wastewater composition.
RBC, rotating biological contactor; SBR, sequencing batch reactor; CSTR, continuous stirred-tank reactor.

(Vlaeminck *et al*., 2010), and hence a lower risk for nitrite accumulation. Wett and colleagues (2010b) took advantage of this fact by installing a cyclone on a SBR, retaining large AnAOB-rich aggregates in the reactor, while discarding the small AerAOB-rich aggregates. SBRs without this type of selective biomass retention, however, can also operate without nitrite accumulation at the long term (Joss *et al*., 2009). For gas-lift and upflow reactors, biomass retention is of utmost importance, because they depend on the continued presence of well settling granules (Abma *et al*., 2010). Distinguishing granules from large flocs remains to some extent subjective, with granules defined for instance by Lemaire and colleagues (2008) as compact and dense aggregates with an approximately spherical external appearance that do not coagulate under decreased hydrodynamic shear conditions and which settle significantly faster than flocs. Overall, a sludge retention time (SRT) of at least 30–45 days is recommended (Wett *et al*., 2010a; Desloover *et al*., 2011; A. Joss, unpublished).

Reviewing literature, the size of the reactor can be dimensioned based on the biomass content in the range of 3–15 g volatile suspended solids (VSS) l^{-1} and a **sludge loading rate** of in the range of 0.05–0.20 mg N g^{-1} VSS day^{-1}.

An important 'hardware' choice is based on the desired level of reactor monitoring and control. Given the delicate steady-state equilibrium between nitritation and anammox, with minimal nitratation, control of the **dissolved oxygen** (DO) **level** is of primary importance. The DO can be kept at one setpoint (e.g. 0.5 mg O_2 l^{-1}) or within a certain range (e.g. 0.3–0.8 mg O_2 l^{-1}), with either continuous or intermittent aeration. Furthermore, anoxic reaction periods (*c.* 0 mg O_2 l^{-1}) can be built in when mixing and aeration are independent (Table 1), allowing removal of occasional nitrite and/or nitrate accumulation. An additional option is to control the start and stop of intermittent aeration with pH values, which is typically a function of ammonia oxidation (Wett, 2006). The effect of different aeration regimes has not been examined extensively so far. Joss and colleagues (2009) compared aeration in continuous and intermittent mode, i.e. 75% of the time aerated, at full scale and DO setpoint of about 0.5 mg O_2 l^{-1}. Continuous aeration was preferred, since this did not result in nitrite accumulation and since the aerators were not continuously switched off and on, allowing also better process monitoring thanks to higher signal/noise levels. Zubrowska-Sudol and colleagues (2011) tested four aeration regimes in batch (100%, 66%, 50% and 33% of the time aerated), at three DO levels (2, 3, 4 mg O_2 l^{-1}), showing for each DO level that 66% aeration obtained the highest nitrogen removal rate but also the highest nitrite accumulation.

Monitoring and control of the **nitrogen removal** has been reported via indirect measurement of the conductivity (Joss *et al*., 2009), or via direct nitrogen measurement with ion-selective ammonium probe (Joss *et al*., 2009), regulating the duration of the SBR operation cycle. Further, automated colorimetric ammonium and nitrite analyses of grab samples every 10–15 min are an additional control mechanism for the DO level (Abma *et al*., 2010).

In case the treated wastewater contains no sufficient alkalinity, additional **pH** control might be necessary. It should be noted, however, that the alkalinity requirements for OLAND and nitrification/denitrification are similar, i.e. 0.073 and 0.065 meq mg^{-1} N removed respectively (Eqs 1 and 2). **Temperature** is another parameter of importance, which is discussed below (Section *Output*).

In semi-continuous or batch-fed reactors such as SBR, the choice of **feeding regime** is mostly chosen *a priori*, and can have an effect on process performance. Design choices include the timing of feeding (pulse versus continuous), and the percentage volume exchanged per cycle. These will determine the concentration range of substrates and intermediates 'experienced' by the sludge. Few studies addressed this aspect specifically, but the findings of De Clippeleir and colleagues (2009) and Schaubroeck and colleagues (2012) indicate that short operational cycles require relatively slow feeding and low volumetric exchange ratio per cycle for successful start-up.

The **shear forces and mixing patterns** in the reactor will be influenced by the aeration regime, applied air flow rates, bubble sizes, positions of the blowers, shape of the reactor and by the additional power input in case of additional mixing. For suspended growth systems, the effect of shear and mixing on biomass architecture, and hence activity and stability, was recently hypothesized (Vlaeminck *et al*., 2010). Research is awaited to deliver a range of desirable shear forces.

Due to slow enrichment of the AnAOB, extremely long start-up periods of 2.5–3.5 years recently demonstrated the reality of this problem (Wett, 2006; van der Star *et al*., 2007). More recently, considerable quantities of OLAND biomass have become available from operating reactors, which can be used as **inocula** for quick reactor start-up (Wett, 2006; Rekers *et al*., 2008; Joss *et al*., 2009; Abma *et al*., 2010). Inoculation is assisted by the possibility to store active OLAND biomass. Over a storage of OLAND biomass 5 months, AnAOB maintained 55%, 30% and 32% of their original activity, depending on the storage conditions at 4°C without nitrate, 4°C with nitrate and 20°C with nitrate respectively (Vlaeminck *et al*., 2007). For safety, it is recommended to supply nitrate, ensuring the suppression of toxic sulfide formation. It is obvious that inoculation of a new reactor is facilitated provided sludge of a similar 'growth mode' is available, e.g. a moving-bed biofilm reactor (MBBR) requires biofilm grown on carrier materials. Note also in case of absent inoculum but suitable physicochemical conditions, a fast start-up is possible, as shown by Jeanningros and colleagues (2010) in 4 months after inoculation with activated sludge.

