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Nitrogen removal bacterial communities characteristics and dynamics at lab-scale reactors

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3.1 Introduction

Human activities in the modern overcrowded world generate more and more wastewater which needs to be treated efficiently each day. The wastewater contains high concentrations of nitrogen, which together with phosphorus, is a crucial biogenic compound which directed to water bodies can cause their eutrophication. The most noticeable effect of eutrophication is algal bloom linked with reduction of water clarity and quality caused by oxygen depletion and structural and functional changes in the ecosystems (Zhou et al., 2018). To protect the aquatic environment from these unfavorable effects of the influence of high nitrogen concentration its effective removal from wastewater is of utmost importance.

3.2 Morphological forms of nitrogen removal bacteria communities in wastewater treatment systems

In nature the nitrogen removal is performed by several groups of microorganisms leading various biochemical processes linked together in a biogeochemical nitrogen cycle. These processes occur in soil and water and they are led by microbes inhabiting different ecological niches in these environments. In the case of wastewater treatment plants (WWTPs)

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nitrogen removal is performed in an aquatic environment in a suspension or as a form of biofilm.

The most often used system of biological wastewater treatment, especially at a large scale of municipal WWTPs is a suspension of microorganisms biomass known as activated sludge. This wastewater treatment method was developed on the basis of observations of water self-purification processes adopted to wastewater treatment in an intensified form. From a microbiological point of view, activated sludge is a flocculated mixture of microorganisms belonging to *Bacteria*, *Archaea*, *Protozoa*, and *Metazoa*, creating technological biocenosis. In this technological system the well-working community requires a convenient sludge age and other technological parameters as loading rate, hydraulic retention time, acceptors of electrons, etc., which as a result ensure high treatment reaction rates (Azizi et al., 2013). Activated sludge microorganisms metabolize wastewater impurities, collaborating in a biological system based on ecological rules. Despite the fact that activated sludge is the most popular biological way for wastewater treatment, biofilm-based systems are regarded to be useful in wastewater purification processes, especially in case of sewage containing recalcitrants harmful for the microbial community.

Biofilm is a three-dimentional microbial structure created on a surface of a solid carrier surrounded by matrix, which consists of extracellular polymeric substances (EPS), usually sugars and/or proteins, but nucleic and humic acids were also reported (Nguyen et al., 2016). EPS are also produced in flocculated activated sludge but in lower quantities. These compounds play an important role in microbial and suspended solids flocculation improvement, metal binding, removal of toxic organic compounds, and further sludge-settling and dewatering (Nouha et al., 2018). The qualitative composition of EPS is highly variable and depends on the community structure, the feeding medium composition, and technological factors such as pH, temperature, and dissolved oxygen (DO) (Nouha et al., 2018). Microbes living in such a form have easier access to nutrients and higher protection against unfavorable environmental factors due to the matrix presence. Active bacteria are able to adhere to any sort of solid surface via gene expression change and alter their form from “swimmers” into “stickers” in a very short time. Linked to the surface, bacteria produce large quantities of EPS. They multiply and diversify to create a mature biofilm irreversibly linked to the surface (Hall-Stoodley et al., 2004). Mature biofilm structure enables the creation of oxic and anoxic zones in one biotope which conduce the coexistence of microorganisms with various oxygen demand (Fig. 3.1A).

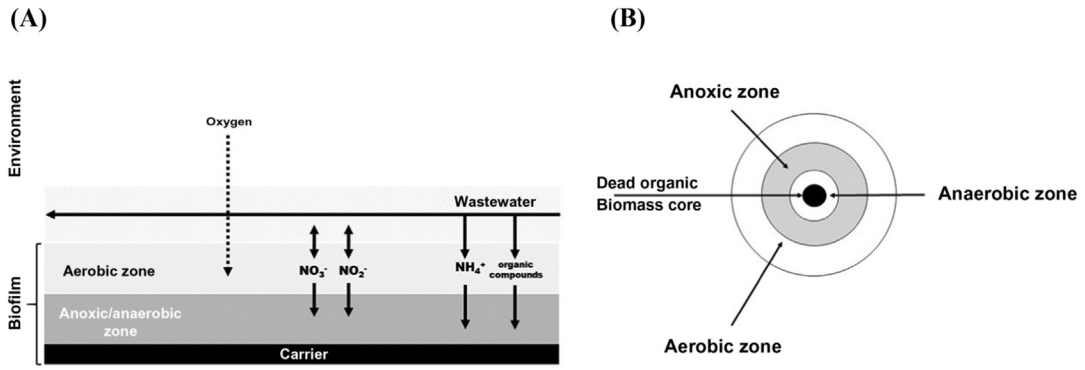

Figure 3.1 Scheme of biofilm (A) and granule (B) structure.

