POLITECNICO DI MILANO
Facoltà di Ingegneria Civile, Ambientale e Territoriale

Corso di Laurea Magistrale in Ingegneria per l'Ambiente e il Territorio

CHALLENGES OF MAINSTREAM ANAMMOX IN A TWO STAGE REACTOR: PARTIAL NITRITATION AND HETEROTROPHIC COMPETITION.

Relatrice: prof. Francesca Malpei Politecnico di Milano

Correlatore: ing. Michele Laureni Eawag (Swiss Federal Institute of Aquatic Science and Technologies)

Correlatore: ing. Adriano Joss Eawag (Swiss Federal Institute of Aquatic Science and Technologies)

Tesi di laurea di: Sara Melziade
Matr. 787009

Anno Accademico 2013-2014

Indice

Abstract

In this thesis the principal challenges in the application of mainstream anammox in municipal wastewater treatment plant are discussed. The study was conducted in a lab scale two-stage nitritation/anammox system.

Partial nitritation in Sequencing Batch Reactor (SBR) was run for four months at ambient temperature (15-20 °C). At the beginning, the effluent reported an increasing nitrite production and, after a period, despite of changes in operational set-up (sludge age and oxygen control), a production of nitrate. It was found that high rate biological COD removal is feasible at ambient temperature and constitute a proper pre-treatment for a subsequent anammox-based process. On the other hand, in the adopted conditions the correct value of sludge age for partial nitritation at ambient temperature has to be serched in the range between 2-4 days. In addition further improvement in oxygen control has to be done to obtain AOB retention and NOB washout.

Through batch experiments, in anammox reactor, it was also investigated the competition for substrate between anammox and heterotrophs and the mechanisms involved in organic matter degradation. In the presence of easy biodegradable organic carbon (COD) it was reported a storage capacity. From the comparison with different sludge fed in (semi-)continuous mode, it is proposed that discontinuous feeding boosts the organics storage potential of the sludge. This hypothesis needs further investigation to be proved.

Regularly samples were taken from reactors, prepared with FISH (Fluorescence In Situ Hybridization) techniques and analysed using Confocal Microscope.

Abstract in italiano

In questa tesi sono stati analizzate e discusse le principali problematiche legate all'utilizzo di anammox applicato alla linea acque nell'ambito del trattamento delle acque reflue urbane. Lo studio e' stato contotto su un sistema a due stadi nitrosazione/anammox a scala di laboratorio.
La nitrosazione in reattore SBR è stata condotta per quattro mesi a temperatura ambiente (15-20°C). Inizialmente, l'analisi degli effluenti ha evidenziato un'incremento della produzione di nitriti. Dopo un certo periodo, indipendentemente dai cambiamenti nelle condizioni sperimentali (età del fando e ossigeno), si è anche riscontrato un'incremento della produzione di nitrati. Si è mostrato che un elevato tasso di rimozione biologica di carbonio organico è fattibile a temperatura ambiente. Ciò rappresenta un adeguato pre-trattamento per il successivo processo nel reattore anammox. Nelle condizioni sperimentali considerate, il valore ideale di età del fango per la nitrosazione a temperatura ambiente è risultato essere compreso tra 2 e 4 giorni. Un ulteriore miglioramento nel controllo dell'ossigeno sembra essere necessario per ottenere un buon mantenimento dei batteri *Nitrosomonas* per l'ossidazione dell'ammoniaca a nitriti e il dilavamento dei batteri Nitrobacter per l'ossidazione dei nitriti a nitrati.
Attraverso esperimenti batch nel reattore, si è investigata, inoltre, la competizione per il substrato tra anammox e eterotrofi. Sono anche stati studiati i meccanismi coinvolti nella degradazione della materia organica. In presenza di carbonio organico rapidamente biodegradabile, si è registrata una certa capacità di storage. Attraverso l'analisi dei risultati ottenuti confrontando differenti fanghi alimentati in modalità (semi-)continua, si è proposto che un'alimentazione discontinua possa incrementarne le capacità di storage. Questa ipotesi, comunque, richiede ulteriori analisi e uno studio più approfondito.
Regolarmente, sono stati estratti campioni dai reattori, che sono successivamente stati preparati con tecniche FISH ed analizzati utilizzando la microscopia confocale.

Estratto in italiano

Nel corso degli ultimi decenni l'uomo, con le sue attività industriali, si è inserito nel ciclo naturale dell'azoto modificandone i flussi. Ciò ha provocato cambiamenti negli ecosistemi con effetti negativi per l'ambiente. L'ecosistema acquatico risente in maniera significativa di concentrazioni eccessive di azoto. Esso, infatti, risulta tossico per le specie viventi, in particolare per i pesci; al contrario, promuove la crescita di alcune piante acquatiche favorendo il fenomeno dell'eutrofizzazione e il consumo di ossigeno utilizzato dai batteri per la decomposizione delle alghe morte. Particolare attenzione, inoltre, deve essere riposta nella presenza di nitrati nelle acque di approvigionamento rischiosi per la salute umana, soprattutto dei bambini. Il decreto legislativo 152/06, in attuazione della direttiva della Comunità Europea in materia di trattamento delle acque, pone alcuni vincoli sulle concentrazioni in uscita dagli impianti di trattamento delle acque reflue. E'prassi consolidata da diverso tempo la presenza di un'unità di rimozione dell'azoto ammoniacale di nitrificazione/denitrificazione in varie declinazioni impiantistiche.

Questo lavoro di tesi si concentra sulla presentazione di un nuovo processo, che consente di operare la rimozione dell'azoto attraverso una scorciatoia rispetto al processo tradizionale. L'ossidazione anaerobica dell'ammoniaca utilizza dei batteri autotrofi (anammox) che ossidano l'ammoniaca presente nei reflui, utilizzando i nitriti come accettori di elettroni, e che producono azoto molecolare. Il processo anammox viene operato per via anaerobica risparmiando il 60% di ossigeno da fornire al processo e il 100% di carbonio organico rispetto ai tradizionali impianti. Inoltre, a causa del lento tempo di duplicazione di questi batteri, la produzione di fango di supero è notevolmente ridotta consentendo di risparmiare il 30-40% dei costi totali.
Questo processo negli ultimi anni è stato impiegato, con successo, per il trattamento delle acque molto concentrate da digestione anaerobica ad alta temperatura. Una delle sfide principali è l'applicazione di anammox sulla linea acque degli impianti di trattamento di reflui urbani a temperatura

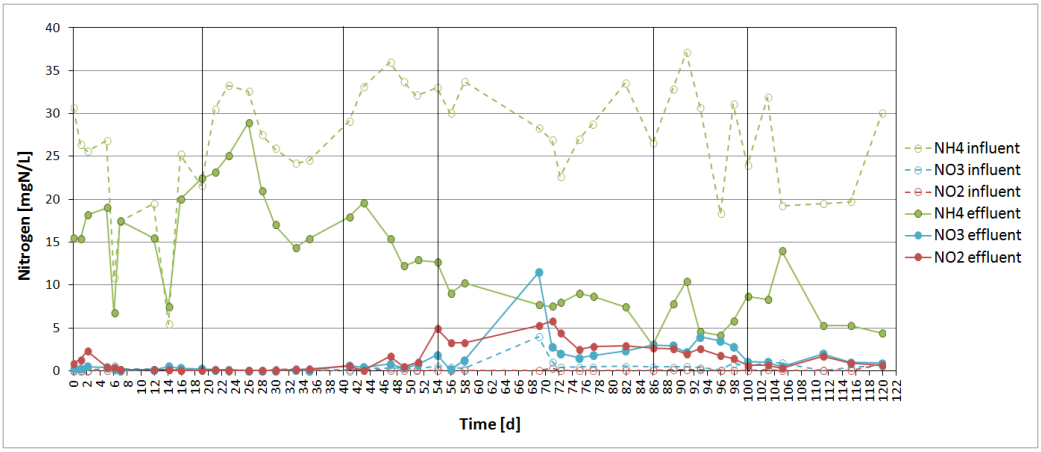

Figura 1: Principali parametri dell'influente ed efflunete del reattore di nitrosazione (R1).

ambiente. Questi ultimi infatti presentano maggiori e più diluiti volumi da trattare, che risulta economicamente poco conveniente riscaldare.
In questo studio è stata scelta una configurazione di reattori a due stadi per la rimozione di azoto ammoniacale. Nel primo reattore avviene la nitrosazione: l'ossidazione di metà dell'azoto ammoniacale a nitriti ad opera di batteri aerobi. Nello step successivo avviene la reazione governata dai batteri anaerobi in cui l'ammoniaca restante viene ossidata ad azoto molecolare utilizzando nitriti come accettori di elettroni.
L'obiettivo iniziale del lavoro era rispondere a due quesiti:

- E' possibile realizzare la nitrosazione a bassa temperatura in un'ottica di applicazione del processo sulla linea acque di un impianto di trattamento di reflui urbani?
- I batteri anammox sono in grado di competere con altre popolazioni, in questo caso eterotrofi, eventualmente presenti nel refluo?

Il primo capitolo tratta gli obiettivi di questo lavoro con una breve introduzione al processo.
Il secondo capitolo, partendo dalla descrizione del ciclo dell'azoto e degli impatti dell'azoto sull'ecosistema acquatico, arriva a descrivere le principali tecnologie attualmente in uso per la rimozione dell'azoto. Si presenta, poi, il processo anammox dalla sua scoperta alla fisiologia, per terminare con la presentazione delle diverse conformazioni impiantistiche studiate.
Nel terzo capitolo si illustra il set-up impiantistico dei reattori utilizzati. Per ogni reattore sono descritte le fasi e il ciclo. Sono anche riportate le descrizioni degli esperimenti batch effettuati nei diversi reattori. Un ultimo

paragrafo viene infine dedicato alle analisi chimiche dei campioni presi durante gli esperimenti e la fase di operativa.
Il quarto e quinto capitolo forniscono l'interpretazione dei dati ottenuti durante il periodo di operazione e le conclusioni.
Il reattore di nitrosazione ha funzionato per quattro mesi a temperatura ambiente. I dati dell'andamento di nitriti, nitrati e ammoniaca nell'influente e nell'effluente sono riportati in Figura 1. Dopo un primo momento di stasi, in seguito all'attivazione del controllo dell'ossigeno basato sulla rimozione di ammoniaca, nell'effluente hanno incominciato a essere rilevati nitriti (fase III). Questo suggerisce che una limitata aerazione, piuttosto che l'aumento dell'età del fango (passato da 2 a 4 giorni tra la fase I e la fase II), rappresenti il fattore limitante per la nitrosazione. In fase IV, in seguito all'incremento della soglia di rimozione dell'ammoniaca, il progressivo aumento di nitriti è stato seguito, a distanza di alcuni giorni, dal manifestarsi di nitrati. La concentrazione di ossigeno è stata quindi ridotta per arginarne la crescita, ma al giorno 75 la tendenza si è comunque invertita e i nitrati sono diventati preponderanti. L'età del fango è stata quindi riportata ai valori iniziali per tornare alla condizione di sola rimozione del COD. A questo proposito fattori chiave, per la corretta operazione del reattore, sono stati individuati nell'età del fango (2-4 giorni) e nella concentrazione di ossigeno, ma l'esperimento è stato interrotto prima che fosse possibile raggiungere un funzionamento stabile.
Nel reattore anammox sono stati condotti esperimenti batch, alimentando carbonio organico rapidamente biodegradabile in assenza di ammoniaca (Figura 2). Nonostante la quasi totale rimozione del carbonio organico aggiunto, si è vericata solamente una lieve riduzione della concentrazione di nitrati. Questo non è compatibile con l'attività dei batteri eterotrofi e il consumo di COD è stato, quindi, ipotizzato attribuibile ad un fenomeno di *storage*. In seguito all'aggiunta di ammoniaca, l'esperimento ha mostrato un incremento nel consumo sdi nitriti. Questo dimostra che durante l'esperimento la principale via di consumo di nitriti era anammox e che i batteri anammox sono in grado di competere con altre popolazioni batteriche come i batteri eterotrofi. Analoghi esperimenti condotti con fanghi alimentati in maniera differente (in continuo) forniscono risultati diversi. L'ipotesi che l'alimentazione discontinua favorisca lo *storage* rimane, tuttavia, da confermare con ulteriori analisi.

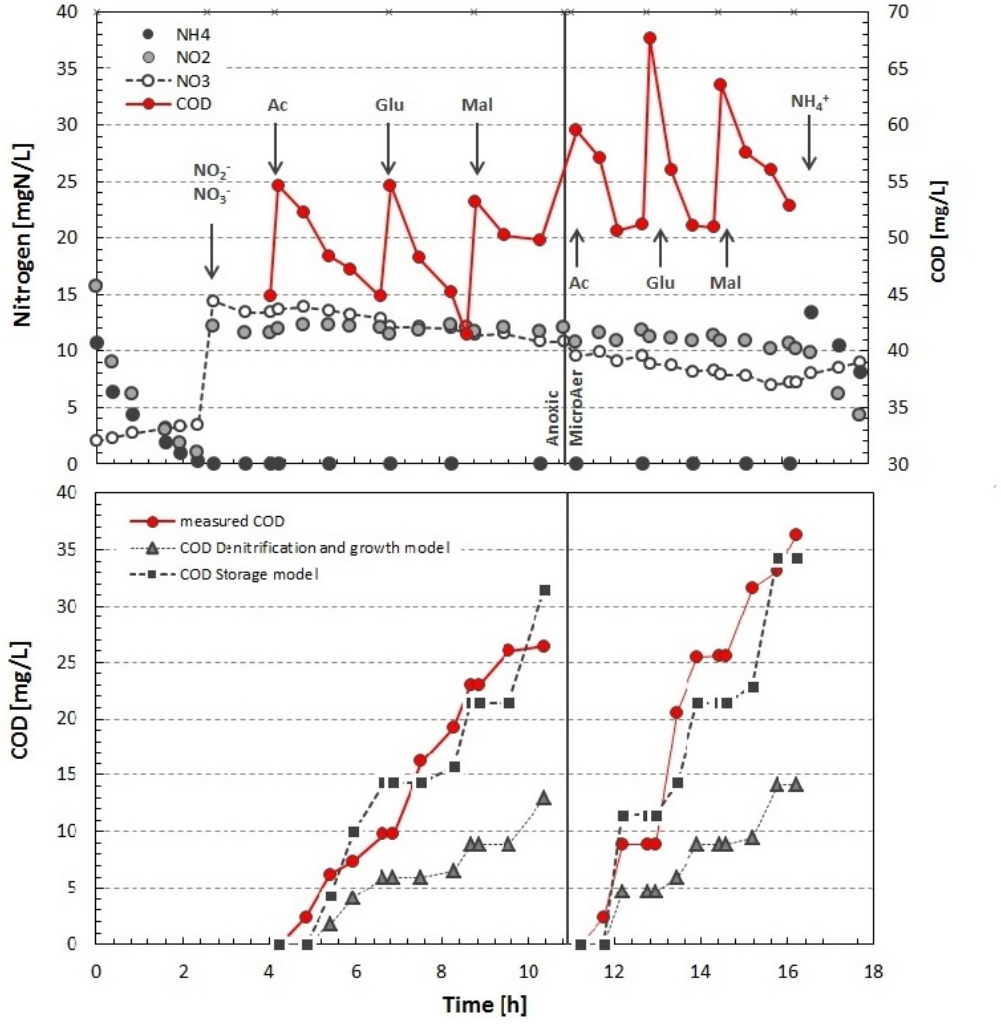

Figura 2: Esperimento batch. Sopra: concentrazioni nel reattore anammox durante l'esperimento; sotto: curve cumulative del COD nell'esperimento.

Capitolo 1

Introduction and objectives

Nitrogen plays an important role in the natural ecosystem. The increasing production of antropogenic nitrogen interfered with the natural cycles. In aquatic ecosystem nitrogen can lead to important problems from eutrophication and fish toxicity to human health hazardous. Recent legislations of Europee Union and Italy regulated the discharge of municipal wastewater including nitrogen concentration limit.
Traditionally nitrogen removal was led in a two steps process called nitrification/denitrification. Ammonium is first oxidized to nitrite and then to nitrate because nitrogen is usually present in the form of ammonium and organic nitrogen. In the second step nitrate is reduced to nitrite and then to nitrogen gas. Anammox process creates a shorcut in the conventional process saving 30-40% of the overall costs. Anaerobic ammonium oxidation bacteria (anammox) are capable of autotrophic ammonium oxidation with nitrite as electron acceptor and CO_2 as carbon source.

The overall topic of the present thesis was the challenge of applying anammox-based processes for the direct treatment of municipal wastewater (mainstream anammox). It is well known how to apply anammox in side-stream configurations (digester supernatant) and for the treatment of industrial wastewaters with low concentration of COD and high total ammonia content. Conversely, several challenges are to be faced with municipal wastewaters (MWW): ammonia concentrations are low (could result in potential growth and rate limitations), COD content is relatively high (might foster the growth of competing heterotrophs) and, due to the large volumes involved, it is not possible to control the temperature.
In this study, a two-stage configuration for partial nitritation-anammox was chosen to investigate the two processes separately. For the first step (partial nitritation) the aim was to test the feasibility of the process at ambient

temperature, with particular focus on the balance between ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) and on the overall nitrogen mass balance. In the second anoxic reactor (anammox) the goal was to understand the microbial community interactions: the competition for substrate (mainly NO_2^-) between anammox and heterotrophs and the mechanisms involved in organic matter degradation.

The study was integrated with microbiological analysis using FISH (Fluorescence In Situ Hybridisation) technique to support a deeper comprehension about the presence of different bacterial population in the sludge.

Capitolo 2

State of the art

2.1 Water pollution and Nitrogen cycle

Nitrogen has an important role in the natural ecosystem because it is present in small amount in the form available for plants. The availability of nitrogen for agriculture use, produced with the industrial process Haber-Bosch, fostered the growth of food production with positive consequences on the population growth. On the other hand the nitrogen produced for food and due to fossil combustion modified the natural nitrogen cycle doubling the turnover rates of the Earth (Figure 2.1) (Galloway *et al.*, 2004). That had repercussion on atmosphere, marine and terrestrial system with a cascade effect that means the same nitrogen atom can be responsible of multiple negative effects (Galloway *et al.*, 2003).
In wastewater the presence of nitrogen can produce negative effects:

- Toxicity of ammonia for fish and other aquatic organisms;
- Nitrogen is a nutrient that can foster extremely the growth of plant. Bacteria uses oxygen for the decomposition of dead algae;
- Eutrophication can lead to algae bloom with a loss in the economical, aesthetic and recreational function of lakes and basins. It can lead to a change in the balance of organism with a loss of diversity;
- Human health hazardous caused by the presence of nitrate in drinking water. Methemoglobinemia is also called blue baby syndrome and it is a disease that stop the mechanism of transportation of oxygen.