A degree of freedom exists in the **addition** of chemicals, such as trace elements, flocculants or others, but these are not strictly required according to practically all literature reports. In case of significant reactor activity loss, for instance due to AnAOB nitrite inhibition, repeated spiking with hydrazine (N_2H_4; 1.4–2.0 mg N l^{-1}) and/or hydroxylamine (NH_2OH; 0.7–3.1 mg N l^{-1}) can be considered (Strous *et al.*, 1999; Bettazzi *et al.*, 2010). Alternatively, reinoculation with previously harvested sludge can also be an option to restore the process. Recently, two interesting, yet costly approaches were reported: (i) the continuous reinoculation with AnAOB, as proposed for the treatment of toxic pharmaceutical wastewater (Tang *et al.*, 2011), and (ii) the spiking with quorum sensing molecules to enhance anammox activity during start-up (De Clippeleir *et al.*, 2011b).

Catalyst

A microbial community comprises, by definition, different microbial populations, which are groups of microorganisms of the same species. These **species** can be identified due to a specific base sequence in their genes. However, given the physiological versatility of many prokaryotes, species identification and community structure generally do not provide much information on the **function or physiology** of the species (Lee *et al.*, 1999). There are only some exceptions in which phylogeny and function are linked, including the autotrophs involved in biological nitrogen removal. In most OLAND applications, AerAOB mostly belong to the β-*Proteobacteria* subphylum, genus *Nitrosomonas*, AnAOB to the *Planctomycetes* phylum, genera '*Candidatus* Kuenenia and Brocadia', and NOB, if any, to the *Nitrospirae* phylum, genus *Nitrospira* (Vlaeminck *et al.*, 2010 and references therein). More work remains to be done to characterize the heterotrophs present in the OLAND process; however, preliminary evidence suggests the possibility of a symbiotic relationship between specific heterotrophic and autotrophic groups (S.E. Vlaeminck, H. De Clippeleir and W. Verstraete, unpubl. results). Recently, microbial 'communication' was found to play a role in the anammox step. De Clippeleir and colleagues (2011b) showed that long-chain acylhomoserine lactones were present in an OLAND biofilm and AnAOB granules, increasing the anammox reaction at low biomass concentrations.

In OLAND, the presence of the required populations in the required community structure is not sufficient to guarantee process functionality. In addition, **an oxygen gradient is needed in space or time** to create the required anoxic microniche for the AnAOB. In perfectly mixed and continuously aerated systems, the presence of anoxic zones relies on the three-dimensional cell organization, in which the AerAOB on the surface protect the AnAOB in the lower layers from oxygen while also providing them with nitrite. The resultant aggregate is a multilayered, three-dimensionally symmetrical granule (Vlaeminck *et al.*, 2010). This strictly layered structure was less pronounced in OLAND biofilms grown in low shear environments, with AerAOB also prevailing in putatively anoxic zones (Pynaert *et al.*, 2003; Vlaeminck *et al.*, 2009b). Furthermore, suspended reactor systems with heterogeneous mixing or intermittent aeration do not rely on a specific microbial organization to create oxygen gradients.

The structural basis of any microbial three-dimensional structure is the **biogenic EPS matrix**, gluing individual cells to form multicellular aggregates (biofilms, flocs or granules). Vlaeminck and colleagues (2010) showed that EPS occupied at least 50% of the granule space in AerAOB and AnAOB zones. Both the EPS amount and composition have important implications. Besides the fundamental structural role, EPS determines the aggregate density and the diffusivity of the substrates. Further, the EPS composition is interlinked with the aggregate morphology and can trigger biofilm or granule formation. It is expected that future contributions on understanding EPS in OLAND will significantly improve our understanding of biofilm and granule formation.

As a result of a selective settling pressure in suspended OLAND reactors, single cells are washed out and only attached biofilm or suspended flocs and granules can maintain themselves in the system. For this biomass, **size and shape have physical and biological consequences**. First, the denser, larger and/or more circular an aggregate is, the faster it will settle. Second, the anoxic volume of larger aggregates or thicker biofilms occupies a larger portion of the biomass, since the oxygen penetration depth is expected to be the same. This results in a lower risk for nitrite accumulation for larger aggregates (Nielsen *et al.*, 2005; Vlaeminck *et al.*, 2009a; 2010).

Output

Both from an economical and from an environmental point of view, it is desirable that an OLAND reactor has a **short start-up period**. As discussed above, inoculation from existing reactors or stored biomass allows for a fast start-up nowadays. It is also expected that future insights in chemical or physical triggering mechanisms for biofilm or granule formation could enhance reactor start-up.

Another objective of optimal OLAND functionality is **high process stability**. High biomass retention is a prerequisite to achieve this. In attached growth systems, the biofilm should be well attached to the carrier material. In suspended growth systems, high settling capacity allows easy separation in the three-phase separator of an upflow reactor, or fast separation during the settling phase in a SBR, allowing for a longer reaction phase and thus higher nitrogen removal rates. A second aspect of process stability is stress resistance, for instance to high oxygen levels, strong shear forces or episodic exposure to toxicants. Assuming anammox as the most fragile step, symmetrically structured granules would be most resistant to stress situations, since the AerAOB shield will prevent direct exposure of the AnAOB to bulk oxygen concentrations or toxins. Greater understanding of the effect of architecture structure on stress resistance is therefore likely to be of practical value.