Microbial ability to aggregate and their EPS production is used in to create granular sludge (Fig. 3.1B). Granules are spherically shaped dense structures with better settleability than floccular activated sludge. It has been observed that granules and flocs often coexist in one bioreactor system (Hubaux et al., 2015). The process of granulation requires direct cell−cell contact and mutual biological, chemical, and physical phenomena occurrence. To obtain granular sludge, a batch-wise operation with feast−famine feeding is required. This process is possible with a large height to diameter ratio of the reactor and a short settling time to select dense microbial aggregates. Also high hydrodynamic shear forces are needed. Granular sludge can be produced in aerobic and anaerobic conditions and possesses excellent settling properties (Wilén et al., 2018). Granules usage enables the performing of aerobic and anaerobic processes of nitrogen and phosphorus removal due to the fact that their structure is similar to biofilm but without the necessity of a solid carrier presence. Thus the granules' microbiological structure can be also highly diversified (Szabó et al., 2017; Wilén et al., 2018). Fig. 3.1 presents a comparison of biofilm and granule structure.

3.3 Nitrogen removal bacteria characteristics

Nitrogen removal processes in wastewater treatment bioreactors are led by a few main groups of microorganisms needed to remove it from the sewage. Fig. 3.2 presents a simplified nitrogen cycle in which nitrogen is removed from the sewage in bioreactors. The most important nitrogen removal microorganisms involved in proper wastewater treatment are nitrifiers, denitrifiers, and anammox bacteria (AMX).

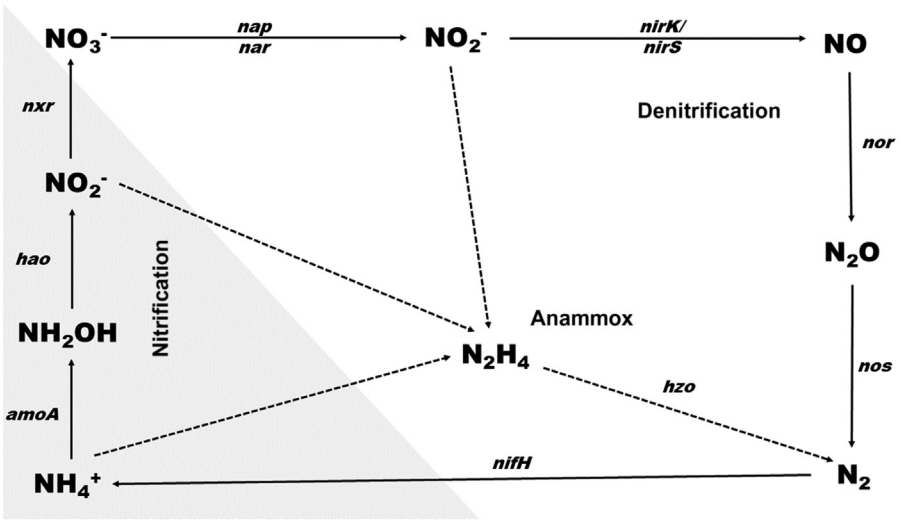

Figure 3.2 Simplified nitrogen removal cycle in wastewater treatment bioreactor with functional genes responsible for the processes (gray zone indicates aerobic processes; organic nitrogen ammonification and ammonia and nitrate assimilation for biomass production not shown).