Nitrogen is present in nature in different forms depending on the oxidation state. It can assume values between -3 for ammonium and organic nitrogen

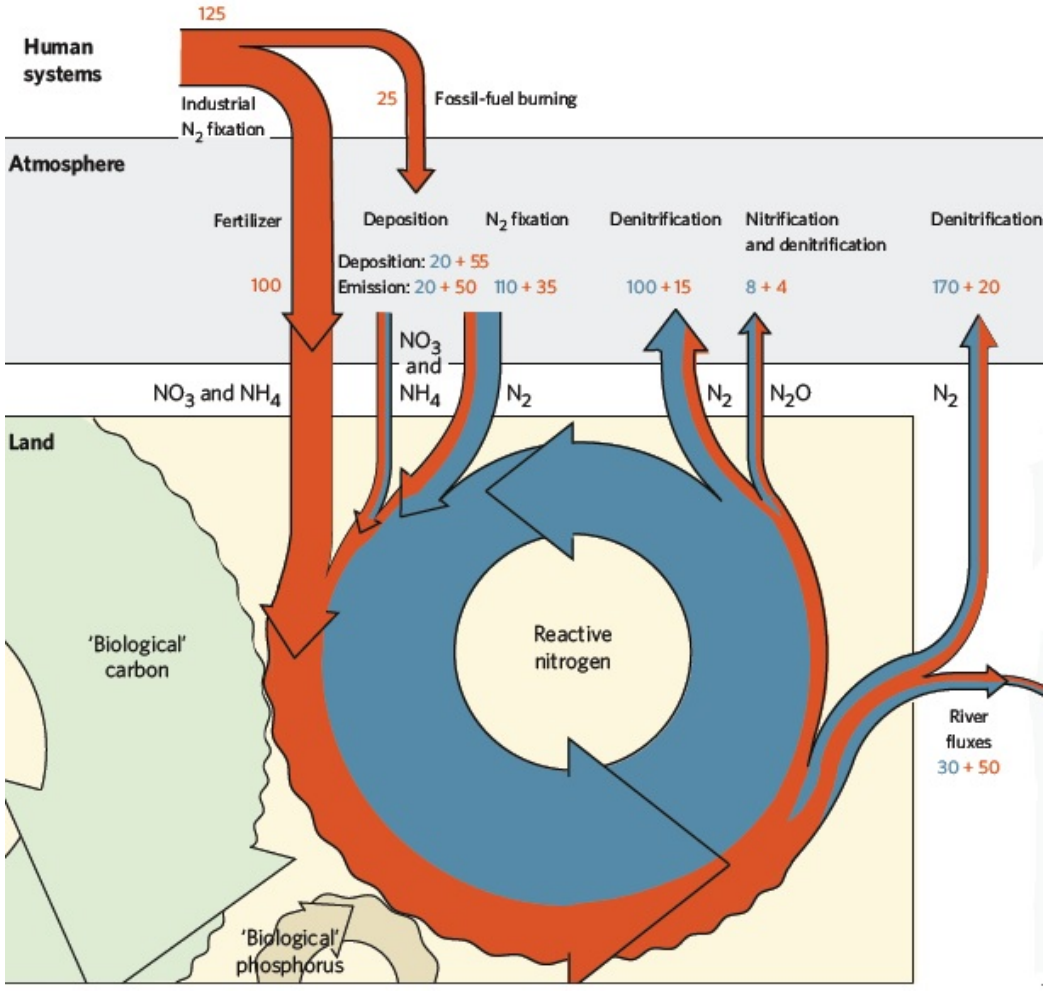

Figura 2.1: Nitrogen cycles (Gruber and Galloway, 2008; Galloway *et al.*, 2003)

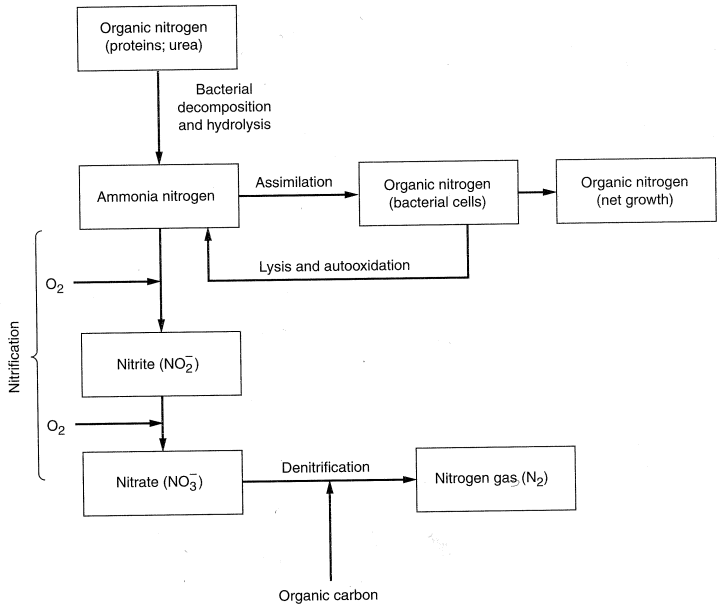

Figura 2.2: Nitrogen transformation in biological treatment processes (Metcalf and Eddy, 2004)

.

and +5 for nitrate.
The organic nitrogen is directly used by some bacteria, leguminous and algae or indirectly used by plant to produce organic nitrogen compound. The nitrogen removed from the environment is returned with death event and bacteria decay. Urine present in animals excretions is hydrolysed by enzymes. All these phenomenons are responsible of ammonia nitrogen production from organic nitrogen.
In wastewater the most common species are organic nitrogen, ammonium and nitrate. In biological treatment systems assimilation of ammonia can occur for the synthesis of new cells. Furthermore in a two steps process, called nitrification, where ammonia is oxidized to nitrate with nitrite as intermediate.
In anoxic condition heterotroph denitrifiers are able to reduce nitrate to nitric oxid (NO), nitrous oxid (N_2O) and nitrogen gas (N_2). This is the so called denitrification that takes nitrogen out of aquatic system and contributes to nitrogen in the atmosphere (Figure 2.2).

2.2 Conventional technologies for Nitrogen removal

In recent period, European Union ratified regulation about sewage treatment (Directive 91/676/CEE and 91/271/CEE). In Italy the legislation for the discharge of urban wastewater is regulated by legislative decree 152/06 (Table 2.1).
To respect this limit values nitrogen removal is needed. The two steps process (nitrification/denitrification) is well established in municipal wastewater treatment plant.

Nitrification is a biological process in which ammonia is oxidized to nitrite and nitrite is oxidized to nitrate. This process is carried out by autotrophic bacteria that use inorganic carbon as carbon source and oxygen as electron acceptor. During the first step of nitrification Aerobic Oxidizing Bacteria (AOB)(Nitrosomonas bacteria and others) oxidise ammonium to nitrite (Reaction 2.1). In the second Reaction (2.2) Nitrite Oxidizing Bacteria (NOB) (Nitrobacter and others) oxidize nitrite to nitrate.

$$NH_4^+ + \frac{3}{2}O_2 \rightarrow NO_2^- + 2H^+ + H_2O \tag{2.1}$$

$$NO_2^- + \frac{1}{2}O_2 \rightarrow NO_3^- \tag{2.2}$$

The total oxidation reaction is:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \tag{2.3}$$

The oxygen required is 4.43 gO_2/gN for ammonium oxidation and 1.12 gO_2/gN for nitrite oxidation not considering ammonia for cell synthesis (0.16 g of new cell for g of ammonia nitrogen oxidized).
The total reaction requires a substantial amount of alkalinity (7.1 g $CaCO_3$ for each gram of NH_4^+-N oxidized).
The NOB growth rate is faster than AOB that usually has slower kinetics. The first step is the limiting one. Under different temperature, pH and dissolved oxygen condition, NOB can be washed-out. Hellinga *et al.* (1998) reported that at temperature below 15 °C, NOB requires a minimum sludge age higher than that for AOB (Figure 2.8).
At low dissolved oxygen value, lower than 0.5 mg/L, AOB have a higher affinity constant for O_2, they are not inhibited by O_2.

Denitrification is a biological process in which nitrate is reduced to nitrite (Reaction 2.4) and nitrite to nitrogen gas (Reaction 2.5). The overall denitrification reaction with methanol as carbon source is reported in Reaction 2.6. Heterotrophic bacteria use nitrite and nitrate as electron acceptor for oxidation of biodegradable organic compound. Heterotrophs prefer free oxygen as electron acceptor when it is available (aerobic metabolism), otherwise nitrite and nitrate are used (anoxic metabolism).
Denitrification requires the availability of an organic carbon source as electron donor. Different alternatives are possible:

- Internal carbon that is present in the wastewater. It assures a sufficient kinetics rate without any external addition and provides organic removal at the same time;
- External carbon easy biodegradable (methanol, acetic acid) when carbon is not present in wastewater;
- Endogenous carbon composed by the biodegradable organic part from the lysis of bacteria cells.

$$3NO_3^- + CH_3OH \rightarrow 3NO_2^- + CO_2 + 2H_2O \tag{2.4}$$

$$2NO_2^- + CH_3OH \rightarrow N_2 + CO_2 + H_2O + 2OH^- \tag{2.5}$$

$$6NO_3^- + 5CH_3OH \rightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^- \tag{2.6}$$

Denitrification produces alcalinity that compensate half of that consumed during nitrification.

2.2.1 Conventional nitrification/denitrification

Biological nitrogen removal can be obtained with a first phase of nitrification (because nitrogen is usually not present in the nitric form) followed by a denitrification phase where nitrate is converted to nitrogen gas. In the first step takes place the removal of the organic carbon that, instead, can be useful in the denitrification process. According to this, different process models are possible. There will be explained in the following paragraphs the two main process: pre-denitrification and post-denitrification.

Tabella 2.1: Discharge limit values into surface water of legislative decree 152/06.

	10000-100000 [AE]		> 100000 [AE]	
	Conc [mgN/L]	Reduction [%]	Conc [mgN/L]	Reduction [%]
Total nitrogen	≤ 15	70-80	≤ 10	70-80

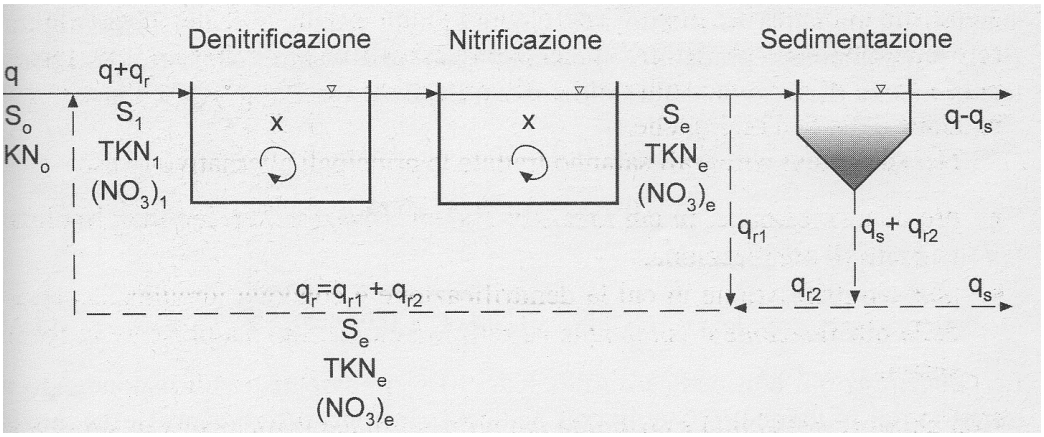

Figura 2.3: Pre-denitrification system for biological nitrogen removal (Bonomo, 2008).

Pre-denitrification

This process uses internal organic carbon that arrives in denitrification with the main flow and the recirculation flow from the second reactor of nitrification. The latter contains nitrate, produced in nitrification, that is reduced to nitrogen gas with consumption of organic carbon in the first reactor. In the aerobic phase occurs the oxidation to nitrate of organic nitrogen and ammonia and the removal of the remaining organic carbon. After the two reactors there is a unique clarifier (Figure 2.3).

Post-denitrification

It is applicable on rich-nitrate wastewater that include:

- special industrial wastewater or contaminated ground-water layer;
- wastewater that contains organic nitrogen and ammonium with organic carbon.

A first step of pre-denitrification using internal carbon is necessary and then post-denitrification to enhance nitrogen removal.
It is possible to use both internal and external organic carbon, but the least is preferable.
In the independent post-denitrification (Figure 2.4) an oxidation reactor allows nitrogen gas to be stripped out because, otherwise, it will prevent the next settling phase. It is also removed the organic carbon dosed in excess.
In the scheme where post-denitrification follows a first step of pre-denitrification (Figure 2.5) the goal is to remove the nitrogen slipped throw the first reactor. The amount of external organic carbon used is less than that used in the first scheme. It is possible to utilize the internal organic carbon, but the kinetics are too low and the volume too wide.
The reactor configuration for internal carbon use can be adopted only to treat low nitrogen flow. This is because only a part of the total inflow is provided to nitrification. The remaining flow is sent directly to post-denitrification for carbon supply (Figure 2.6).

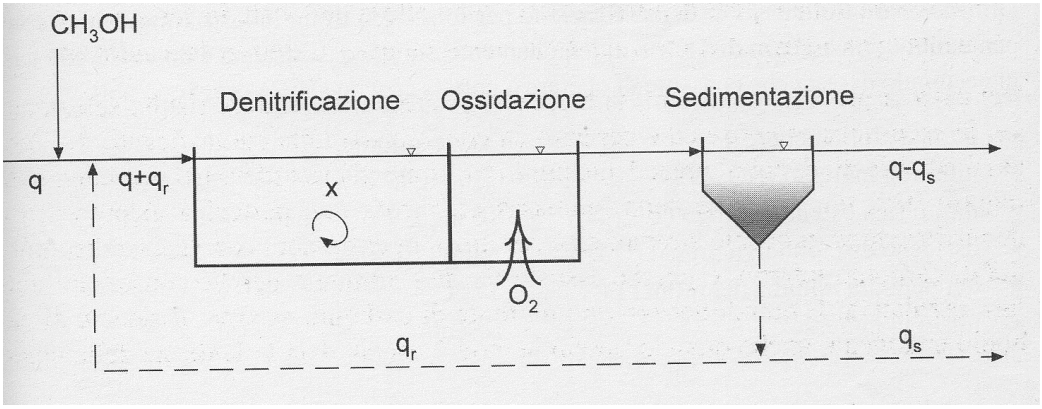

Figura 2.4: Post-denitrification system for biological nitrogen removal with external carbon source (Bonomo, 2008).

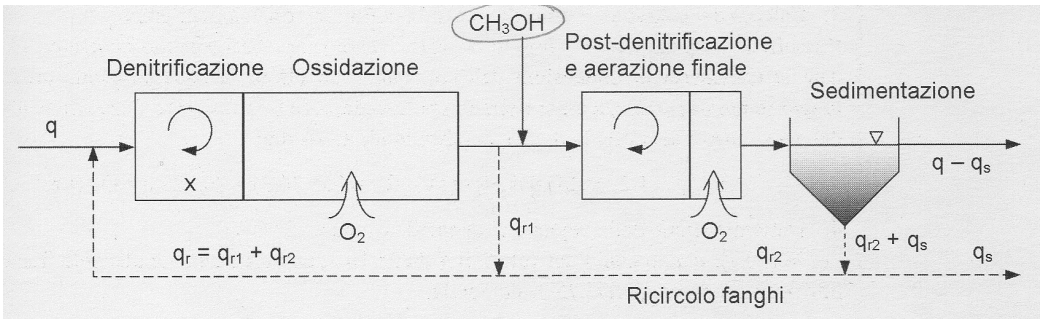

Figura 2.5: Pre and post-denitrification system for pushed biological nitrogen removal with external carbon source (Bonomo, 2008).

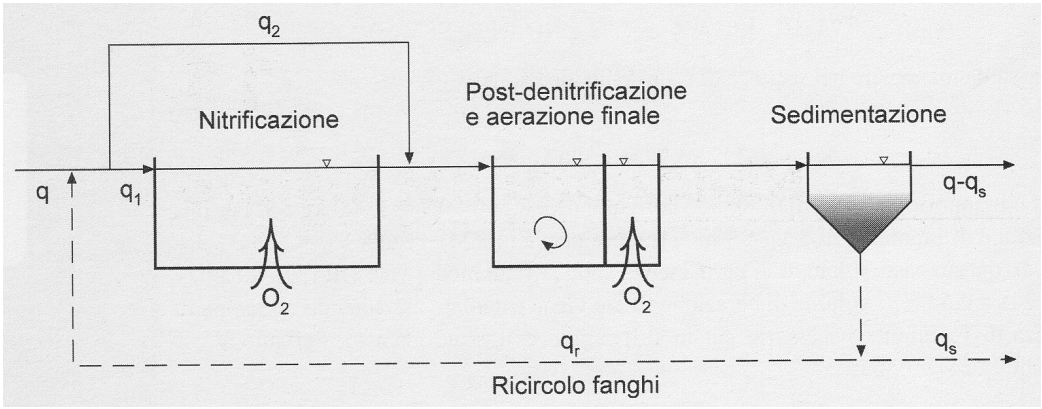

Figura 2.6: Post-denitrification system for biological nitrogen removal with internal carbon source (Bonomo, 2008).

2.3 Anammox

2.3.1 Anammox process

Anammox bacteria are capable of ammonium oxidation, coupled with nitrite reduction and nitrous oxide production, using only CO_2 as carbon source.

$$\begin{aligned} &NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \\ &\rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \end{aligned} \tag{2.7}$$

From the nitrogen balance the ratio between ammonium-nitrite-nitrate is 1:1.32:0.26. Anammox reaction is a three steps process. Ammonium is difficult to activate without oxygen, but it is proposed by Kartal that the molecolar pathway proceed throw hydrazine. First (Reaction 2.8) nitrite is reduced to nitric oxide (NO) that reacts with ammonium to form hydrazine (N_2H_4) (Reaction 2.9) oxidized to N_2 (Reaction 2.10) (Kartal *et al.*, 2011).