Obviously, **low nitrogen removal costs** are desirable. A precise comparison of the overall costs of the different reactor technologies is practically impossible, yet Table 1 offers a tentative cost estimate. OLAND operational costs are dominated by personnel costs (Fux and Siegrist, 2004). Similarly, for regular sewage treatment these vary heavily depending on the country, constituting between 30% and 70% of the operational costs (Kemper *et al*., 1994; Zessner *et al*., 2010). Energy consumption constitutes the other main operational cost, predominantly dependent on the type of aeration: active aeration in sequencing batch reactors requires about 1.2 kWh kg^{-1} N (Wett *et al*., 2010a), whereas passive aeration in rotating biological contactors (RBC) requires down to 0.4 kWh kg^{-1} N (Mathure and Patwardhan, 2005). Costs should of course be scored against the requirements. If for instance an effluent is required which is free of suspended particles, the overall high costs of an OLAND MBR might be quite acceptable.

A **high nitrogen removal efficiency** is likely the main OLAND output objective, and is elaborated in detail in Section *Maximizing nitrogen removal efficiency*.

Besides the emission to the environment comprised in the effluent and produced sludge, **direct and indirect gaseous emissions** form an important part of a sustainable process. Direct emissions include CH_4, NO and N_2O, and are treated in detail below (Section *Minimizing harmful gas emissions*). Indirect emissions are derived from the consumption of electrical or heat energy, which are in our fossil-based energy economy proportionally related to CO_2 emissions. The electricity use is mainly dependent on the type of aeration, as discussed higher. Concerning heating, most reactors are currently operated between 25°C and 30°C, yet since mostly mesophilic effluents are treated (typically 30–35°C), no heating is applied. Absence of large wastewater buffer and thermal isolation of the reactor should be sufficient to maintain the temperature, and the metabolic heat of the OLAND treatment even rises the temperature by several degrees (Schmid *et al*., 2003), in combination with the heat from compressed air, in case of active aeration. So far, long-term OLAND or anammox activity has been reported below 20°C (Hippen *et al*., 2001; Dosta *et al*., 2008; Isaka *et al*., 2008), but the focus in these studies was not on maximum nitrogen removal. It is anticipated that OLAND treatment of colder waste streams (10–20°C) is possible also at high performance, as elaborated below (Section *OLAND enabling energy-positive sewage treatment*).

MRM framework elaborated for three OLAND challenges

Maximizing nitrogen removal efficiency

The maximum nitrogen removal efficiency that can be obtained in a balanced OLAND system without additional denitrification is 89% (Eq. 1). Lower removal efficiencies are mainly caused by hampered nitritation resulting in residual ammonium, by an imbalance between nitritation and anammox resulting in nitrite accumulation, or by increased nitratation resulting in a higher nitrate production. For most OLAND applications treating high-strength nitrogenous wastewaters, a post-treatment is obligatory to meet discharge limits. For sewage sludge reject water treatments (Fux and Siegrist, 2004) or source-separated black/grey-water systems (Verstraete and Vlaeminck, 2011), the OLAND effluent is sent to the diluted treatment stream for polishing. For industrial applications, the effluent can be sent to a sewage treatment plant (Abma *et al*., 2010), or can be polished by additional separate stage nitrification and denitrification (Desloover *et al*., 2011; Tokutomi *et al*., 2011). The latter techniques are also used to polish OLAND-treated landfill leachate, and can be complemented with an activated-carbon stage (Hippen *et al*., 2001; Denecke *et al*., 2007). A possibility which has not been explored so far, is the inclusion of an anoxic reaction phase in the OLAND reactor to denitrify the nitrate produced with either autochtonous or added COD to further increase the removal efficiency. Given the low COD/N required to remove the remaining 11% of the nitrogen load, it is anticipated that denitrifying bacteria would not outgrow the AnAOB.

AerAOB activity should be high enough to deliver nitrite to the AnAOB, otherwise **residual ammonium** prevails (Fig. 2). An increase in AerAOB activity can be obtained by adjustment of the oxygen supply and level, yet care should be taken not to use DO levels above 0.5 mg O_2 l^{-1}, since this will favour the development of NOB (Wett, 2006; Joss *et al*., 2009). It should be noted that in systems with larger aggregates (granules), higher DO setpoints can be applied (Volcke *et al*., 2010). Under more extreme conditions, high free ammonia (8–120 mg N l^{-1}) could decrease AerAOB activity at high ammonium concentrations, high pH and elevated temperatures, or high nitrous acid concentrations (0.2–2.8 mg N l^{-1}) could be inhibitory at high nitrite concentrations, low pH and low temperatures