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \tag{2.8}$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \tag{2.9}$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \tag{2.10}$$

The overall catabolic equation is:

$$NH4^+ + NO_2^- \rightarrow N_2 + 2H_2O \tag{2.11}$$

Anammox bacteria are able to reduce nitrate to ammonium to provide for it in case of ammonium lack. This is called Dissimilatory Reduction of Nitrate to ammonium (DRNA). Kartal *et al.* (2007) found that phisically purified *kuenenia stuttgartiensis* cells are able to reduce NO_3^- to NH_4^+ with NO_2^- as intermediate.
The presence of organic matter can result in competition because anammox are not able to compete heterotrophic for nitrite. However it was shown that anammox bacteria are able to use organic acid as electron donors to reduce nitrite and nitrate and outcompete heterotrophs for these compound (Güven *et al.*, 2005). Heterotrophic denitrifiers reduce NO_3^- via NO_2^- to molecular nitrogen. The competition can be due to the growth rate of denitrifiers that is higher than anammox (Strous *et al.*, 1999b). Moreover comparing the Gibbs free energy of both reactions denitrification is thermodynamically favourite.

Anammox are found to be inhibiteted also by low concentration of methanol and ethanol. Other organic matter, like propionate and acetate, are indeed not able to influence anammox activity. Van de Graaf *et al.* (1996) stated that anammox bacteria are able to compete heterotrophs in batch experiments.
Anammox bacteria are inhibited not only by external substance, but also by their substrates and products.
Nitrite concentration strongly affects anammox process. Different studies were conducted, but there is no agreement on the threshold value for nitrite inhibition. Strous *et al.* (1999a) reported a total inhibition over 100 mgNO_2-N/L based on batch tests and nitrogen gas production; Dapena-Mora *et al.* (2007) found 50% inhibition at 350 mgNO_2-N/L; Fux *et al.* (2004) described that after days at a concentration of 40 mgNO_2-N/L anammox lost completely the activity. A different behaviour toward nitrite was also found with different genera of anammox. The inhibition could be put back at the previous activity adding intermediate as hydrazine.
Ammonium does not play a inhibitor role till concentration of 1 gNH_4^--N/L (Strous *et al.*, 1999a).
Since anammox uses inorganic carbon source, bicarbonate concentration is a critical parameter. It was observed that low concentrations bring to low anammox activity, but high concentrations cause inhibition.
Similarly phosphate and sulphide inhibition appears with different anammox species and at different concentration. There is no agreement between different studies.
Anammox bacteria are inhibited by dissolved oxygen both at low and high levels.
The temperature range for anammox activity is between 30-40 °C with 37 °C as optimum. They were found to live in a lot of environments with different temperatures. Several studies in the last years were conducted to investigate anammox at low temperature with high nitrogen wastewater. Nitrogen removal from low strength wastewater was reported by Hendrickx *et al.* (2012) at 20 °C; De Clippeleir *et al.* (2011) at 25 °C; Winkler *et al.* (2012) at 18 °C and Ma *et al.* (2013) at 16-25 °C.
The pH interval for anammox is 6.7-8.3 with optimum of 8 (Strous *et al.*, 1999a).
It is also reported the inhibition by flocculants used in the process before anammox reactor.

2.3.2 Discovery and physiology

The existence of anaerobic bacteria was first postulated in 1977 by Broda (Broda, 1977). Ten years later they were discovered in a denitrifying pilot plant for wastewater treatment from a yeast factory in Delft (Mulder *et al.*, 1995).
The first obstacle was that the classical microbiological techniques were not able to isolate anammox microorganisms. The main reason is the very slow division time (11 d, Strous *et al.* (1999b)) that don't allow to culture the microorganisms, but only obtain an enriched culture in a Sequencing Batch Reactor (SBR). It seemed to be possible to purify the cells with density gradient centrifugation (Van de Graaf *et al.*, 1996; Strous *et al.*, 2002). From DNA and RNA extracted from the purified cells, it is obtained the 16S rRNA gene sequence in clone libraries. That was used to design specific gene probes for Fluorescence In Situ Hybridisation (FISH).
Annamox bacteria belong to five genera (Candidatus: *Brocadia, Kuenenia, Scalindua, Anammoxoglobus and Jettenia*) in the phylum of *Planctomycetes*. Their characteristics are red colour, crateriform structure on the surface and intracellular compartment anammoxosome containing ladderan lipid. Ammonium and nitrite are converted to denitrogen gas in a membrane-bound organelle using hydrazine (N_2H_4) as intermediate. Ladderan lipid are found to support hydrazine to enter in the organelle (Damsté *et al.*, 2002).

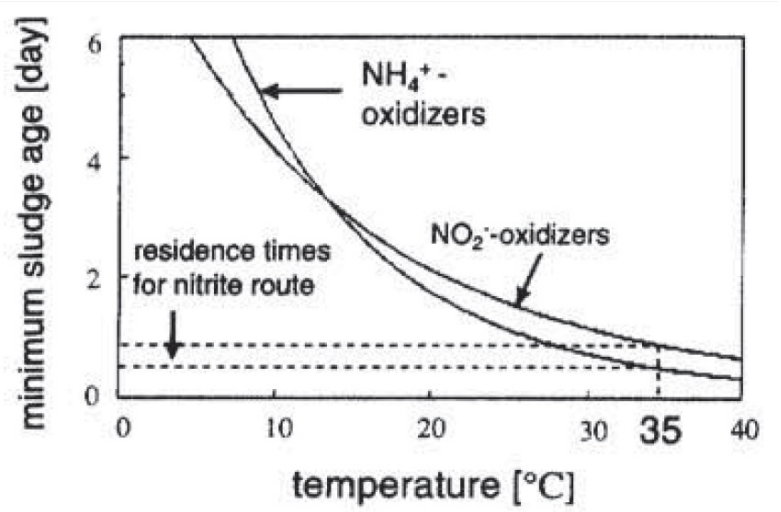

Figura 2.7: AOB and NOB curves as minimum sludge age in function of temperature (Hellinga *et al.*, 1998).

2.3.3 Technologies

The traditional nitrification/denitrification scheme is energy consuming, leads to a significant amounts of sludge and greenhouse gases production. The new anammox process represents a shortcut in the ammonium removal directly converted to nitrogen gas. The process does not require oxygen that is only used by AOB to produce nitrite (60% reduction). However, half of the ammonium is converted (partial nitritation) instead of a full conversion to nitrate. Furthermore the yield of the bacteria is low and permits a reduced sludge production. In addition, low or no organic carbon source is used. The overall cost of this technology (1 €/kg$N_{removed}$) compared with the conventional one is reduced to 50-75% (2-4 €/kg$N_{removed}$) (Van Dongen *et al.*, 2001).

Partial nitritation/anammox in two stage reactor

Partial nitritation/anammox technology divides the process in two steps. In the first aerobic reactor ammonium is partial nitrified to nitrite by AOB. In the second anaerobic reactor nitrite, produced in the first step, is reduced to nitrogen gas by anammox.
The challenge of the first reactors is to obtain an annammox-influent with a ratio of 1:1.32 ammonium:nitrite. Three type of reactors were studied for this purpose. Completely stirred tank reactor (CSTR) (Van Hulle *et al.*, 2005; Van Dongen *et al.*, 2001; Udert *et al.*, 2003), membrane bioreactor (MBR)

(Udert *et al.*, 2003; Wyffels *et al.*, 2004; Feng *et al.*, 2007) and sequencing batch reactor (SBR) (Udert *et al.*, 2003; Yamamoto *et al.*, 2008; Ganigué *et al.*, 2007). In the MBR the sludge age is difficult to regulate, CSTR and SBR with suspended biomass are preferable. In the last two reactors the sludge retention time (SRT) can be uncoupled from hydraulic retention time (HRT) giving more flexibility in the reactor operational. Different name for the reactor are used in literature as SHARON (Sustainable High rate Ammonium Removal Over Nitrite); in this study the first reactor is called partial nitritation reactor and the second anammox reactor.
There are factors that have to be optimize to achieve proper partial nitritation:

- sludge retention time: to enable AOB retention and NOB wash out. Van Kempen *et al.* (2001) reported successfully experience at full scale with 1 and 2.5 d; Gali *et al.* (2007) describe a SBR with 5 d of sludge age;

- temperature: to obtain a proper retention Hellinga *et al.* (1998) found as optimum value 38 °C for AOB and 35 °C for NOB. In Figure 2.8 AOB and NOB growth curves are reported with minimum SRT as a function of temperature. The graph shows that at temperature > 25 °C the sludge age is minimal and at lower temperature it is necessary to increase the sludge retention time.;

- dissolved oxygen: under oxygen limitation AOB are favourite because the half saturation coefficient (0.3 mg/L) is lower than NOB coefficient (1.1 mg/L) (Blackburne *et al.*, 2008).

- pH plays a strong role to favourite the stability of the partial nitritation. At pH value > 7 the conversion of nitrite to nitrous acid (HNO_2) is limited and the concentration of free ammonia (NH_3) increases inhibiting NOB. NOB are predicted to grow faster than AOB at lower pH range. Free ammonia is the substrate and nitrous acid is the inhibitor for AOB. With pH increase, free ammonia increments and nitrous acid decreases.
  Different values of free ammonia are reported in literature to inhibit AOB; Anthonisen *et al.* (1976) reported a inhibition range for AOB between 10-150 mgNH_3-N/L and from 0.2-2.8 mgHNO_2/L starting the NOB inhibition.
  The ratio between alcalinity and ammonium is guaranteed by the buffer capacity of HCO_3^- and if pH increases the equilibrium to the nitrification reaction switches to ammonium and it stops.

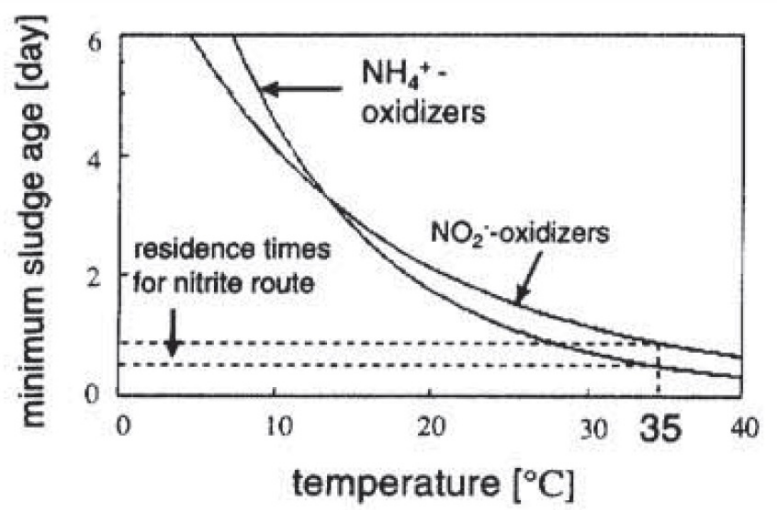

Figura 2.8: AOB and NOB curves as minimum sludge age in function of temperature (Hellinga *et al.*, 1998).

Partial nitritation/anammox in one-stage reactor or Deammonification

In a single stage reactor anammox and AOB co-exist in a microaerobic system. In the reactor NOB are inhibited due to lower affinity to oxygen compared to AOB and to nitrite in respect to anammox. For this process different reactors were used: SBR (Ahn, 2006), air-lift (Sliekers *et al.*, 2003), Rotating Biological Contactor (RBC) (Pynaert *et al.*, 2003), Moving Bad Biofilm Reactors (MBBR) (Gong *et al.*, 2007).
When biomass is in biofilm or granule, anammox bacteria are surrounded by AOB that avoids the permeation of oxygen. On the other hand, AOB uses oxygen to produce nitrite that are immediately available for anammox reaction. For suspended growth system alternation of aeration and low oxygen is provided.
Different names are used to describe the one-stage reactor: OLAND-process (Oxygen Limited Autotrophic Nitrification and Denitrification), CANON (completely Autotrophic Nitrogen removal Over Nitrite), aerobic/anoxic deammonification DEMON.

Mainstream and side-stream anammox

Side-stream anammox after anaerobic digestion is applied at full scale in many WWTP worldwide. In figure 2.9 it is described the process. Sidestream from anaerobic digestion, after anammox treatment, is recirculated to the

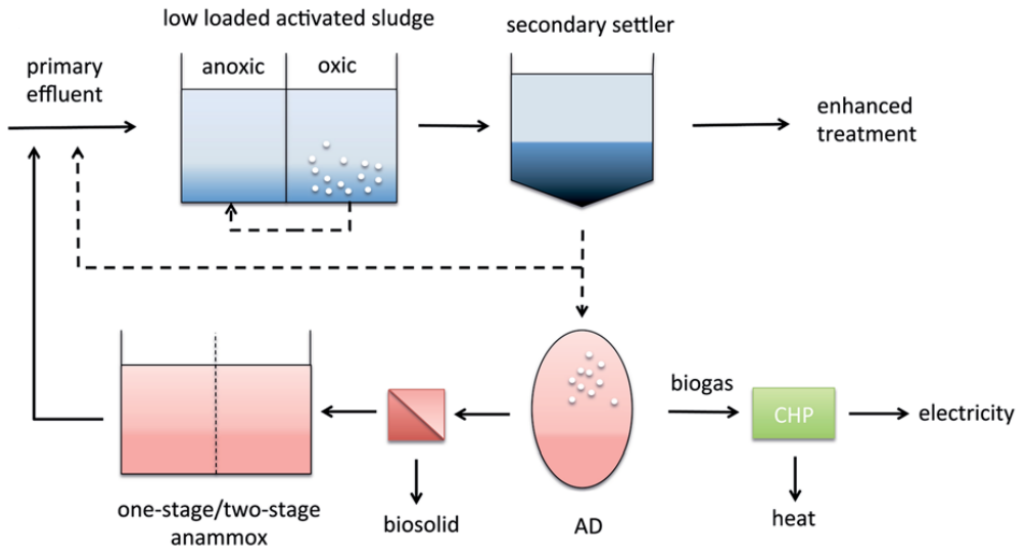

Figura 2.9: Side-stream anammox.

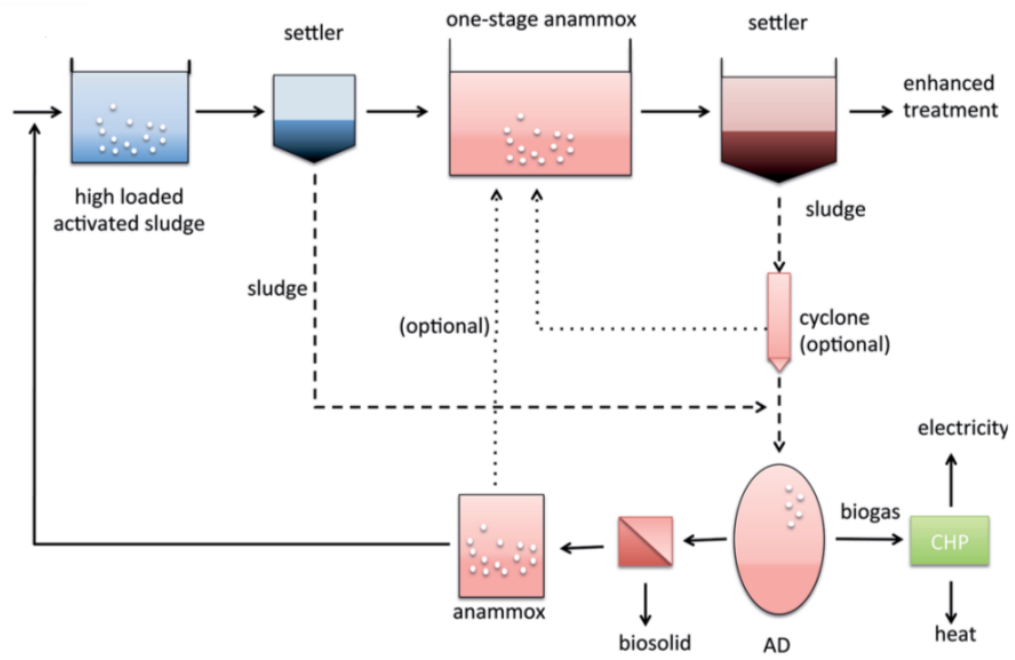

Figura 2.10: Mainstream anammox combined with anaerobic digestion.

mainstream. This stream represents 1% of the total volume and 15-20% of the nitrogen loading in a WWTP.
In figure 2.10 is presented the configuration for the application of anammox in mainstream. The one stage nitritation/anammox reactor is located after a high rate activated sludge unite for COD removal. The sidestream from anaerobic digestion, after the anammox reactor, is recirculated in the mainstream.

2.3.4 Main challenges

The main challenges for future application of anammox process can be summarized in the fallowing topics.

- Application of anammox at low temperature and low nitrogen concentration to integrate the process in the main stream of a wastewater treatment plant. Now the process is used for sludge digester rejection and for industrial anaerobic wastewater with both high nitrogen and temperature;
- Selection of anammox biomass retention to compensate slow anammox growth rate;
- In the effluent of anammox process, nitrate are present as co-product of the reaction. The ability of anammox to oxidise organic matters and out-compete heterotroph can be used to complete the reduction of residual nitrate.

In the last years the number of studies of anammox application at low temperature increased considerably. In table 2.2 are summarized some of that studies, but this still represents a challenges for the application of anammox in mainstream.

Tabella 2.2: Comparison of different reactor configurations for mainstream anammox at low temperature.

Configuration	Volume [L]	Nitrogen influent	T [°C]	HRT [d]	Reference
Granular SBR (2 units/1 unit)	1.0 /1.5+1	400-700mg$NH_4 - N/L$	20	0.25+1/0.25	[1]
A lab-scale anammox UASB	4.5	69± 5mgN/L	20	0.22	[2]
An anammox UASB	8	16.87± 2.09mg$NH_4^+ - N$	30-16	0.12-0.26[h]	[3]
		20.57± 2.31mg$NO_2^- - N$			
		13.97± 3.99mg$NO_3^- - N/L$			
A one stage nitritation-anammox SBR	5	70mg$NH_4^+ - N$	12	0.5	[4]
A one stage nitritation-anammox Rotating Biological Contactor (RBC)	2.5	60mg$NH_4^+ - N$	15	1.09-1.57[h]	[5]
A pilot scale MBBR nitritation-anammox	200	715-837mg$NH_4^+ - N$	10-19	1.7-4.1	[6]

[1] Vazquez-Padın *et al.* (2011)
[2] Hendrickx *et al.* (2012)
[3] Ma *et al.* (2013)
[4] Hu *et al.* (2013)
[5] De Clippeleir *et al.* (2013)
[6] Persson *et al.* (2014)

2.4 FISH (Fluorescence In Situ Hybrizidation)

FISH technique consists in the use of fluorescent probes to detect DNA sequences on chromosomes. A labeled DNA or RNA sequence is used, as a probe, to recognize or quantify the naturally analogue of the sequence. First of all a copy of the sequence, that will be later rendered fluorescent, is made. The target and the probe are denaturated to allow the formation of hydrogen bonds between the target and the probe during next phase. The probe and the target are mixed together and the probe is hybridized to its complementary sequence. Therefore, it is possible to detect the hybrids formed between the probe and their chromosomal targets using a confocal microscope.
Traditional methods to identify the presence of microbial structure generally require a prior cultivation and isolation (Moter and Göbel, 2000).
According to FISH method this phase is not required and allows to conserve the morphology of the cells and their ribosomal RNA content.
Cultivation can lead to an alteration in the structure of the population and permit the growth only of specific microorganism that are favourite in some conditions.
With the fixation it is possible to permeate the cells wall in order to let the oligonucleotide in. So it is possible to analyse the intern of the cell without modify microorganism.
Thus, thanks to FISH it is possible to get information about the spatial distribution and the number of the organism (Langendijk *et al.*, 1995; Amann *et al.*, 1990).
Although, several problems occur using this technique (Figure 2.11).
First not all bacteria can be permeated by oligonucleotides probes (Amann *et al.*, 1995). Problems for the identification of procaryotes are reported when there is a low ribosome content per cell. Moreover the quantification accuracy is obtained mostly by time-consuming manual analysis. In addition no information about physiological activity is supplied.
FISH quantification can be improved using Catalyzed Reporter Deposition (CARD-FISH) when there is a low ribosome content in the target organism (Pernthaler *et al.*, 2002). In addition, FISH-FCM (Flow Cytometry) technique overcomes the limitation of Polymerase Chain Reaction, that can not accurately counts microorganisms. On the other hands, FCM allows a quantitative evaluation at single-cell level (Cui *et al.*, 2012). Furthermore, non RNA based methods can answer the limitations of the use of only RNA based methods. One of this approach is FISH targeting Intergenic Spacer Region (ISR) between the 16S and the 23S rRNA. For slowing growth bacteria the

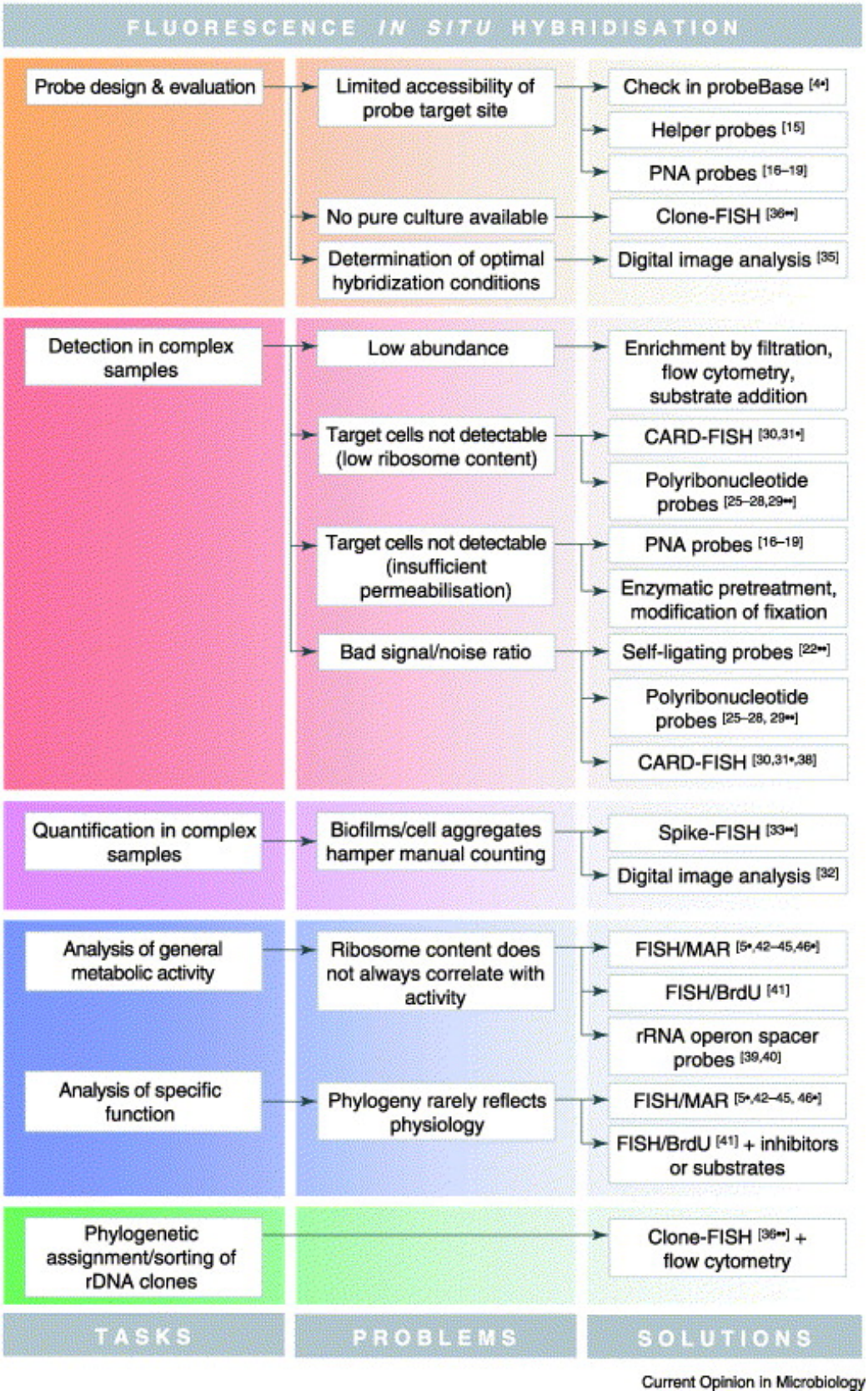

Figura 2.11: Problems and solution of FISH tecnique, Wagner *et al.* (2003).

precursor rRNA concentration is a direct signal of growth activity of bacteria and it can be used in start-up phase to monitore anammox reactor. FISH-MAR (MicroAutoRadiography) connects the detection with the activity of anammox bacteria. The method is based on bacteria assimilation of radioactive substrate. To identify the radioactive cells MAR is used in connection with FISH (Lee *et al.*, 1999).

Capitolo 3

Materials and methods

3.1 Experimental set-up

3.1.1 Reactors

A conventional pilot-scale wastewater treatment plant (200 PE) is operated, at Eawag, for experimental purposes. The raw municipal wastewater comes from the municipality of Dübendorf, Switzerland. The partial nitritation reactor (R1), discussed in the present study, uses as influent this wastewater, after primary settling. The anammox reactor (R2) was inoculated with suspended biomass from a full scale wastewater treatment plant (Werdhölzli, Zurich) and fed with the effluent of R1. Both reactors were operated as sequencing batch reactors (SBR) with a working volume of 12 L (Figure 3.1). R1 and R2 had the same design: peristaltic pump for the influent, a valve for effluent discharge (by gravity), a vertical stirrer, a rotameter to control aeration and a water jacket for temperature control. The inflow pipe was connected close to the bottom of the reactor while the outflow pipe was connected at the head part of the reactor. R2 was also provided with a pump for pH control (5M HCl). R1 was operated at ambient temperature (15-20 °C) and R2 at 28 °C.
A third pilot-scale reactor, named Vayu (Figure 3.2) was considered in the study. The reactor has a working volume of 400 L, it is fed with supernatant liquor from Werdhölzli wastewater treatment plant in Zurich and operated at 30 °C. It is equipped with a stirrer, an aeration unit, a heater, four feed pumps, two valves for effluent and excess sludge and an offgas measuring system.

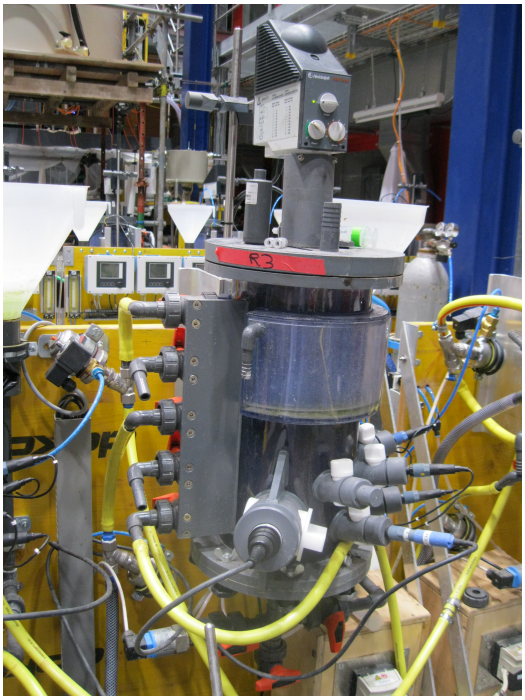

Figura 3.1: Partial nitritation (R1) reactor. Same design also for the anammox reactor (R2).

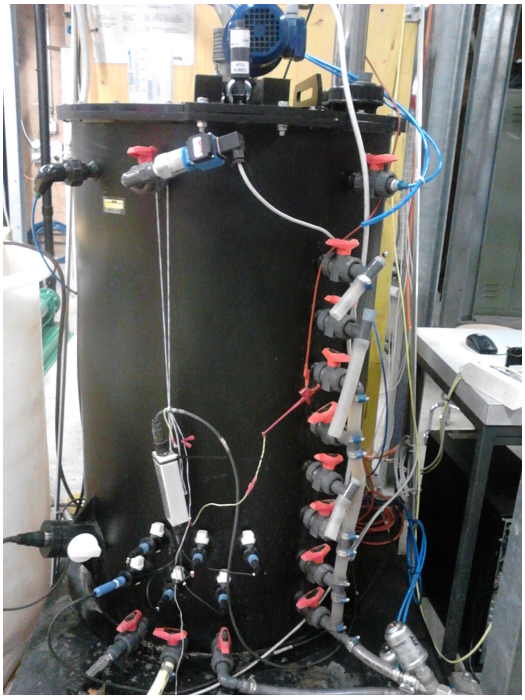

Figura 3.2: Nitritation/anammox reactor (Vayu).

3.1.2 Sensors

All the reactors are equipped with online sensors (Figure 3.3) for process control: fill level sensor, dissolved oxygen (DO), pH, temperature, ammonium (NH_4^+), nitrate (NO_3^-) for R1 and R2 and, in addition potassium (K^+) for Vayu. All sensor are provided by Endress & Hauser. In Table 3.1, the operating ranges of sensors given by Endress & Hauser, are reported.

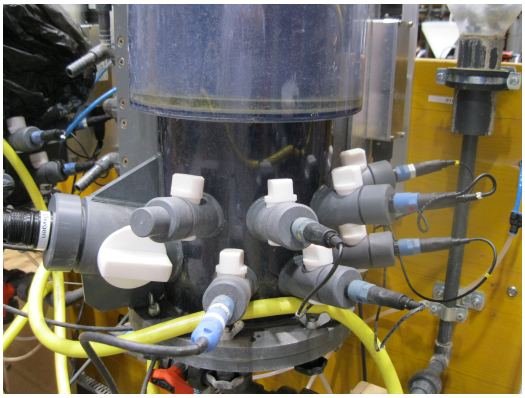

Figura 3.3: Detail of the online sensors for the partial nitritation and anammox reactors.

Tabella 3.1: Operating ranges of sensors.

Sensor	Range
DO	0-20mg/L
NH_4^+	0.1-1000mg/L
NO_3^-	0.1-1000mg/L
Temperature	2-40°C
pH	2-10

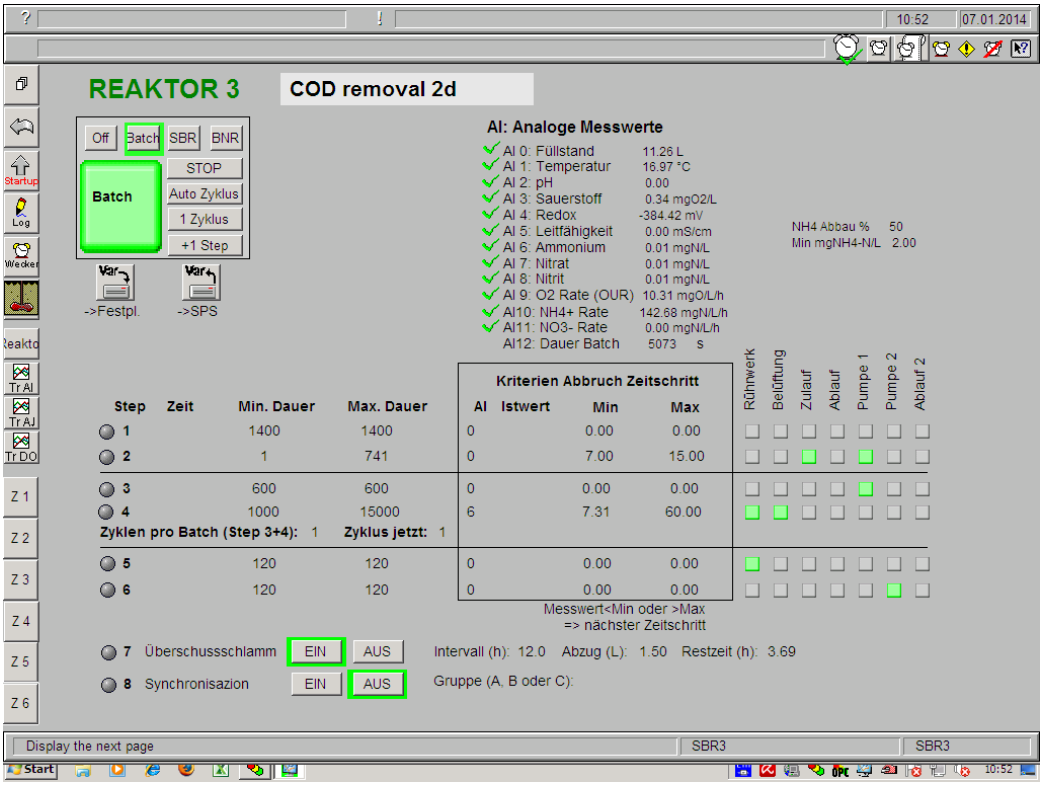

Figura 3.4: Partial nitritation reactor control interface (Phase IV, see text).

3.2 Reactors operation

3.2.1 R1 (COD removal and partial nitritation reactor, MWW)

SBR CYCLE DESIGN

R1 was fed with real MWW, after primary settling (26.9±6.6 mgNH_4-N/L; 0.1±0.1 mgNO_2-N/L; 0.4±0.6 mgNO_3-N/L; 154±54 mgCOD/L) . An example of the SBR cycle design for R1 (during Phase IV, see next section) is presented in Figures 3.4 and 3.6 and detailed in this section. In the first step the sludge is settled for 1400 s. Then the reactor is fed with real MWW for maximum 741 s (equivalent to 4L; Step 2, Zulauf) and the effluent valve (Pumpe 1) is activated to allow effluent discharge by gravity. In step 3, effluent valve is kept open to allow volume equilibration. Simultaneous aeration and mixing is provided during Step 4 for a minimum of 1000 s to a maximum of 15000 s. This step is controlled by ammonium sensor. Ammonium concentration is measured 10 minutes after the starting of the cycle and the

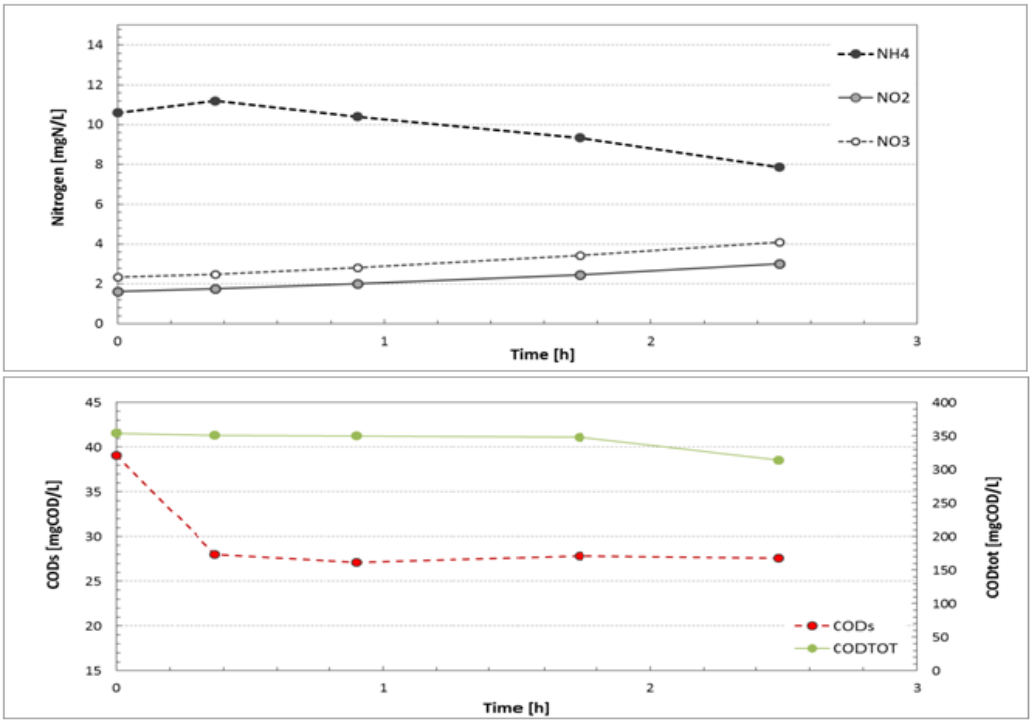

Figura 3.5: Trend of parameters during a single cycle (in phase V).