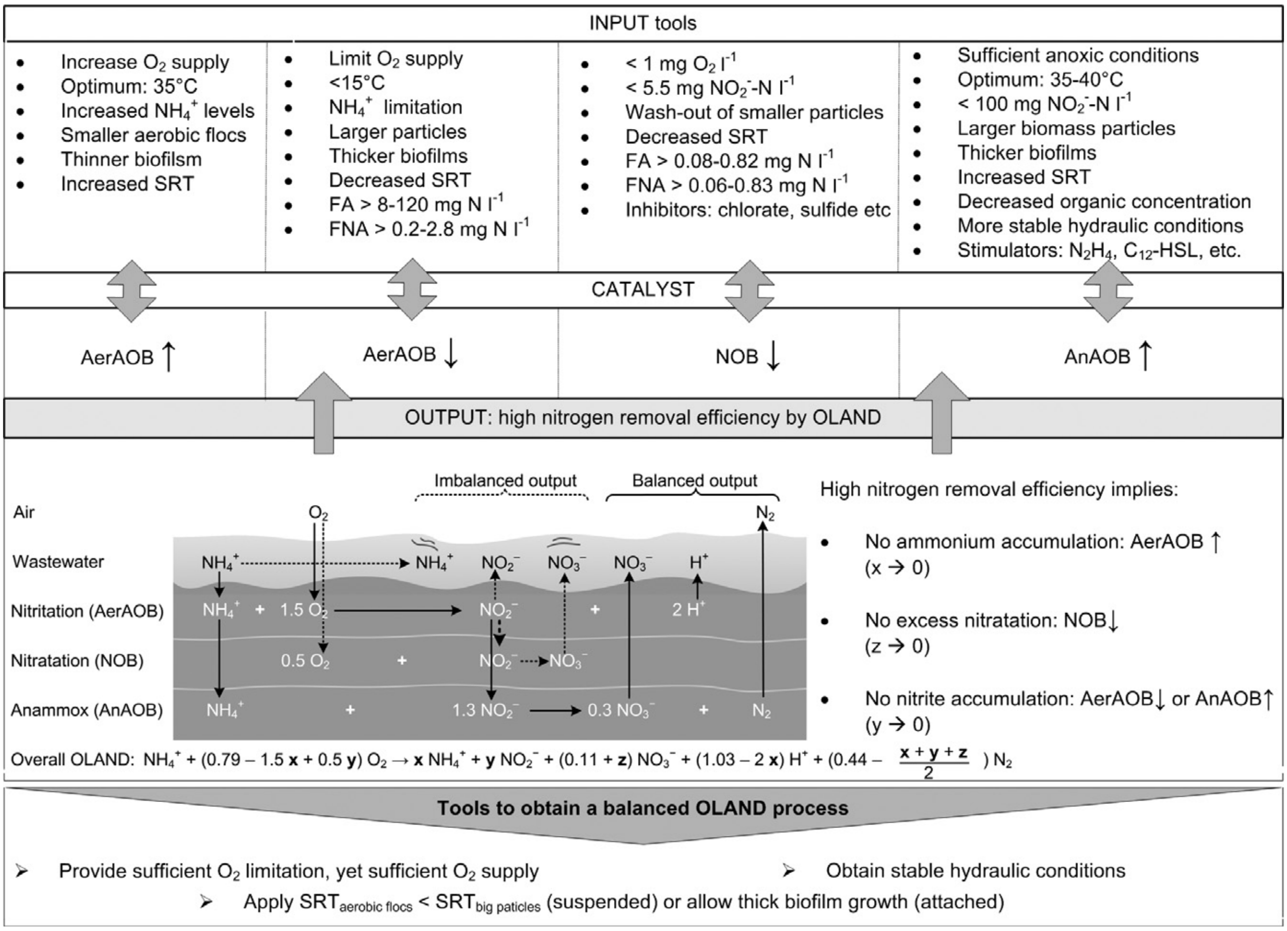

Fig. 2. OLAND MRM framework highlighting tools to obtain high nitrogen removal efficiency. FA, free ammonia; FNA, free nitrous acid; SRT, sludge retention time.

(Anthonisen *et al.*, 1976; Fig. 2). However, these conditions are not likely for OLAND reactors.

If the AnAOB are not able to consume the formed nitrite or AerAOB leave not enough ammonium to combine with nitrite, **nitrite accumulation** will occur, which in a more extreme case (> 100–250 mg NO_2^--N l^{-1}, Strous *et al.*, 1999; Egli *et al.*, 2001; Dapena-Mora *et al.*, 2007) can inhibit AnAOB. Besides lowering the AerAOB activity by operational parameters such as a lower oxygen supply and level, one of the main factors to discounter the difference in growth rate between AerAOB and AnAOB is the separation of the sludge retention of small flocs, containing mainly AerAOB, and larger biomass particles, containing mainly AnAOB (Vlaeminck *et al.*, 2010). Different selection methods are available to decrease the aerobic activity, depending on the applied reactor technology. In SBR systems, selection is based on the selective removal of smaller particles, which have a lower density and hence lower settling velocity. Higher critical particle settling velocities can be imposed by applying a lower settling time and/or a higher volumetric exchange ratio (De Clippeleir *et al.*, 2009). Typical critical settling velocities applied in SBR systems are 0.3–3 m h^{-1} (Wett, 2006; De Clippeleir *et al.*, 2009; Joss *et al.*, 2009). In granular upflow systems, removal of smaller, nitrifying granules at the top of the sludge bed led to higher biomass-specific conversion rates (Winkler *et al.*, 2011). In floccular systems, the use of hydrocyclones has been initiated to selectively maintain AnAOB-containing granules (Wett *et al.*, 2010a). As the AnAOB are the slowest growers in the OLAND system, they should be maximally maintained in the system and stimulated as much as possible. It has been shown in several studies that the AnAOB are sensitive for oxygen (Strous *et al.*, 1997; Egli *et al.*, 2001). The presence of anoxic zones can also be promoted by the use of suspended carrier material in a MBBR (Beier and Schneider, 2008) or by biomass immobilization in a gel matrix (Section *Input*). Moreover, depending on the reactor technology applied, anoxic reactor zones can be created in space or time. It should be noted that methanol, commonly used as exogenous carbon source for denitrification, is detrimental for anammox (Güven *et al.*, 2005;

Dapena-Mora *et al.*, 2007). Besides prevention of anammox inhibition, anammox can also be stimulated with components such as hydrazine, and dodecanoyl homoserine lactone (De Clippeleir *et al.*, 2011b). Moreover, it was shown in lab-scale SBR tests that only sufficient AnAOB activity could be obtained in OLAND reactors when stable semi-continuous hydraulic conditions were applied (De Clippeleir *et al.*, 2009; Schaubroeck *et al.*, 2012). The latter is in congruence with successful full-scale SBR systems, which apply low volumetric loading rates and semi-continuous feeding (Wett, 2006; Joss *et al.*, 2009). Note that stable hydraulic conditions also minimize the accumulation of substrates or intermediates, probably resulting in lower NO and N_2O emissions (Section *Minimizing harmful gas emissions*).