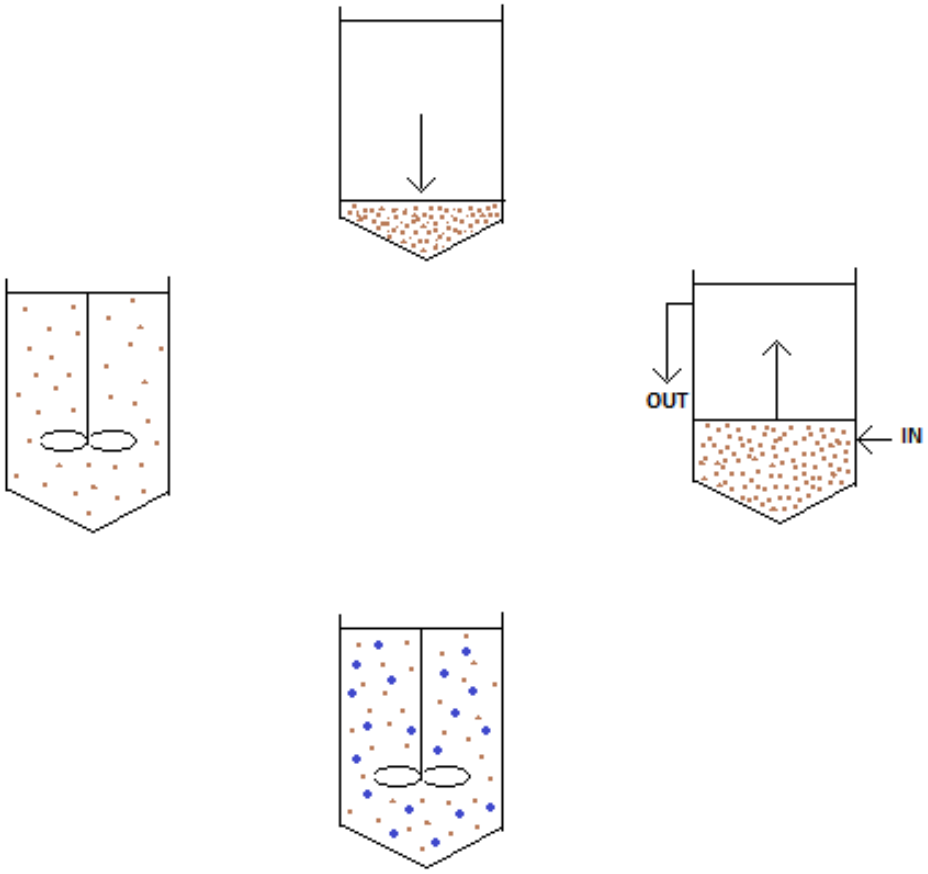

Figura 3.6: Single cycle of partial nitritation reactor (R1).

aeration continues until the desired consumption of the initial concentration is reached (in this case 50%). During this phase the oxygen concentration is kept in a fixed range (here 1.8-2.2 mg/L). To avoid complete ammonium depletion and, consequently, the risk of further NO_2^- oxidation to NO_3^-, a minimum ammonium concentration of 2 mgN/L was set. The aeration step is over, either when it has lasted the maximum time allowed or the value of ammonium concentration is reached. In Step 5 the sludge is mixed for another 120 s. Figure 3.4 shows how is the cycle in the computer interface and Figure 3.5 reports the concentration of different parameters during a single cycle.
Influent and effluent composite samples were taken two or three times in a week.

PHASES OF OPERATION

During the study, the operational conditions of R1 have been progressively changed from COD removal to combined COD removal and partial nitritation (ideally half of the NH_4^+ oxidized to NO_2^-). Seven different phases, each with specific operational conditions, can be defined as follows:

- Phase 0: the reactor was run only for COD removal.
- Phase I (day 1-18): as first step towards partial nitritation, to favour the growth of AOB, the sludge age was set at 2 d and, after COD consumption, an additional fixed aeration phase of 1000 s (0-2 mgO_2/L) was introduced.
- Phase II (day 19-39): as no NO_2^- was detected in the effluent, on day 19, the SRT was further increased from 2 to 4 d and the temperature decreased to ambient temperature. At the same time, to mimic full scale conditions, the temperature was decreased to ambient temperature (no control).
- Phase III (day 40-53): from day 40, aeration was controlled by ammonium sensor: aeration, set between 1.8-2.2 gO_2/L, stops when the initial concentration of ammonium decreased by 30 %. A minimum of 2 $mgNH_4$-N/L was set to avoid formation of nitrate (nitratation) in absence of ammonium.
- Phase IV (day 54-85): on day 54 ammonium consumption threshold was further increased from 30% to 50% to reach partial nitritation.

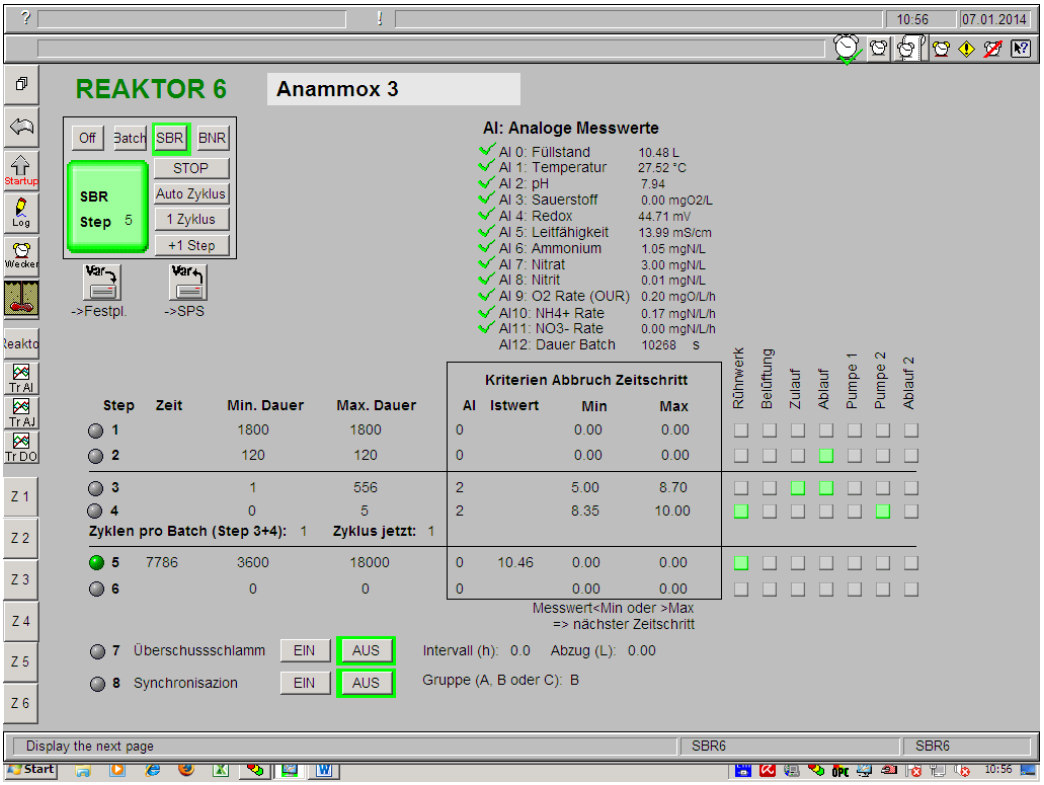

Figura 3.7: Anammox reactor interface (R2).

- Phase V (day 86-99): from day 86, due to the significant NO_3^- production, the air flow was reduced from 2.5 to 2 L/min and the limited for oxygen reduced to 0.5-0.7 gO_2/L.
- Phase VI (day 100-120): on day 100 the sludge age was reduced back from 4 to 2 d, to test whether NOB could be washed out.

3.2.2 R2 (main-stream anammox reactor, MWW)

SBR CYCLE DESIGN

R2 was fed with the effluent of R1 (31.1±8.5 mgCOD/L) amended with NH_4^+ and NO_2^- up to 20 mgN/L each (to avoid the effects of natural fluctuations in MWW concentrations). As no aeration was required, cycle length was controlled by time. Depending on the actual activity, the maximum time was set to allow for complete NO_2^- depletion and a "safety" anoxic mixing time (to avoid sludge washout due to flotation with N_2 bubbles). An example of the control cycle is presented in Figure 3.7. The sludge is first decanted

for 1800 s (Step 1) to minimize sludge loss and, thus, favour the slow growing anammox bacteria. In the second step, the discharge valve at the top of the reactor is opened to allow volume equalization. During the third step the influent is fed while the discharge valve remains open (effluent discharged by gravity). Due to the slightly basic characteristics of the influent, this step is controlled by pH: feeding is stopped if pH is higher than 8.7 (NOTE: there was a constant offset of one pH unit, thus the actual upper limit was at 7.7). Step four is the pH control: if the pH value is more than 8.35 the acid pump (here Pump 2, 5% HCl) is activated along with the stirrer and acid is added for at most 5 s. In the last step (Step 5) the stirrer is turned on and the anammox reaction takes place for 18000 s (variable length during the experiment). To characterize anammox activity, two samples during one cycle were taken almost every day.

PHASES OF OPERATION

During the study, R2 was always fed with the effluent of R1 and, thus, the same phases could be defined. Herein, however, only the following two phases are defined.

- Phase 0: The reactor was inoculated with biomass from a full scale reactor (Zurich, Werdhölzli) and run as anammox for over 200 days, on pre-treated MWW, before the start of this experiment.
- Phase I: The reactor feeding was switched to the effluent of R1, following all its operational changes (see previous section).

3.2.3 VAYU (side-stream nitritation/anammox reactor, supernatant)

SBR CYCLE DESIGN

The pilot scale reactor (named Vayu) treats digester supernatant from a full scale WWTP (Werdhölzli, Zurich; 700±50 mgNH_4-N/L; <0.2 mgNO_2-N/L; <0.2 mgNO_3-N/L; 300±50 mgCODs/L; 630±50 mgCODtot/L). The overall goal of the reactor is to work in a semi-continuous mode at low ammonium concentrations (always between 2-8 mgNH_4-N/L). The feed is introduced in steps during aeration as opposed to the common practice of a single feeding step at the beginning of the cycle. The operational cycle is presented in Figure 3.6.
The first phase is a short feeding (Step 2). The maximum volume that it

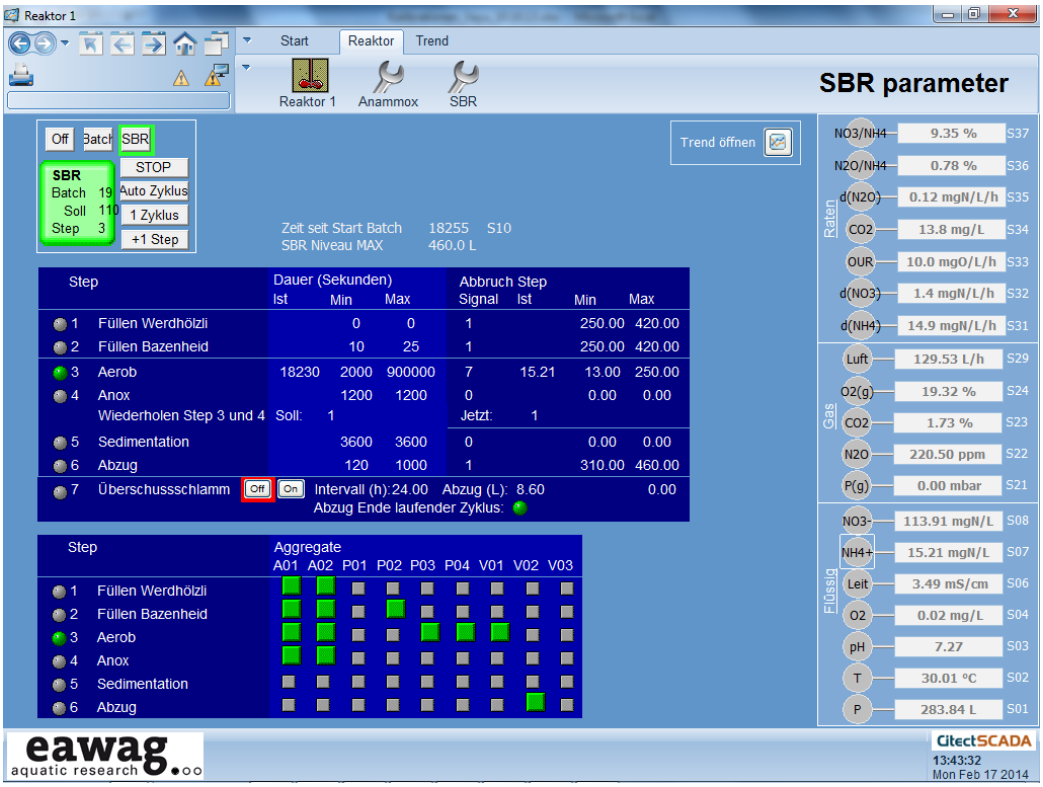

Figura 3.8: Vayu reactor interface.

is possible to reach is 420 L. During the following aeration phase (Step 3) each time ammonium concentration reach the value of 13 mg/L, the feeding is activated again for 20 s (NOTE: due to constant NH_4^+ sensor offset, this value corresponds to an actual concentration of 2 mgNH_4-N/L). This is repeated until the maximum volume of 420 L is reached (NOTE: the step feeding cannot be seen from the control panel presented in Figure 3.6). The aeration flow is kept at 100-200 L/h to prevent NOB formation. Next, in Step 4 the reactor is anaerobically stirred for 1200 s to consume residual NO_2^-, before the sedimentation (1 h, Step 5). Finally, in Step 6, 90 L (20% v/v) of reactor content are discharged. pH is controlled with the addition of sodium carbonate and maintained in the range 7.15-7.25. The temperature is kept at 30 °C. Antifoam is used to limit foam formation.

3.3 Batch experiments (heterotrophic potential)

The feasibility of anammox application for the treatment of MWW relies on anammox capabilities to outcompete, on the long term, other microbial species in the presence of complex mixtures of different carbon sources and potential toxics. To verify the presence of heterotrophic activity (denitrification) in R2, the consumption of NO_2^- and NO_3^- was monitored in the absence of NH_4^+ and with the subsequent addition of different carbon sources. To elucidate potential differences between main- and side-stream reactors, similar experiments were repeated with the sludge from Vayu reactor. A detailed description of the performed experiments is given in the following sections.

3.3.1 Experiment 1 (main-stream anammox reactor, R2)

In this experiment, the behaviour of R2 in presence of different type of COD was tested.
First, about 15 mgNO_2-N/L (as $NaNO_2$) were added to allow for the complete depletion of the ammonium present in the system. Next, the system was left with non-limiting concentrations (10-15 mgN/L) of NO_2^- and NO_3^-: 10 mgNO_3-N/L (as KNO_3) were added and, since anammox reaction had consumed all nitrites, other 12 mgNO_2-N/L were added. In the absence of ammonium, three different COD sources were added to test the heterotrophic potential of the system. In subsequent pulses, 10 mgCOD/L acetate, glucose and maleate were added. Around 1.5-2 hours were left for their complete consumption. The same experiment was repeated under anoxic (N_2/CO_2 bubbling) and microaerobic (reactor left open to the atmosphere) conditions to test weather oxygen availability enhances COD consumption. CO_2 was provided both to control the pH and to avoid anammox to be limited by the availability of inorganic carbon (CO_2 stripped during N_2 bubbling). Finally, to further confirm the presence of anammox activity, 12 mgNH_4-N/L (NH_4Cl) were added to consume the residual NO_2^-.

3.3.2 Experiment 2 (side-stream nitritation/anammox sludge, Vayu; November)

All experiments with Vayu sludge followed the same rationale of Experiment 1. Experiments were performed in 12 L reactors, under continuous

mixing and anoxic conditions, and only acetate was tested. The reactor was bubbled with N_2 to avoid the presence of oxygen, before and during sampling and during the introduction of the different substrate. Before the experiment was started, the sludge was left overnight (15h) to consume the residual NH_4^+ and easily degradable COD. After 20 mgN/L of NO_2^- and NO_3^- and 200 mgCOD/L were added to the system. The reactor was run till almost all nitrites were consumed.

3.3.3 Experiment 3 (side-stream nitritation/anammox sludge, Vayu; November)

Vayu sludge was diluted with reactor effluent (1:1 vol.) to reduce residual COD concentrations. In analogy to the previous experiments the reactor was provided with N_2 pipe at the bottom and N_2/CO_2 pipe for bubbling the head space of the reactor. In addition pH was controlled also by adding HCl 5%. First of all, 39 mgNO_2-N/L (as $NaNO_2$) to consume during the night all ammonium (28.5 mg/L). Next, when all ammonium was consumed, 100 mgCOD/L as acetate were added to remove all nitrite and nitrate. When both were consumed, in the presence of negligible concentrations of ammonium, nitrite and nitrate, the experiment was started. 150 mgCOD/L as acetate were added along with 35 mgNO_3-N/L. After, once all NO_3^- was consumed, 20 mgNO_2-N/L were added and the experiment was run until its complete consumption.

3.3.4 Experiment 4/5 (side-stream nitritation/anammox sludge, Vayu; December)

These experiments were conducted twice with the same sludge from Vayu reactor around one month later (December). The set-up was the same as the experiment in November (Experiment 2/3).
All ammonium was first consumed. COD, nitrite and nitrate were added. Samples were taken regularly to follow the trend of COD consumption.

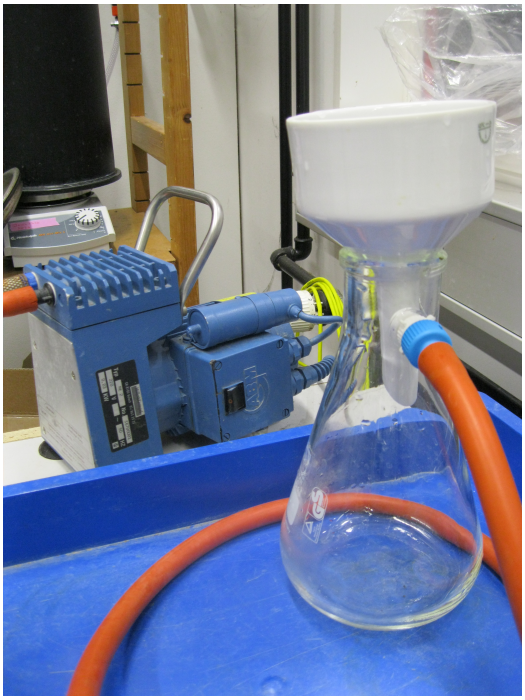

Figura 3.9: Vacuum pump and filter-holder.

3.4 Analytical methods

Total suspended solids

Samples were taken when the sludge in the reactor was stirred. The filter-holder (Figure 3.9) was cleaned with distilled water. The filter (Macherey-Nagel MN 640 dd, diameter of 90 mm) was numbered, washed and put in the oven at 105 °C overnight to remove water. The filter paper was put in the desiccator for 3 minutes and then was weighted with METTLER AE 260 DeltaRange scale. An appropriate volume of sample was purged into the filter-holder and vacuum filtered (Figure 3.9) until no water could be seen. The filter was removed with tweezers and folded four times. Bended filters were than dried in the oven at 105 °C (Figure 3.11) for at least 24 hours and then cooled in desiccator for 3 minutes again and reweighted. The difference between the clean filter weight and the dried filter with biomass represent the total suspended solids (TSS).

Figura 3.10: Porcelaine cup.

Figura 3.11: 550 °C (left) and 105 °C (right) ovens.

Volatil suspended solids

Porcelain cups were left in the 105 °C oven (about 20 min) and then, after having been cooled down in the desiccator, they were weighted (tara). Each filter was placed in a separate cup and left for minimum 2 hours in the 550 °C oven (Figure 3.11, left). The porcelain cup and the filter were weighted: the difference between the obtained weight and the tara represents the inerts. By subtracting the inerts from the TSS (obtained in the precedent step) the volatile suspended solids (VSS) are obtained.

Manual chemical analysis

Prior to chemical analysis, samples from all reactor were filtered with Membranfilter Macherey 0.7 μm. In most cases, commercial photochemical test kits (Hach Lange GmbH, Düsseldorf, Germany; spectrophotometer type LASA 26) were used to measure Soluble COD, total COD, NH_4^+, NO_3^- and NO_2^-. Sometimes samples were analysed using a 881 Compact IC Pro ion chromatograph from Metrohm.

Capitolo 4

Results and discussion

To tackle the challenge of direct municipal wastewater with anammox-based systems, a two stage partial nitritation/anammox was chosen to study the two main processes separately. First, COD is aerobically removed and nitrite production by Ammonium Oxidizing Bacteria (AOB) is promoted. Next, as second step, nitrogen is removed anoxically via anammox with the oxidation of ammonium with nitrite as electron acceptor.
For the first step (COD removal and partial nitritation) the aim was to test the feasibility of the process at ambient temperature, with particular focus on the balance between ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) and on the overall nitrogen mass balance. In the second anoxic reactor (anammox) the goal was to understand the microbial community interactions: the competition for substrate (mainly NO_2^-) between anammox and heterotrophs and the mechanisms involved in organic matter degradation.

4.1 Partial nitritation

The partial nitritation reactor (R1), fed with fresh wastewater after a primary settling, was shifted from an initial phase of only COD removal to the combined COD removal and partial nitritation. The goal was to evaluate the feasibility of running a partial nitritation reactor at ambient temperature as a pre-treatment for the subsequent anammox (R2) reactor.

Reactor (R1) was run for more than three months in a temperature range

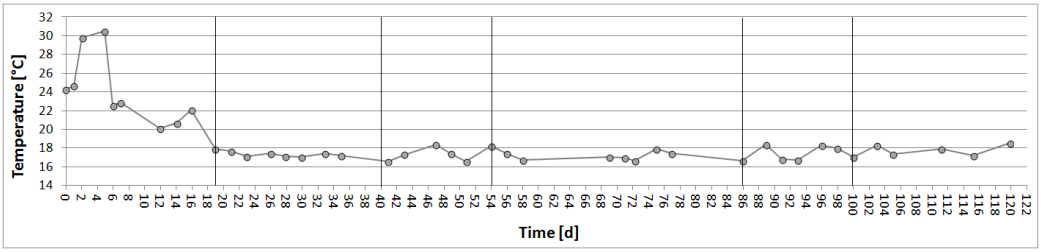

Figura 4.1: Temperature trend for the partial nitritation reactor (R1).

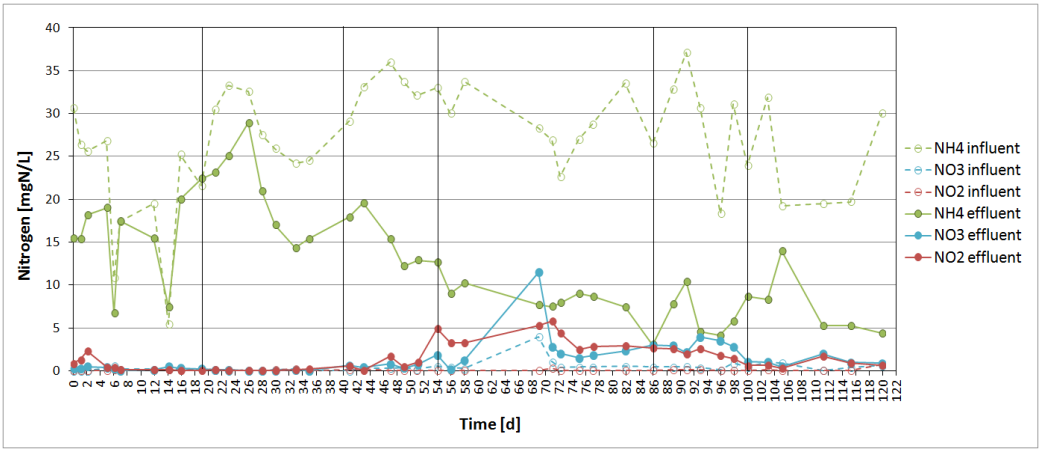

Figura 4.2: Main influent and effluent parameters for the partial nitritation reactor (R1).

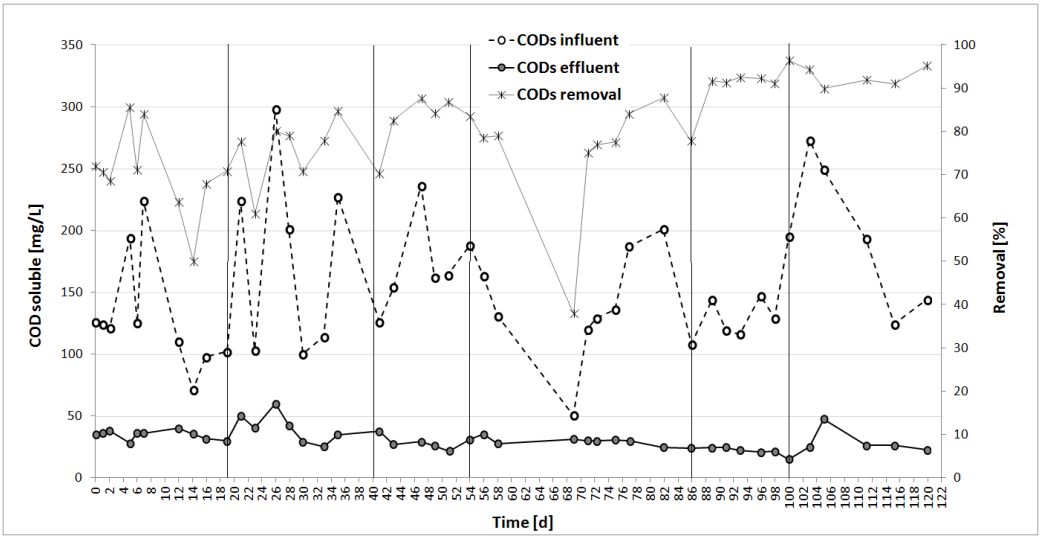

Figura 4.3: COD influent, effluent and removal fraction of the partial nitritation reactor (R1).

between 15 and 20 °C (trend is reported in Figure 4.1). The evolution of influent and effluent parameters are summarized in Figures 4.2 for nitrite, nitrate and ammonium and in Figure 4.3 for COD.
During the first two phases (Phase I and II) there was no evidence of nitritation with negligible production of nitrite (NO_2^-) and nitrate (NO_3^-). Furthermore, the change in the sludge age, from 2 to 4 d (Phase II), did not result in any significant process change. Conversely, when aeration started to be controlled based on ammonium removal (Phase III), nitrites and, in smaller amount, nitrates started to be measured in the effluent (Figure 4.2). This suggest that in the previous phases limited aeration, rather than a short SRT, was the limiting factor for partial nitritation to occur. Effluent average values in phase III were 0.9 mgNO_2-N/L and 0.6 mgNO_3-N/L, with 52% and 82% of ammonium and COD removal respectively. During the following phase IV, the threshold for ammonium consumption was incremented to 50% and, consequently, nitrite and nitrate concentrations in the effluent increased (average of 3.8 mgNO_2-N/L and 2.7 mgNO_3-N/L) along with the removal rate of ammonium and COD reached 69% and 76%, respectively.
As nitrites and nitrates were produced in almost equal amounts, in an attempt to reduce the NO_3^- production (not a primary substrate for anammox), the working oxygen concentration was reduced to 0.5-0.7 mgO_2/L as well as the air-flow from 2.5 to 2 L/min. In fact, AOB are reported in literature to have a higher affinity for oxygen than NOB (Hellinga *et al.*, 1998). Therefore, it was expected that a reduction in the overall oxygen available would have reduced the further oxidation of nitrites. However, as no apparent reduction in nitrification was observed, the sludge age was shifted back to 2 d on day 100, to test whether by washing away AOB and NOB it was possible to get back to the initial condition of only COD removal. According to the expectations, a significant reduction in NO_2^- and NO_3^- production was observed. Unfortunately, the experiment had to be stopped on day 120 before stable operation could be reached.

To further understand the observed reactor performance, six representative biomass samples (day 8, 23, 34, 56, 90, 98) were characterized by FISH and Confocal microscope. As can be seen in Figure 4.4, the shift from only COD removal to combined partial nitritation and COD removal resulted in major biomass changes. In particular, filamentous bacteria, predominant in the first phases (Figure 4.4 a, b, c), where almost completely washed out in the following phases (Figure 4.4 d, e, f). (NOTE: only AOB-mix and NOB-mix gene probes were used and, thus, the staining of filamentous bacteria most likely resulted from unspecific staining; however, as all samples were processes on the same slide, relative comparison is still possible). Furthermore, during the

period of increasing nitrite in the effluent, AOB population seems to be more abundant than NOB population (Figure 4.4 d). In the final period (Figure 4.4 e, f), the overall size of granules/flocs decreased, with an apparent reduction of AOB relative abundance. Finally, despite the fact that no quantitative analysis was performed, a positive correlation between reactor performance and qualitative FISH results could be found. Several conclusions can be drawn from the discussed results. First, high rate biological COD removal is feasible at ambient temperature and constitute a proper pre-treatment for a subsequent anammox-based process (see also next session for further discussion). Secondly, two main challenges are to be faced when combining COD removal and partial nitritation in a single reactor. On one side, control of NOB at low temperature requires particular attention: in the present study NO_2^- and NO_3^- were always produced in almost equal amounts and the latter could not be reduced. The identification of the optimal combination of SRT and oxygen control will be further investigated. Surprisingly, on the other side, about half of the consumed ammonium could not be found as NO_2^- or NO_3^-. Most likely, due to the high COD content of MWW, this behavior could be ascribed to simultaneous denitrification and nitrogen incorporation into biomass. In the perspective of the implementation of anammox-based systems for MWW treatment, the occurrence of denitrification is to be avoided: it reduces, in fact, the COD available for energy production in anaerobic digestion. It is therefore suggested that COD removal and partial nitritation should be performed in separate reactors in future installations. Finally, to this end, nitrogen losses in the form of N_2O, a powerful greenhouse gas, cannot be ruled out (no specific measurement were performed). N_2O is produced, in fact, mainly in the presence of low dissolved oxygen concentration and increased nitrite concentration (Kampschreur et al. 2009), conditions that are present in the partial nitritation reactor. This aspect has to be further evaluated: even low N_2O emissions (1-2 %) could completely nullify the environmental benefits of anammox-based systems.

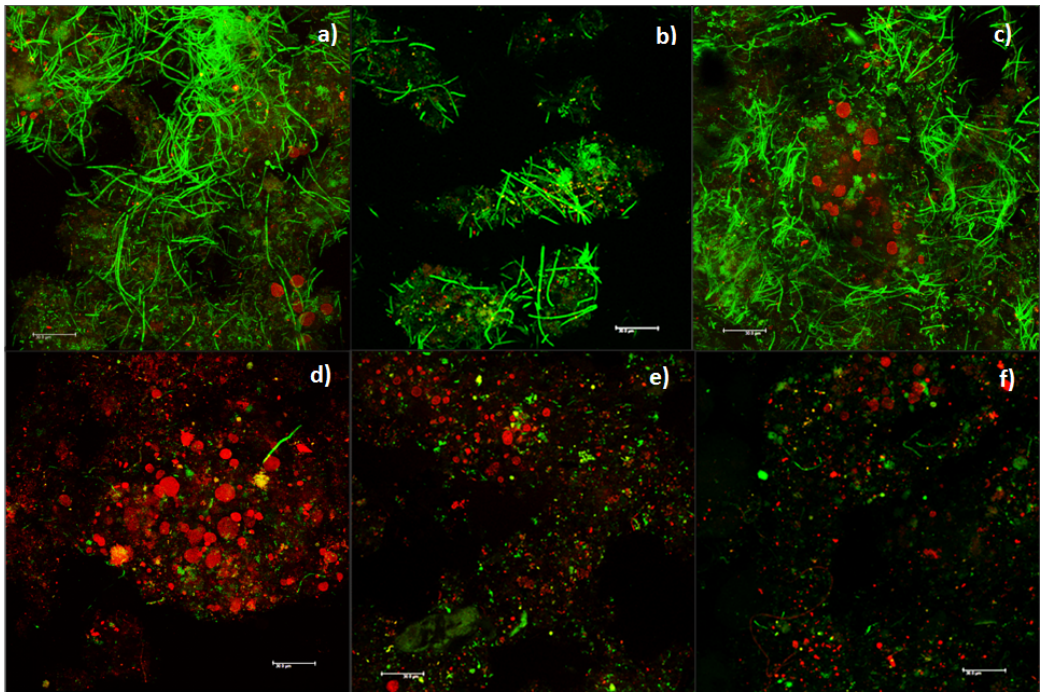

Figura 4.4: FISH images of different samples from partial nitritation reactor; AOB in red and NOB in green. a)day 8; b)day 23; c)day 34; d)day 56; e)day 90; f)day 98. Scale bar 30 μm.

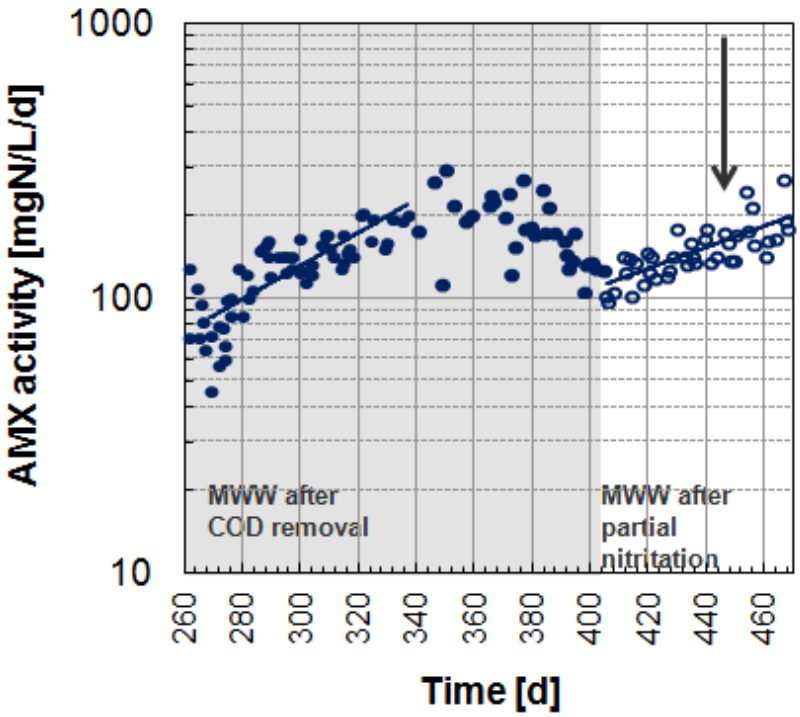

Figura 4.5: Anammox activity in R2 (anammox reactor).

4.2 Organics consumption in anammox sludges

Low nitrogen concentration and relatively high amounts of COD are typical characteristics present in municipal wastewaters.
Consequently, successful application of anammox-based systems to MWW requires anammox bacteria to be able to prevail, on the long term, over potentially competing species (i.e. heterotrophs and NOB) in the presence of complex mixtures of organics (as in MWW, even after a pre-treatment). Specifically, in the studied system (no O_2; NH_4^+ and NO_2^- in the feeding) anammox and heterotrophs are expected to be the two main species involved, where the latter could potentially grow on decay products (Ni *et al.*, 2012) or be carried over from the preliminary COD removal step (the influent itself is assumed to have only hardly degradable COD). Coexistence of anammox and heterotrophs can result in reduced process efficiencies and losses in anammox activity, due to competition for NO_2^- (Lackner *et al.*, 2008) and/or to the potential decrease in SRT due to poor sludge settleability (Jenni *et al.*, 2014). Direct toxicity of some organic compounds has been reported as well (Güven *et al.*, 2005). On the other side, the presence of heterotrophs in anammox-

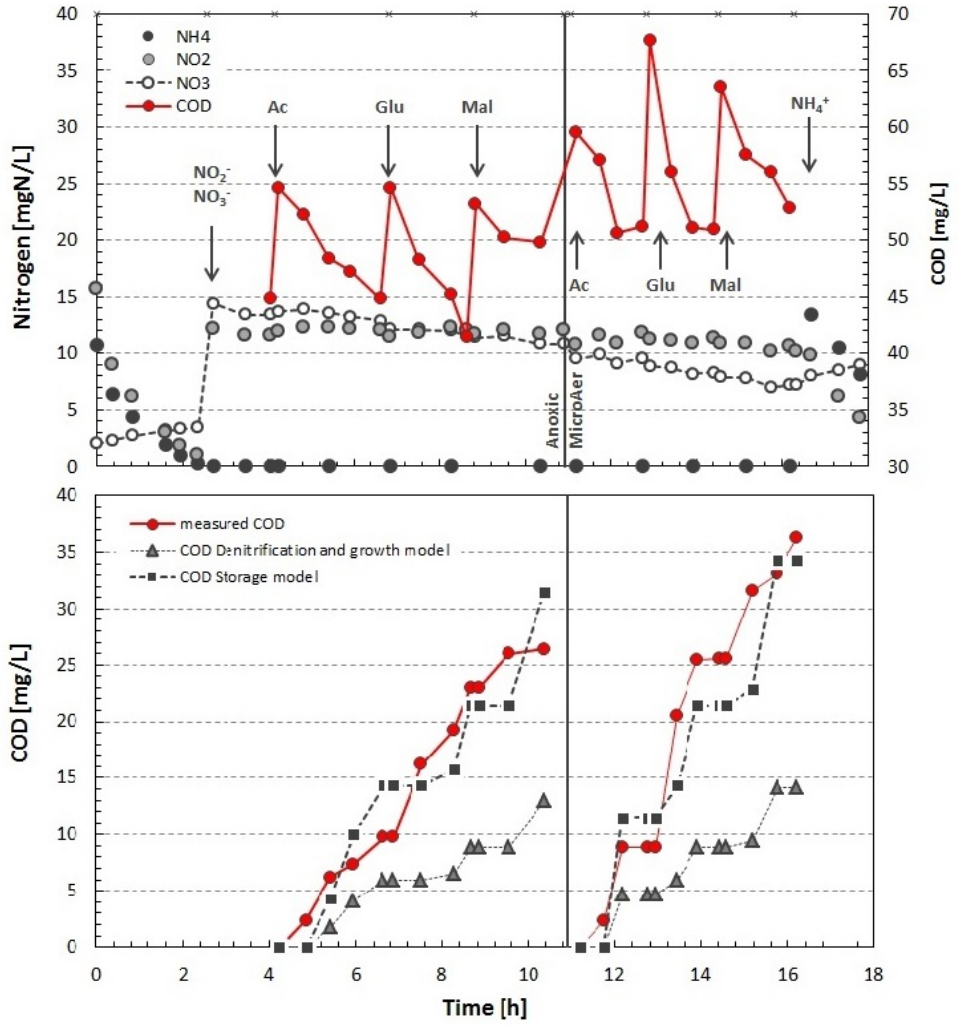

Figura 4.6: Batch experiment (heterotrophic potential). Above: concentrations in anammox reactor during experiment 1; below: COD cumulative curve in batch experiment 1.