Nitrate accumulation due to NOB should be avoided at all time. For high-strength wastewaters followed by a post-treatment, NOB can be suppressed in the OLAND system at high free ammonia concentrations (> 5 mg N l^{-1}) and low oxygen concentrations (Vlaeminck *et al.*, 2009b). In the latter case, the AerAOB will have a competitive advantage over the NOB for substrate and space. In the case of diluted wastewater systems which have to reach effluent quality standards, free ammonia levels will not be sufficient anymore to suppress NOB and other methods should be searched especially for application at low temperatures (Section *OLAND enabling energy-positive sewage treatment*). One option is the addition of compounds such as sulfide at concentrations of 20–80 mg S l^{-1} (Erguder *et al.*, 2008) or chlorate at concentrations of 83–830 mg l^{-1} (Belser and Mays, 1980), which have been shown to inhibit NOB activity. However, as long-term addition of these compounds could result in adaptation and could also affect AerAOB or AnAOB, this should be avoided as much as possible. Although *Nitrospira* lacks the common protection mechanisms for reactive oxygen species (Lücker *et al.*, 2010), the addition of peroxide (up to 1.0 g H_2O_2 l^{-1}) had no influence on the nitratation rate of a nitrifying culture with *Nitrospira*. In contrast, already at 0.5 g H_2O_2 l^{-1}, the nitritation rate was significantly inhibited, rendering peroxide addition as a useful strategy to suppress nitratation (T. Vanslambrouck, unpublished). A close interaction between AerAOB and AnAOB could also play a role in avoiding nitratation, as the affinity of the AnAOB for nitrite is higher than the affinity of NOB for nitrite (Lackner *et al.*, 2008). It should be however noted that until now, only limited knowledge exists about the genus/species dependence of these inhibition factors and it is therefore not always straightforward to avoid nitratation.

In general, it is suggested that to obtain a balanced OLAND system with maximum nitrogen removal efficiency, sufficient DO limitation, separation between the SRT of small aerobic flocs and larger anoxic particles as well as stable hydraulic conditions are desired (Fig. 2).

Minimizing harmful gas emissions

In terms of gaseous emissions, sustainability mainly includes minimal emissions of nitric oxide (NO), an ozone degrader, and nitrous oxide (N_2O) and methane (CH_4), two potent greenhouse gases (GHG).

Methane can be expected in the OLAND influent when treating anaerobic digestates (dissolved at 11 g m^{-3} at 35°C), and small quantities might be in a non-aerated phase if all oxygen and nitrate are consumed (Desloover *et al.*, 2011). Aeration causes stripping of this methane. Although this can have a non-negligible contribution to the overall carbon footprint of the process (Desloover *et al.*, 2011), it is difficult to prevent the emission of dissolved influent methane, unless bubbleless aeration would be used for OLAND, as for instance in a membrane aerated biofilm reactor (Pellicer-Nacher *et al.*, 2010).

In contrast to methane, the formation of **N_2O and NO** occurs *in situ* (Fig. 3). As mentioned above, for three monitored full-scale OLAND-type of systems, 0.4–1.3% of the nitrogen load was emitted as N_2O (Joss *et al.*, 2009; Kampschreur *et al.*, 2009b; Weissenbacher *et al.*, 2010). These values can be considered acceptable, since they do not significantly exceed the N_2O emission values from nitrification/denitrification (Kampschreur *et al.*, 2009b). NO emissions are normally ranging from negligible to 0.01% of N load (Joss *et al.*, 2009; Kampschreur *et al.*, 2009b; Weissenbacher *et al.*, 2010), but NO is due to its low water solubility easily emitted when formed. The formation of N_2O and NO is complex and often difficult to predict due to the interplay of many parameters and contributors (Fig. 3).

AerAOB are probably the predominant responsibles for N_2O/NO emissions in OLAND, through so-called ‘nitrifier denitrification’. The dominant energy generation method by AerAOB is via aerobic metabolic pathways (Chain *et al.*, 2003). However, under oxygen limitation or anoxic conditions AerAOB, including *Nitrosomonas europaea* and *N. eutropha*, can use NO_2^- or N_2O_4 as electron acceptors and NH_3 or H_2 as electron donors to produce NO and N_2O, but no N_2 (Ritchie and Nicholas, 1972; Poth and Focht, 1985; Schmidt *et al.*, 2004). The oxygen level and regime (i) have profound effects on N_2O/NO emissions. At oxygen concentrations below 1 mg O_2 l^{-1}, N_2O productions up to 10% of the nitrogen load were observed (Goreau *et al.*, 1980). While NO can be produced under both aerobic and complete anoxic conditions (Ritchie and Nicholas, 1972; Yu *et al.*, 2010), N_2O formation by AerAOB was only detected at aerobic or microaerophilic conditions. The N_2O production by AerAOB mainly occurs at the transition from anoxic to aerobic conditions and is coupled to the presence of accumulated ammonium (Yu *et al.*, 2010). Besides oxygen, nitrite concentrations (ii) play an important role in AerAOB NO and N_2O emission