Tabella 4.1: Stoichiometric and composition parameters used in the model Gujer *et al.* (1999)

Parameters		
$Y_{HB,NO}$	0.54	gCOD/gCOD
$i_{N,XB}$	0.07	gN/gCOD
$Y_{STO,NO}$	0.80	gCOD/gCOD

Component	S_S	S_{NH4}	S_{NO2}	S_{NO3}	S_{N2}	X_{HB}	X_{STO}	X_{AMX}
Process	gCOD m^{-3}	g N m^{-3}	g N m^{-3}	g N m^{-3}	g N m^{-3}	gCOD m^{-3}	gCOD m^{-3}	gCOD m^{-3}
Growth (anoxic)								
HET on NO2	$-\frac{1}{Y_{HB}} - 3.43 \cdot i_{NXBM}$		$-\frac{1}{1.71}\left(\frac{1-Y_{HB}}{Y_{HB}}\right) - i_{NXBM}$		$\frac{1}{1.71}\left(\frac{1-Y_{HB}}{Y_{HB}}\right)$	1		
HET on NO3	$-\frac{1}{Y_{HB}} - 4.57 \cdot i_{NXBM}$			$-\frac{1}{2.86}\left(\frac{1-Y_{HB}}{Y_{HB}}\right) - i_{NXBM}$	$\frac{1}{2.86}\left(\frac{1-Y_{HB}}{Y_{HB}}\right)$	1		
Storage (anoxic)								
HET on NO2	-1		$-\frac{1}{1.71}(1-Y_{STO})$		$\frac{1}{1.71}(1-Y_{STO})$		Y_{STO}	
HET on NO3	-1			$-\frac{1}{2.86}(1-Y_{STO})$	$\frac{1}{2.86}(1-Y_{STO})$		Y_{STO}	
Composition Matrix								
gTOD	1		-3.43	-4.57	-1.71	1	1	1
gN		1	1	1	1	i_{NXB}		i_{NXAMX}

Conversion factors used for the mass balances		
$\frac{Y_{COD}}{Y_{NO2}}$	3.7	gCOD/gNO2-N
$\frac{Y_{COD}}{Y_{NO3}}$	5.9	gCOD/gNO3-N
$\frac{Y_{COD}}{Y_{NO2}}$	8.6	gCOD/gNO2-N
$\frac{Y_{COD}}{Y_{NO3}}$	14.3	gCOD/gNO3-N

Figura 4.7: Stoichiometric and composition matrix of the model (left) and conversion factors used in the COD mass balance (right).

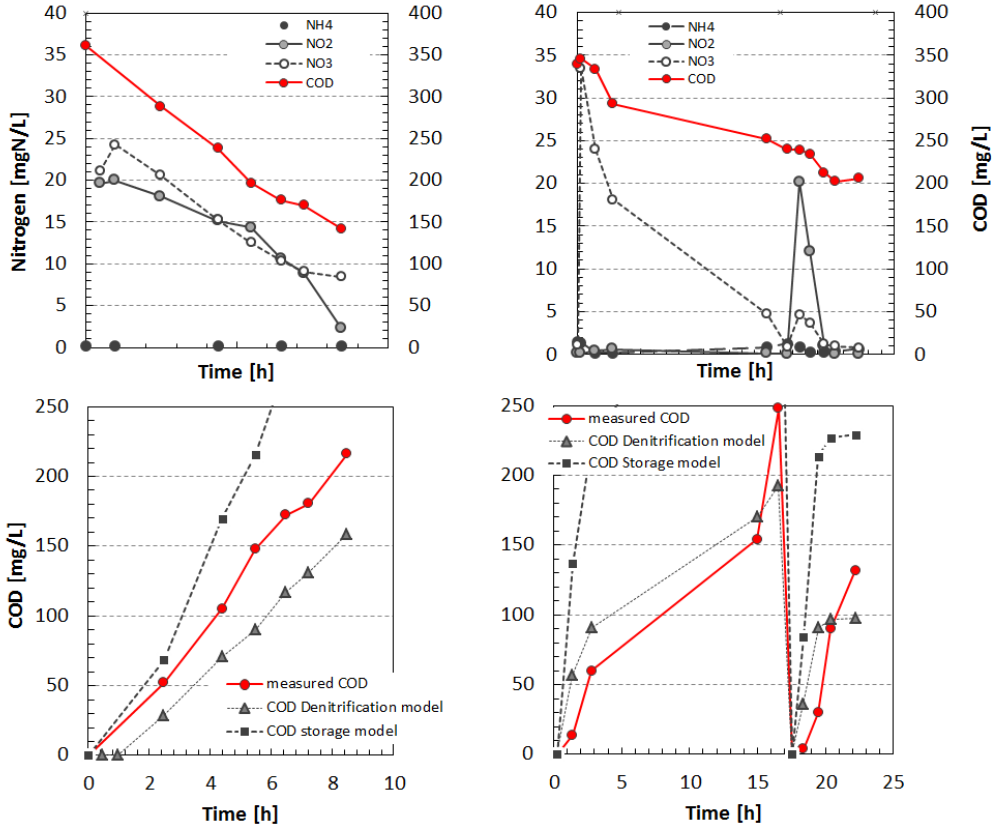

Figura 4.8: Batch experiment (heterotrophic potential). Above: concentrations in batch experiment 2 and 3; below: COD cumulative curve in batch experiment 2 and 3.

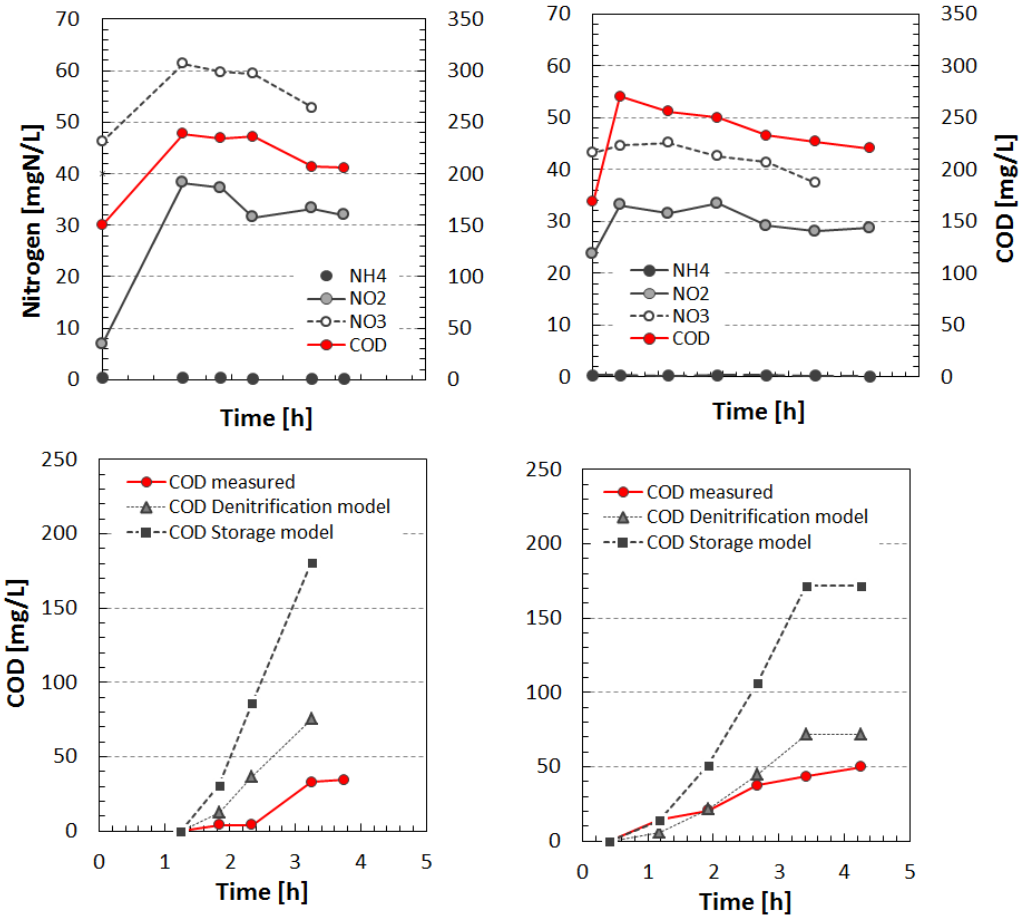

Figura 4.9: Batch experiment (heterotrophic potential). Above: concentrations in batch experiment 4 and 5; below: COD cumulative curve in batch experiment 4 and 5.

based systems can concur in the improvement of overall nitrogen removal efficiency, by consuming the NO_3^- produced by anammox metabolism (Udert *et al.*, 2008; Ni *et al.*, 2012). Furtehrmore, anammox are known to be able to oxidize organic compounds (e.g. acetate) using nitrate and nitrite as electron acceptors. The experiments conducted in this study aimed at understanding the competition for substrate (mainly NO_2^-) between anammox and heterotrophs and the mechanisms involved in organic matter degradation. The tests were conducted by adding easy biodegradable COD in the presence of NO_2^- and NO_3^- as electron acceptors, after complete depletion of ammonia (no direct anammox metabolis is possible).

In Figure 4.5 is reported anammox activity of R2. The vertical arrow represents the day of the batch experiment 1 (T=28°C).
Three sources of COD were tested in subsequent pulses: acetate, glucose and maleate. As can be seen in Figure 4.6, first ammonium and nitrite were consumed according to anammox stoichiometry (c.a. 1.3 gNO_2-N/gNH_4-N). Next, in the absence of ammonium, despite the almost complete consumption of the added organics, only limited NO_3^- depletion was observed. With regard to NO_2^-, hardly any consumption was observed, apart from small variations after each organics pulse (considered here as artefacts).
In the final part of the experiment, when NH_4^+ was added, the marked increase in NO_2^- consumption rate confirms that anammox bacteria were active during all experiment and that anammox was the dominant NO_2^- consumption route in the system. This evidence is further supported by recent studies. Jenni *et al.* (2014) reported that anammox were able to stay in the system in the presence of high acetate and glucose concentrations, up to 1.4 COD_{bio}/NH_{tot}–N, and outcompete heterotrophs.

To further understand the observed COD consumption a stoichiometric model, based on ASM3 Activated Sludge Model no.3 (Gujer *et al.*, 1999) was developed.
In the absence of oxygen and NH_4^+, anoxic growth, on NO_2^- and NO_3^- (denitrification), and anoxic storage have been modelled according to the stoichiometric and composition matrix presented in Figure 4.7. Anoxic storage of readily biodegradable substrates consists in the storage of S_S as cell internal storage products X_{STO}. The composition matrix defines the variables in terms of TOD (Theoretical Oxygen Demand) and N (Nitrogen). The coefficients of the stoichiometric matrix are derived from by applying TOD and N conservation laws for each defined reaction.
The variables included in the model are the following:

- S_S: readily biodegradable organic substrates (COD). It is the fraction of soluble COD directly available for heterotrophs;
- S_{NO2}: nitrite nitrogen;
- S_{NO3}: nitrate nitrogen;
- S_{N2}: denitrogen. S_{N2} is assumed as the only product of denitrification;
- X_H: heterotrophic organisms;
- X_{sto}: a cell internal storage product of heterotrophic organisms. It occurs only associated with X_H.

The stoichiometric parameters (Table 4.1) assumed for the calculation are (Gujer *et al.*, 1999):

- $Y_{HB,NO}$: anoxic yield of heterotrophic biomass;
- $Y_{STO,NO}$: anoxic yield of stored product per readily biodegradable organic substrates;
- $i_{N,XB}$: nitrogen content in the heterotrophic biomass.

From the model equations, it is possible to calculate the "COD equivalents" of NO_2^- and NO_3^-, per each reaction (e.g. how many grams of COD are required per gram of NO_2^- consumed via denitrification). The conversion factors used are presented in Figure 4.7. According to these assumptions, it is possible to compare the actual measured COD consumption with the one expected from the measured NO_2^- and NO_3^- depletion. The COD cumulative curves are presented in Figure 4.6. Interestingly, denitrification could explain only 49% of the consumed COD (13.0 mgCOD/L out of a total of 26.4 mgCOD/L consumed). Conversely, if anoxic storage is considered, most of the COD depletion could be explained. Comparable results were obtained under micro-aerobic conditions, suggesting that oxygen plays only a minor role in the studied system. Previous studies showed that biomasses adapted to long term discontinuous feeding regimes, i.e. acetate pulse under anoxic conditions, develop significant storage capacities as opposed to biomasses fed continuously (Çığgın *et al.*, 2007). As R2 was fed once per cycle and, thus, the biomass was subject to instant relatively high nitrogen and COD concentrations, it is therefore speculated that the feeding regime triggered the observed storage potential. This hypothesis seems to be further supported by the results obtained with Vayu sludge and presented in Figure 4.8 and

4.9. The nitritation/anammox sludge used as inoculum for Vayu came from a full scale SBR reactor (fed discontinuously, once at the beginning of each cycle) whereas Vayu reactor is operated semi-continuously (small and frequent pulses). One month after the inoculation, about 75% of the acetate consumption could be explained by denitrification (with the remaining 25% ascribed to storage) (Experiment 2 and 3, Figure 4.8). In the following experiment 4 and 5 (Figure 4.9), after one month of semi-continuous feeding, no storage was observed anymore and even the simple denitrification seems to overestimate the expected COD consumption. The experiment will be repeated and, to better follow the evolution of the observed changes in COD consumption pattern, the inoculum will be characterized as well. The correlation between feeding regime and storage potential, however, needs further confirmations, preferably via independent methods (e.g. labelled substrates).

Based on the results presented in this section, the following conclusions can be drawn. It is possible to treat MWW in an anammox-based system at 28°C, provided that an appropriate pre-treatment is applied (here: biological COD removal and, for the moment, external NO_2^- addition). In the perspective of full scale implementation, operation temperature needs to be decreased: for the sake of completeness it is mentioned that, in a follow up study on the same reactor discussed herein, lower but stable activities were obtained down at 15°C (data are still preliminary, not shown). Furthermore, after over four months of stable operation with MWW, only limited heterotrophic denitrification was observed and anammox remained the dominant nitrogen consumption route in the system. Interestingly, in addition, in the presence of easily degradable organics the sludge revealed a significant anoxic storage capacity. From the comparison with a different sludge, a tentative correlation between the applied feeding regime and the storage capacity was proposed: discontinuous feeding, as opposed to (semi-)continuous, boosts the organics storage potential of the sludge.
Consequently, it is suggested that anoxic storage should be included when modelling anammox-based systems, especially when discontinuous feeding is applied. Finally, to this end, the direct involvement of anammox bacteria in COD storage/depletion cannot be ruled out: this would represent a clear competitive advantage for them. Therefore, this hypothesis will be tested with the combination of micro-autoradiography and FISH (MAR-FISH), with radioactively labelled substrates.

Capitolo 5

Conclusion and further developments

In this study the feasibility of municipal wastewater (MWW) treatment with anammox bacteria was studied in a two stage process. MWW was first treated in a highly aerated reactor (COD removal and partial nitritation) and then, in a second reactor, nitrogen was removed via anammox. The main conclusions are here discussed along with the planned and possible further developments.
With regard to the first step (COD removal and partial nitritation) the following conclusions can be drawn. First, high rate biological COD removal is feasible at ambient temperature and constitute a proper pre-treatment for a subsequent anammox-based process (see also next session for further discussion). Secondly, two main challenges are to be faced when combining COD removal and partial nitritation in a single reactor. On one side, control of NOB at low temperature requires particular attention: in the present study NO_2^- and NO_3^- were always produced in almost equal amounts and the latter could not be reduced. The identification of the optimal combination of SRT and oxygen control (e.g. total supply, working concentration etc.) will be further investigated. Surprisingly, on the other side, about half of the consumed ammonium could not be found as NO_2^- and NO_3^-. Most likely, due to the high COD content of MWW, this behavior could be ascribed to simultaneous denitrification and nitrogen incorporation into biomass. In the perspective of the implementation of anammox-based systems for MWW treatment, the occurrence of denitrification is to be avoided: it reduces, in fact, the COD available for energy production in anaerobic digestion. It is therefore suggested that COD removal and partial nitritation should be performed in separate reactors in future installations. Finally, to this end, nitrogen losses in the form of N_2O, a powerful greenhouse gas, cannot be ruled

out (no specific measurement were performed). N_2O is produced, in fact, mainly in the presence of low dissolved oxygen concentration and increased nitrite concentration (Kampschreur *et al.*, 2009), conditions that are present in the partial nitritation reactor. This aspect has to be further evaluated: even low N_2O emissions (1-2%) could completely nullify the environmental benefits of anammox-based systems.
Concerning the second step (anammox), the following concluding remarks are highlighted. It is possible to treat MWW in an anammox-based system at 28°C, provided that an appropriate pre-treatment is applied (here: biological COD removal and, for the moment, external NO_2^- addition). In the perspective of full scale implementation, operation temperature needs to be decreased: for the sake of completeness it is mentioned that, in a follow up study on the same reactor discussed herein, lower but stable activities were obtained down at 15°C (data are still preliminary, not shown). Furthermore, after over four months of stable operation with MWW, only limited heterotrophic denitrification was observed and anammox remained the dominant nitrogen consumption route in the system. Interestingly, in addition, in the presence of easily degradable organics the sludge revealed a significant anoxic storage capacity. From the comparison with a different sludge, a tentative correlation between the applied feeding regime and the storage capacity was proposed: discontinuous feeding, as opposed to (semi-)continuous, boosts the organics storage potential of the sludge. Consequently, it is suggested that anoxic storage should be included when modelling anammox-based systems, especially when discontinuous feeding is applied. Finally, to this end, the direct involvement of anammox bacteria in COD storage/depletion cannot be ruled out: this would represent a clear competitive advantage for them. Therefore, this hypothesis will be tested with the combination of micro-autoradiography and FISH (MAR-FISH), with radioactively labelled substrates.