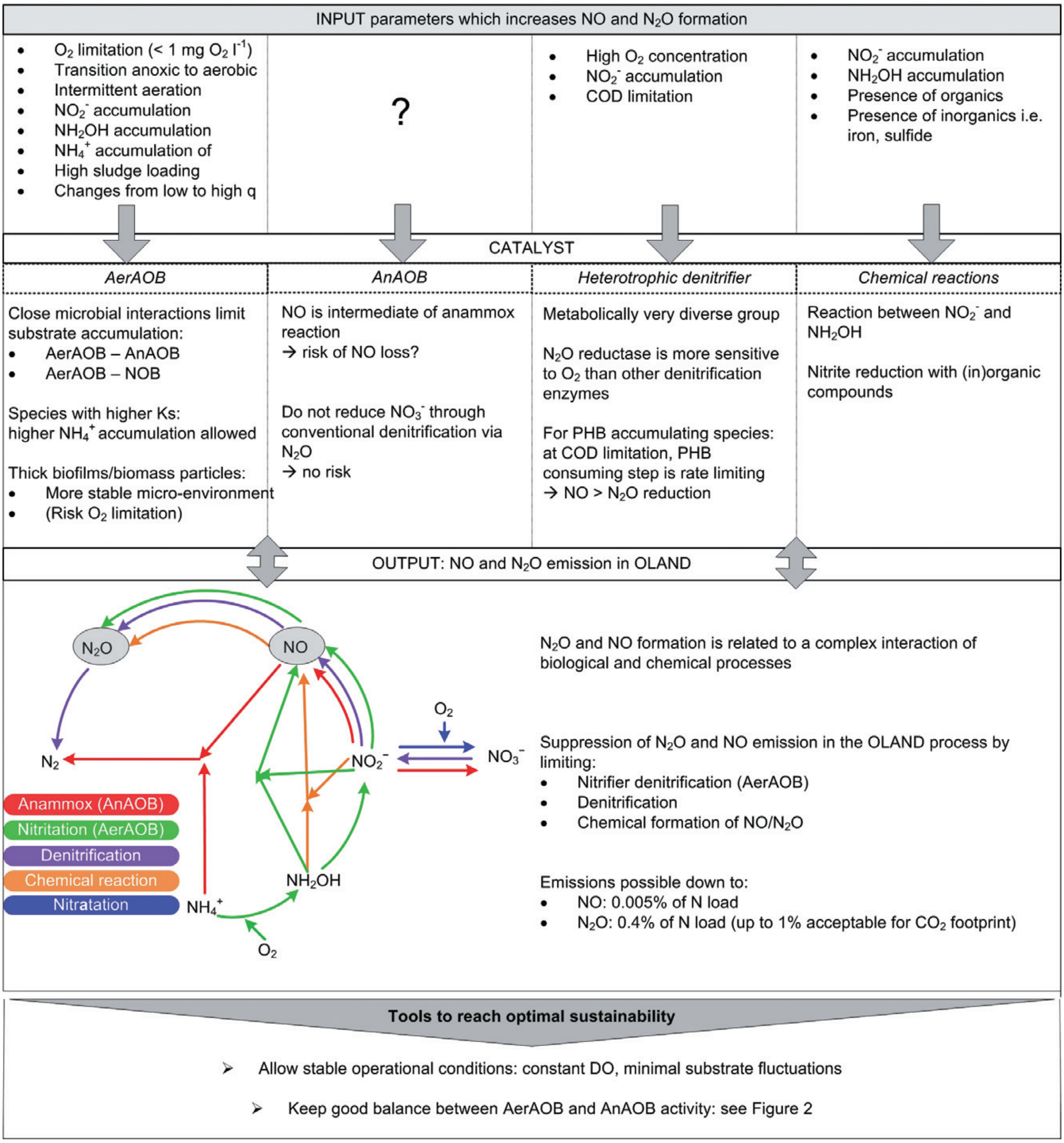

Fig. 3. OLAND MRM framework elaborated for the risk of N_2O and NO emissions in OLAND systems. q: specific microbial activity.

(Kampschreur *et al*., 2009a). Nitrite accumulation is a common malfunctioning in OLAND reactors (Section *Maximizing nitrogen removal efficiency*), and significantly increases AerAOB N_2O emissions (Colliver and Stephenson, 2000). High N_2O production is additionally linked to high specific activity or alternatively high metabolic rates (iii) during periods with high nitrogen flux through the catabolic pathways (Yu *et al*., 2010). Imbalanced enzyme expression in AerAOB performing close to their maximum specific activity (Yu *et al*., 2010), would suggest that, according to the Monod kinetics, working with an AerAOB community with lower substrate affinities (higher K_s) would yield a bigger risk of N_2O emission at lower substrate accumulations. Therefore, process configurations that work under constant specific activity values, which are linked to uniform DO and ammonium concentrations in the reactor, are expected to produce less N_2O. In this aspect, discontinuous technologies such as SBR systems have more potential for N_2O formation due to more frequent transitions. Slow and long feeding during the reaction phase would result in more stable nitrogen concentrations in the liquid phase (Wett, 2006) and could therefore potentially lower the risk of N_2O formation.

Although ammonium-oxidizing archaea (**AOA**) have recently been shown to produce N_2O (Santoro *et al.*, 2012), so far no AOA have been detected in OLAND systems, rendering their contribution to N_2O emissions likely nihil.

Chemical formation of NO/N_2O is another, potentially important pathway. An important factor is the accumulation of the AerAOB intermediate hydroxylamine. If this compound accumulates, it can either biochemically by AerAOB (Yu *et al.*, 2010) or purely chemically (van Cleemput, 1998) react with nitrite and form NO and N_2O. Moreover, chemical nitrite reduction at neutral pH can occur with ferrous iron (van Cleemput, 1998), sulfide (Grossi, 2009) or organic compounds (van Cleemput, 1998) and will also result in the formation of NO and N_2O.

It should be noted that N_2O/NO emissions can also be lowered by a decrease of **stripping**. It was described that NO and N_2O emissions increased with the air flow rate because the concentration of both gases remained constant in the gas phase. Therefore NO and N_2O emissions can be minimized by minimizing the airflow rate under optimal conditions (Kampschreur *et al.*, 2008) or by using bubbleless aeration in a MABR (Pellicer-Nacher *et al.*, 2010).