Appendix

day		CODs	CODtot	NH_4^+-N	NO_2^--N	NO_3^--N
d		mg/l	mg/l	mg/l	mg/l	mg/l
0		126	327	30,6	0,00	0,0
1		124	326	26,4	0,00	0,00
2		121	288	25,6	0,00	0,00
5		194	432	26,8	0,00	0,30
6		125	242	10,8	0,10	0,60
7		224	367	17,4	0,10	0,17
12		110	255	19,5	0,10	0,20
14		71	361	5,44	0,10	0,20
16		97,7	200	25,2	0,00	0,20
19		102	264	21,6	0,20	0,20
21		224	392	30,5	0,10	0,10
23		103	281	33,3	0,11	0,00
26		298	580	32,6	0,00	0,00
28		201	384	27,5	0,00	0,00
30		100	360	25,9	0,10	0,10
33		114	257	24,2	0,18	0,20
35		227	407	24,5	0,00	0,00
40		171	331			
41		126	283	29,1	0,13	0,01
43		154	311	33,1	0,23	0,31
47		236	407	36	0,02	0,30
49		162	357	33,7	0,01	0,37
51		164	311	32,1	0,01	0,17
54		188	353	33	0,05	0,53
56		163	346	30	0,02	0,38
58		131	296	33,7	0,01	0,27
69		50,4	236	28,3	0,01	4,00
71		120	232	26,9	0,31	1,06
72		129	316	22,6	0,02	0,39
75		136	255	27	0,04	0,45
77		187	308	28,7	0,02	0,46
82		201	358	33,50	0,02	0,53
86		108	358	26,50	0,02	0,44
89		144,0	290	32,80	0,09	0,44
91		119,0	281	37,10	0,12	0,50
93		116,0	293	30,60	0,12	0,36
96		147,0	266	18,30	0,04	0,11
98		129,0	236	31,10	0.107	0,90
100		195,0	402	23,90	0,03	0,45
103		273,0	432	31,90	0,08	1,00
105		249,0	467	19,20	0,07	0,92
111		193,0	322	19,50	0,03	0,05
115		124	290	20	0,0	0,4
120		144	463	30	0,7	0,6
AVERAGE						
Phase 1		132,5	310,9	20,9	0,0	0,2
Phase 2		171,1	361,8	27,5	0,1	0,1
Phase 3		168,8	333,3	32,8	0,1	0,2
Phase4		145,0	300,0	29,3	0,1	0,9
Phase5		164,4	336,1	27,9	0,1	0,6

Figura 5.1: Influent values of partial nitritation reactor (R1).

day		CODs	NH_4^+-N	NO_2^--N	NO_3^--N
d		mg/l	mg/l	mg/l	mg/l
0		35,3	15,5	0,86	0,2
1		36,5	15,4	1,30	0,2
2		38,1	18,2	2,30	0,5
5		28	19	0,43	0,4
6		36,2	6,7	0,40	0,2
7		36	17,5	0,11	0,0
12		40	15,5	0,10	0,1
14		35,5	7,45	0,10	0,5
16		31,4	20	0,00	0,3
19		30	22,4	0	0,2
21		50,1	23,1	0,10	0,1
23		40,2	25,1	0,00	0,1
26		59,7	28,9	0	0
28		42,4	21	0	0
30		29,3	17	0	0
33		25,3	14,3	0,14	0,1
35		34,8	15,4	0,24	0
40		21			
41		37,6	17,9	0,53	0,6
43		27	19,6	0,034	0,398
47		29,3	15,4	1,66	0,805
49		25,8	12,2	0,44	0,331
51		21,7	12,9	0,98	0,76
54		31	12,7	4,96	1,82
56		35	9	3,26	0,123
58		27,6	10,2	3,28	1,22
69		31,3	7,69	5,26	11,5
71		30	7,48	5,8	2,8
72		29,8	7,92	4,41	2,02
75		30,6	9,04	2,46	1,5
77		29,9	8,65	2,84	1,79
82		24,6	7,45	2,94	2,34
86		24	3,03	2,67	2,99
89		24,4	7,75	2,54	2,94
91		24,6	10,4	1,97	2,16
93		22,1	4,59	2,53	3,92
96		20,7	4,14	1,78	3,51
98		21,1	5,8	1,46	2,79
100		14,9	8,69	0,60	1,06
103		25	8,29	0,67	1,02
105		47,4	14	0,31	0,49
111		26,3	5,25	1,72	1,99
115		26	5,24	0,92	0,96
120		22,5	4,4	0,68	0,89
AVERAGE					
Phase 1		35,2	15,0	0,6	0,3
Phase 2		37,0	20,9	0,1	0,1
Phase 3		27,1	15,6	0,7	0,6
Phase4		30,0	8,9	3,9	2,8
Phase5		24,9	7,4	1,6	2,3
Phase6		27,0	7,6	0,8	1,1

Figura 5.2: Effluent values of partial nitritation reactor (R1).

day d		NH4-N Rem %	COD rem %	T °C
0		49,3	72	24,3
1		41,7	71	24,7
2		28,9	69	29,8
5		29,1	86	30,5
6		38,0	71	22,6
7		-0,6	84	22,9
12		20,5	64	20,1
14		-36,9	50	20,7
16		20,6	68	22,1
19		-3,7	71	17,9
21		24,3	78	17,7
23		24,6	61	17,1
26		11,3	80	17,5
28		23,6	79	17,2
30		34,4	71	17,0
33		40,9	78	17,4
35		37,1	85	17,2
40			88	18,2
41		38,5	70	16,6
43		40,8	82	17,3
47		57,2	88	18,4
49		63,8	84	17,4
51		59,8	87	16,6
54		61,5	84	18,2
56		70,0	79	17,5
58		69,7	79	16,7
69		72,8	38	17,0
71		72,2	75	17,0
72		65,0	77	16,7
75		66,5	78	17,9
77		69,9	84	17,4
82		77,8	88	16,5
86		88,6	78	16,7
89		76,4	92	18,4
91		72,0	91	16,8
93		85,0	92	16,7
96		77,4	92	18,3
98		81,4	91	18,0
100		63,6	96	17,1
103		74,0	94	18,3
105		27,1	90	17,4
111		73,1	92	17,9
115		73,4	91	17,2
120		85,3	95	18,5
AVERAGE				
Phase 1		21,2	70,3	24,2
Phase 2		24,1	76,6	17,5
Phase 3		52,0	83,1	17,4
Phase4		69,5	75,6	17,2
Phase5		77,3	90,9	17,5
Phase6		66,1	93,1	17,7

Figura 5.3: Removal and temperature values of partial nitritation reactor (R1).

day		TSS/VSS
d		
2		1,82
5		1,31
8		1,22
12		1,24
16		1,25
19		1,31
23		1,28
28		1,20
34		1,22
49		1,21
56		1,33
72		1,29
77		1,24
85		1,25
90		1,25
98		1,30
106		1,30

Figura 5.4: Solids values of partial nitritation reactor (R1).

Elenco delle figure

Elenco delle tabelle

References

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

Amann, R. I., Krumholz, L., and Stahl, D. A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of bacteriology*, **172**(2), 762–770.

Amann, R. I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews*, **59**(1), 143–169.

Anthonisen, A., Loehr, R., Prakasam, T., and Srinath, E. (1976). Inhibition of nitrification by ammonia and nitrous acid. *Journal (Water Pollution Control Federation)*, pages 835–852.

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

Bonomo, L. (2008). *Trattamenti delle acque reflue*. McGraw-Hill.

Broda, E. (1977). Two kinds of lithotrophs missing in nature. *Zeitschrift für allgemeine Mikrobiologie*, **17**(6), 491–493.

Çığğın, A. S., Karahan, Ö., and Orhon, D. (2007). Effect of feeding pattern on biochemical storage by activated sludge under anoxic conditions. *Water research*, **41**(4), 924–934.

Cui, C., Zhang, Y., Han, H., and Zheng, S. (2012). Improvement of fish–fcm enumeration performance in filamentous yeast species in activated sludge by snailase partial digestion. *Yeast*, **29**(3-4), 111–117.

Damsté, J. S. S., Strous, M., Rijpstra, W. I. C., Hopmans, E. C., Geenevasen, J. A., van Duin, A. C., van Niftrik, L. A., and Jetten, M. S. (2002). Linearly concatenated cyclobutane lipids form a dense bacterial membrane. *Nature*, **419**(6908), 708–712.

Dapena-Mora, A., Fernandez, I., Campos, J., Mosquera-Corral, A., Mendez, R., and Jetten, M. (2007). Evaluation of activity and inhibition effects on anammox process by batch tests based on the nitrogen gas production. *Enzyme and Microbial Technology*, **40**(4), 859–865.

De Clippeleir, H., Yan, X., Verstraete, W., and Vlaeminck, S. E. (2011). Oland is feasible to treat sewage-like nitrogen concentrations at low hydraulic residence times. *Applied microbiology and biotechnology*, **90**(4), 1537–1545.

De Clippeleir, H., Vlaeminck, S. E., De Wilde, F., Daeninck, K., Mosquera, M., Boeckx, P., Verstraete, W., and Boon, N. (2013). One-stage partial nitritation/anammox at 15 c on pretreated sewage: feasibility demonstration at lab-scale. *Applied microbiology and biotechnology*, **97**(23), 10199–10210.

Feng, Y.-J., Tseng, S.-K., Hsia, T.-H., Ho, C.-M., and Chou, W.-P. (2007). Partial nitrification of ammonium-rich wastewater as pretreatment for anaerobic ammonium oxidation (anammox) using membrane aeration bioreactor. *Journal of bioscience and bioengineering*, **104**(3), 182–187.

Fux, C., Marchesi, V., Brunner, I., and Siegrist, H. (2004). Anaerobic ammonium oxidation of ammonium-rich waste streams in fixed-bed reactors. *Water Science & Technology*, **49**(11-12), 77–82.

Gali, A., Dosta, J., Van Loosdrecht, M., and Mata-Alvarez, J. (2007). Two ways to achieve an anammox influent from real reject water treatment at lab-scale: Partial sbr nitrification and sharon process. *Process Biochemistry*, **42**(4), 715–720.

Galloway, J. N., Aber, J. D., Erisman, J. W., Seitzinger, S. P., Howarth, R. W., Cowling, E. B., and Cosby, B. J. (2003). The nitrogen cascade. *Bioscience*, **53**(4), 341–356.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C., Green, P., Holland, E., *et al.* (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry*, **70**(2), 153–226.

Ganigué, R., López, H., Balaguer, M., and Colprim, J. (2007). Partial ammonium oxidation to nitrite of high ammonium content urban landfill leachates. *Water Research*, **41**(15), 3317–3326.

Gong, Z., Yang, F., Liu, S., Bao, H., Hu, S., and Furukawa, K. (2007). Feasibility of a membrane-aerated biofilm reactor to achieve single-stage autotrophic nitrogen removal based on anammox. *Chemosphere*, **69**(5), 776–784.

Gruber, N. and Galloway, J. N. (2008). An earth-system perspective of the global nitrogen cycle. *Nature*, **451**(7176), 293–296.

Gujer, W., Henze, M., Mino, T., and Van Loosdrecht, M. (1999). Activated sludge model no. 3 (vol 39, pg 183, 1999). *Water Science and Technology*, **39**(12), AR1–AR1.

Güven, D., Dapena, A., Kartal, B., Schmid, M. C., Maas, B., van de Pas-Schoonen, K., Sozen, S., Mendez, R., den Camp, H. J. O., Jetten, M. S., *et al.* (2005). Propionate oxidation by and methanol inhibition of anaerobic ammonium-oxidizing bacteria. *Applied and environmental microbiology*, **71**(2), 1066–1071.

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

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

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

Jenni, S., Vlaeminck, S. E., Morgenroth, E., and Udert, K. M. (2014). Successful application of nitritation/anammox to wastewater with elevated organic carbon to ammonia ratios. *Water research*, **49**, 316–326.

Kampschreur, M. J., Temmink, H., Kleerebezem, R., Jetten, M. S., and van Loosdrecht, M. (2009). Nitrous oxide emission during wastewater treatment. *Water research*, **43**(17), 4093–4103.

Kartal, B., Kuypers, M. M., Lavik, G., Schalk, J., Op den Camp, H. J., Jetten, M. S., and Strous, M. (2007). Anammox bacteria disguised as denitrifiers: nitrate reduction to dinitrogen gas via nitrite and ammonium. *Environmental microbiology*, **9**(3), 635–642.

Kartal, B., Maalcke, W. J., de Almeida, N. M., Cirpus, I., Gloerich, J., Geerts, W., den Camp, H. J. O., Harhangi, H. R., Janssen-Megens, E. M., Francoijs, K.-J., *et al.* (2011). Molecular mechanism of anaerobic ammonium oxidation. *Nature*, **479**(7371), 127–130.

Lackner, S., Terada, A., and Smets, B. F. (2008). Heterotrophic activity compromises autotrophic nitrogen removal in membrane-aerated biofilms: results of a modeling study. *Water research*, **42**(4), 1102–1112.

Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M., and Welling, G. W. (1995). Quantitative fluorescence in situ hybridization of bifidobacterium spp. with genus-specific 16s rrna-targeted probes

and its application in fecal samples. *Applied and Environmental Microbiology*, **61**(8), 3069–3075.

Lee, N., Nielsen, P. H., Andreasen, K. H., Juretschko, S., Nielsen, J. L., Schleifer, K.-H., and Wagner, M. (1999). Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. *Applied and Environmental Microbiology*, **65**(3), 1289–1297.

Ma, B., Peng, Y., Zhang, S., Wang, J., Gan, Y., Chang, J., Wang, S., Wang, S., and Zhu, G. (2013). Performance of anammox uasb reactor treating low strength wastewater under moderate and low temperatures. *Bioresource technology*, **129**, 606–611.

Metcalf, L. and Eddy, H. P. (2004). *Wastewater Engineering: treatment and reuse*. McGraw-Hill.

Moter, A. and Göbel, U. B. (2000). Fluorescence in situ hybridization (fish) for direct visualization of microorganisms. *Journal of microbiological methods*, **41**(2), 85–112.

Mulder, A., Graaf, A., Robertson, L., and Kuenen, J. (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology*, **16**(3), 177–184.

Ni, B.-J., Ruscalleda, M., and Smets, B. F. (2012). Evaluation on the microbial interactions of anaerobic ammonium oxidizers and heterotrophs in anammox biofilm. *Water research*, **46**(15), 4645–4652.

Pernthaler, A., Pernthaler, J., and Amann, R. (2002). Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Applied and Environmental Microbiology*, **68**(6), 3094–3101.

Persson, F., Sultana, R., Suarez, M., Hermansson, M., Plaza, E., and Wilén, B.-M. (2014). Structure and composition of biofilm communities in a moving bed biofilm reactor for nitritation–anammox at low temperatures. *Bioresource technology*, **154**, 267–273.

Pynaert, K., Smets, B. F., Wyffels, S., Beheydt, D., Siciliano, S. D., and Verstraete, W. (2003). Characterization of an autotrophic nitrogen-removing biofilm from a highly loaded lab-scale rotating biological contactor. *Applied and Environmental Microbiology*, **69**(6), 3626–3635.

Sliekers, A. O., Third, K., Abma, W., Kuenen, J., and Jetten, M. (2003). Canon and anammox in a gas-lift reactor. *FEMS Microbiology Letters*, **218**(2), 339–344.

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

Strous, M., Fuerst, J. A., Kramer, E. H., Logemann, S., Muyzer, G., van de Pas-Schoonen, K. T., Webb, R., Kuenen, J. G., and Jetten, M. S. (1999b). Missing lithotroph identified as new planctomycete. *Nature*, **400**(6743), 446–449.

Strous, M., Kuenen, J., Fuerst, J., Wagner, M., and Jetten, M. (2002). The anammox case – a new experimental manifesto for microbiological eco-physiology. *Antonie van Leeuwenhoek*, **81**(1-4), 693–702.

Udert, K., Fux, C., Mnster, M., Larsen, T., Siegrist, H., and Gujer, W. (2003). Nitrification and autotrophic denitrification of source-separated urine. *Water Science & Technology*, **48**(1), 119–130.

Udert, K. M., Kind, E., Teunissen, M., Jenni, S., and Larsen, T. A. (2008). Effect of heterotrophic growth on nitritation/anammox in a single sequencing batch reactor. *Water Science & Technology*, **58**(2).

Van de Graaf, A. A., de Bruijn, P., Robertson, L. A., Jetten, M. S., and Kuenen, J. G. (1996). Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology*, **142**(8), 2187–2196.

Van Dongen, U., Jetten, M., Van Loosdrecht, M., *et al.* (2001). The sharon®-anammox® process for treatment of ammonium rich wastewater. *Water science and technology*, **44**(1), 153–160.

Van Hulle, S., Van Den Broeck, S., Maertens, J., Villez, K., Donckels, B., Schelstraete, G., Volcke, E., and Vanrolleghem, P. A. (2005). Construction, start-up and operation of a continuously aerated laboratory-scale sharon reactor in view of coupling with an anammox reactor. *Water SA*, **31**(3), p–327.

Van Kempen, R., Mulder, J., Uijterlinde, C., and Loosdrecht, M. (2001). Overview: full scale experience of the sharon® process for treatment of rejection water of digested sludge dewatering. *Water science and technology*, **44**(1), 145–152.

Vazquez-Padın, J., Fernandez, I., Morales, N., Campos, J., Mosquera-Corral, A., and Mendez, R. (2011). Autotrophic nitrogen removal at low temperature. *Water Science & Technology*, **9**(63.6), 9.

Wagner, M., Horn, M., and Daims, H. (2003). Fluorescence¡ i¿ in situ¡/i¿ hybridisation for the identification and characterisation of prokaryotes. *Current opinion in microbiology*, **6**(3), 302–309.

Winkler, M.-K., Kleerebezem, R., and Van Loosdrecht, M. (2012). Integration of anammox into the aerobic granular sludge process for main stream wastewater treatment at ambient temperatures. *Water research*, **46**(1), 136–144.

Wyffels, S., Van Hulle, S. W., Boeckx, P., Volcke, E. I., Cleemput, O. V., Vanrolleghem, P. A., and Verstraete, W. (2004). Modeling and simulation of oxygen-limited partial nitritation in a membrane-assisted bioreactor (mbr). *Biotechnology and Bioengineering*, **86**(5), 531–542.

Yamamoto, T., Takaki, K., Koyama, T., and Furukawa, K. (2008). Long-term stability of partial nitritation of swine wastewater digester liquor and its subsequent treatment by anammox. *Bioresource technology*, **99**(14), 6419–6425.