Although **denitrification** is limited in OLAND systems, typical OLAND conditions promote NO/N_2O emissions by denitrifiers. A high nitrite concentration during denitrification suppresses the denitrification rate and therefore leads to NO and N_2O accumulation (von Schulthess *et al.*, 1995). Also COD limitation during denitrification is a known cause for NO or N_2O accumulation (von Schulthess and Gujer, 1996; Chung and Chung, 2000). Moreover, as oxygen inhibits both the synthesis and activity of denitrifying enzymes and N_2O reductase is the most oxygen-sensitive denitrifying enzyme (Otte *et al.*, 1996), the low DO values typical for OLAND can lead to N_2O emission by denitrifiers.

Although NO is a likely one of the **AnAOB** intermediates (Strous *et al.*, 2006), it is unlikely that AnAOB leak NO, and therefore AnAOB probably do not contribute to NO emissions. Due to the absence of N_2O reductase in the AnAOB genome, N_2O production is not expected during anammox.

Overall, stable conditions allowing for constant specific microbial activities and avoiding accumulation of nitrite and ammonium likely lead to lower NO and N_2O emissions from OLAND systems (Fig. 3). However, the oxygen-limited conditions needed to avoid NOB activity or caused by well settling sludge remain a risk factor. Note that preliminary measurements of intermittent versus continuous aeration could not point out lower N_2O emissions for the latter (Joss *et al.*, 2009). It is expected that future long-term, on-line measurements will reveal the best aeration level and regime to minimize NO/N_2O emissions.

OLAND enabling energy-positive sewage treatment

Until now, the OLAND process has been successfully applied for medium and high-strength nitrogen wastewaters (> 0.2 g N l^{-1}) such as landfill leachate and digestates from sewage sludge, specific industrial streams and concentrated black water. For centralized domestic wastewater treatment, the inclusion of OLAND to treat sludge digestates in the side stream of a conventional wastewater treatment plant (WWTP) lowered the overall plant energy requirements with about 50% (Siegrist *et al.*, 2008). Furthermore, Wett and colleagues (2007) demonstrated energy autarky by including OLAND in the side-stream of a two-stage activated-sludge (AS) process ('AB Verfahren'). In the mainstream, the first AS unit (stage A) has a very high loading rate (SRT $\approx$ 0.5 day), and the second AS unit (stage B) has a low loading rate (SRT $\approx$ 10 days). Besides these energy saving options with OLAND in a side stream, a novel treatment scheme was recently proposed, bringing OLAND to the main treatment stream substituting the previous B stage (Wett *et al.*, 2010a; Verstraete and Vlaeminck, 2011). This even allows the electrical energy recovery and savings to exceed the electrical energy input. Moreover, instead of a biological concentration of the sewage, an enhanced physicochemical concentration step can be applied, involving enhanced sedimentation, dissolved air flotation and/or membrane filtration, separating more than 75% of the COD load from the main stream (Verstraete *et al.*, 2009).

A first difference between treatment of the main or side stream is the **lower nitrogen concentration** to be treated by OLAND (Fig. 4). Domestic wastewater after advanced concentration will still contain most of the nitrogen while around 75% of the COD is removed and sent to the digester, resulting in main stream wastewater with around 30–100 mg N l^{-1} and 113–300 mg COD l^{-1} (Metcalf and Eddy, 2003; Tchobanoglous *et al.*, 2003; Henze *et al.*, 2008). Taking into account the affinity constant of the AerAOB and AnAOB for ammonium i.e. 2.4 and 0.07 mg N l^{-1} respectively and the AnAOB affinity constant for nitrite of 0.05 mg NO_2^--N l^{-1} (Lackner *et al.*, 2008), these low concentrations as such should not be a problem. However, these low substrate conditions could imply that the microbial community will have to work at lower metabolic and lower growth rates compared with side stream processes which allow higher concentrations in the reactor.

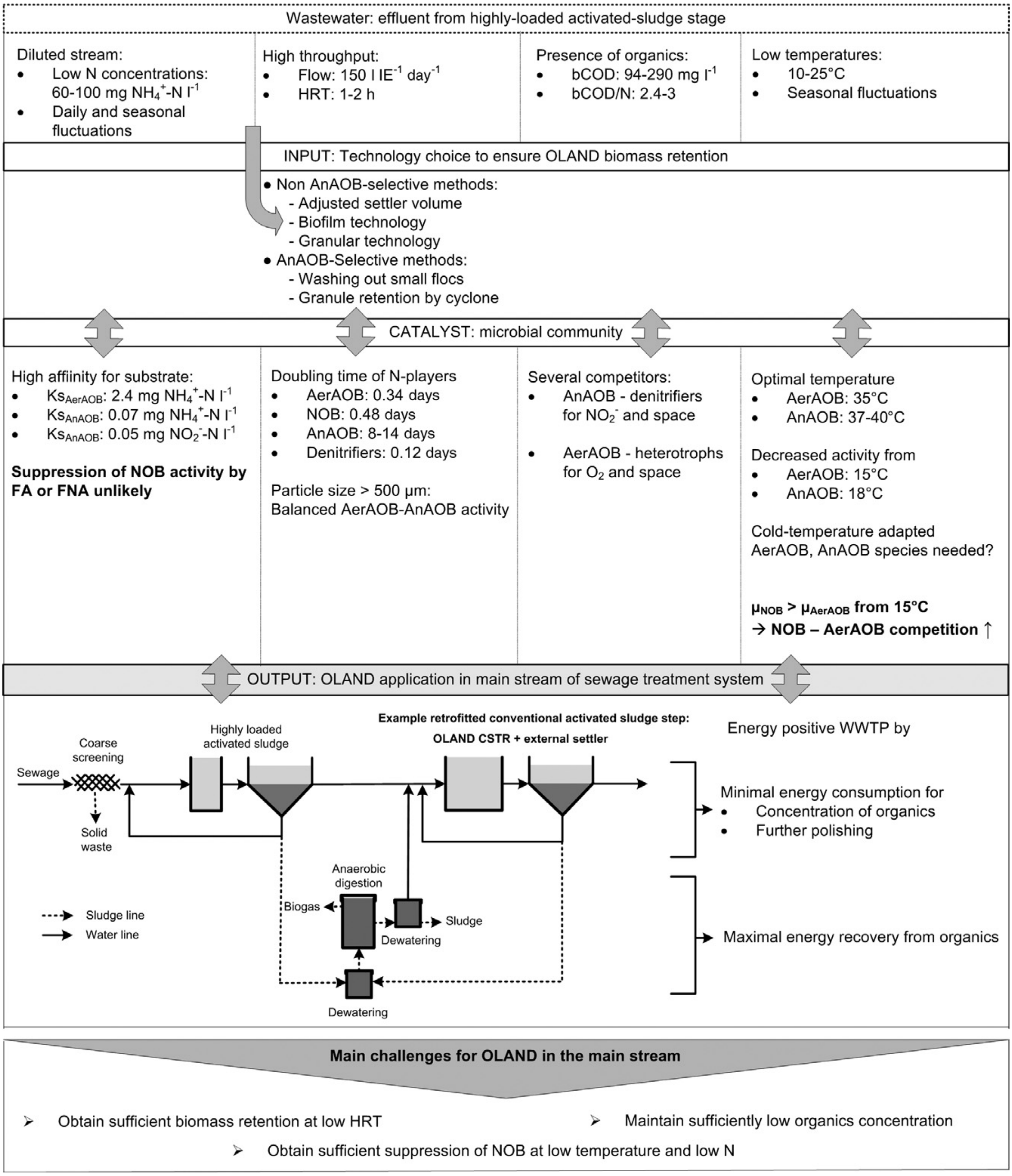

Fig. 4. OLAND MRM framework elaborated to elucidate challenges for application of OLAND in the main stream of a sewage treatment plant.

To obtain high nitrogen removal rates at low concentrations, **low hydraulic residence times** (HRT) are needed for main stream treatment, in the order of hours and hence about 24 times lower than for side stream treatment (Joss *et al*., 2009; Weissenbacher *et al*., 2010). Given the slow biomass growth of the AnAOB, good biomass retention is a prerequisite for OLAND activity under low HRT. Sufficient AnAOB retention can be obtained by separating the retention of small aerobic and larger anoxic particles, which selectively will favour the AnAOB retention (see challenge 1). On the other hand, by increasing the external settler volume, applying a granular technology (Abma *et al*., 2010) or using biofilm-based technology (De Clippeleir *et al*., 2011a), the total SRT can be increased.

Besides the survival of the AnAOB under low hydraulic retention times, an important challenge is to obtain a good microbial balance and activity at **low temperature**. Some studies already described the effect of lower temperatures on the separate activity of AnAOB, AerAOB and NOB. However, limited information exists about the microbial balance of these three groups under OLAND conditions at low temperature. Although AerAOB activity decreased with 50% at a temperature interval from 27°C to 15°C, limited aerobic ammonium oxidation could be observed at 5°C (Guo *et al*., 2010). For AnAOB the critical temperature at which it was difficult to obtain AnAOB activity was 18°C (Dosta *et al*., 2008), although several AnAOB species are found in nature at −1°C to 15°C (Dalsgaard *et al*., 2005). It is not clear whether other AnAOB species, more related to the cold-temperature marine genus '*Candidatus* Scalindua', will take over from the WWTP types '*Candidatus* Kuenenia and Brocadia' at colder temperatures. For inoculation purposes it is important to elucidate if the same AerAOB and AnAOB species do the job at cold temperatures or other species take over. In the latter case, the first start-ups will be slower again due to the absence of appropriate inoculation sources. The possible loss of both AerAOB and AnAOB activities compared with higher temperatures will result in the accumulation of nitrite and a decrease in oxygen uptake (Wett *et al*., 2010b). It will therefore be important to adjust the oxygen regime to impose oxygen-limited conditions to the AerAOB and by this avoid inhibition of AnAOB by nitrite. However, due to the decreased total activity, longer HRT or higher biomass concentrations will be necessary to obtain the same volumetric nitrogen removal rates. Beside the microbial balance between AerAOB and AnAOB, the lower temperature will have an effect on the NOB–AnAOB balance. At temperatures lower than 15°C, the growth rate of NOB will become higher than the growth rate of AerAOB (Hellinga *et al*., 1998) and it will therefore not be possible to wash out NOB based on overall or even selective sludge retention. The main challenge in this application will therefore be the suppression of NOB at low temperature and low nitrogen concentration (low free ammonia and low nitrous acid).

The last point of attention concerning new inputs in this application domain is the presence of **organics**, i.e. moderate levels of bCOD (90–240 mg l^{-1}) in the wastewater. Depending on the raw sewage strength, COD/N ratios between 2.4 and 3 are expected after the concentration step, which is on the edge of the described limit for successful OLAND (Lackner *et al*., 2008). On one hand, the presence of organics will facilitate DO control at low DO levels due to heterotrophic aerobic activity. On the other hand, competition between heterotrophic denitrification and anammox will take place for nitrite. These processes have already been demonstrated to successfully coexist at a COD/N ratio of 2.2 (Desloover *et al*., 2011). It is anticipated that higher nitrogen sewage levels together with the higher sewage temperature which will facilitate OLAND treatment in the main stream, will exist in the main stream due to further dilution preventions (Henze, 1997; Brombach *et al*., 2005).

Finally, according to this MRM approach (Fig. 4), to be able to apply OLAND in the main stream of the WWTP, the challenges of biomass retention at low HRT and NOB suppression at low temperature should be first encountered.

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