Nitrification can be performed by both autotrophic and heterotrophic bacteria. Together with denitrification, it is the most often used way to remove nitrogen from wastewater in classical wastewater treatment plant systems. Autotrophic nitrification is a two-step process of ammonia nitrogen conversion into nitrate via nitrite ions. In the case of autotrophic nitrification its first step is performed according to Eq. 3.1:

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ \quad (3.1)$$

The second step of nitrification presents Eq. 3.2:

$$2NO_2^- + O_2 \rightarrow 2NO_3^- \quad (3.2)$$

The first step of nitrification (called also nitritation) is performed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing *Archaea* (AOA). Although AOA and AOB are both autotrophic, there is a difference between inorganic carbon assimilation path and organic carbon usage. AOB use the Calvin cycle, while AOA use a modified 3-hydroxypropionate/4-hydroxybutyrate cycle (3HP/4HB) (Hatzenpichler, 2012). This difference in carbon usage has consequences in microbial adaptation to environment. Moreover, AOA can function as mixotrophs (Berg, 2011). While AOA belong to one phylum *Thaumarchaeota* (He et al., 2018), AOB are a monophyletic group belonging mainly to β- and γ-*Proteobacteria*. The

most investigated representative of this group is *Nitrosomonas*, while the other cultivable AOB are *Nitrosospira*, *Nitrosolobus*, and *Nitrosococcus*.

The most popular functional molecular marker used for this group's research is the α subunit of ammonia monooxygenase coding gene (*amoA*). The AMO enzyme is responsible for the conversion of ammonia into NH_2OH, both in the case of bacteria and *Archaea*. The second enzyme—hydroxylamine oxidoreductase, HAO—is responsible for hydroxylamine to nitrite conversion and the *hao* coding gene can be also used for AOB analysis (Fig. 3.2).

The second step of nitrification (also called nitratation) is performed by nitrite-oxidizing bacteria (NOB), relatively diverse as a microbial group. Systematically, such genera as *Nitrobacter*, *Nitrospira, Nitrotoga*, *Nitrococcus*, *Nitrospina*, *Nitrolancetus* belong to α-, β-, γ-, and δ-*Proteobacteria*, respectively. They represent two main phyla: *Chloroflexi* and *Nitrospirae* (Han et al., 2018). This group's research is usually based on the nitrite oxidoreductase coding gene (*nxr*) (Fig. 3.2).

It is worth mentioning that there is a group of heterotrophic nitrifiers often present in WWTP communities performing oxidation of inorganic and organic reduced forms of nitrogen (Prosser, 2005). Among this group such genera as *Providencia rettgeri*, *Pseudomonas stutzeri*, *Acinetobacter* sp., *Achromobacter* sp., *Diaphorobacter* sp., *Bacillus* sp., *Lysinibacillus* sp., and *Rhodococcus* sp. can be mentioned as being often present in wastewater treatment systems (Srivastava et al., 2016).

Since its discovery, nitrification was considered to be a two-step process. The first step requires ammonia, while the second requires nitrite as a substrate. Such separate performance of nitrification at two different microorganisms with their substrate-product exchange was the reason why the coexistence of AOB, AOA, and NOB microorganisms in nitrifying communities was observed (Daims et al., 2015; Arp and Bottomley, 2006). There was no known microorganism capable of performing both steps of nitrification in one cell, despite the fact that more energy could be gained from such a process. This would be also the reason that this microorganism would gain prevalence over the ammonia and nitrite oxidizers in the environment (Daims et al., 2015). Previous authors (Costa et al., 2006; Daims et al., 2015), however, pointed that such prevalence would be possible only under particular environmental conditions favoring this microbe's growth. These conditions would be spatial biomass aggregations in biofilms or flocs together with slow, substrate influx-limited growth (Costa et al., 2006; Daims et al., 2015).

In 2015 Daims et al. (2015) and van Kessel et al., 2015 described bacteria, representatives of genus *Nitrospira*, performing a complete ammonia oxidation (comammox) in a single cell. This genus is the most known among NOB, present in various natural and engineered ecosystems. *Nitrospira* has at least six phylogenetic sublineages and lineage II seems to be the most distributed among different environments (Daims et al., 2001).

In the case of Daims et al.'s (2015) study, the microorganism was obtained from a sample of microbial biofilm located on the walls of a pipe under the flow of hot water (56°C, pH 7.5) raised from a 1200 m deep oil exploration well in Russia. The authors proposed the name Candidatus *Nitrospira inopinata* for that isolate. In van Kessel et al., 2015 research, the source of the microbes was the anaerobic part of a trickling filter linked to recirculation of the aquaculture system. The names proposed for these two isolates from the enrichment were Candidatus *Nitrospira nitrosa* and C. *Nitrospira denitrificans*.

Comammox *Nitrospira* representatives in the aforementioned studies grow in biofilm systems, but often these genus representatives grow also as microcolonies or flocs (Schramm et al., 1999; Daims et al., 2015). According to the research performed on nitrite-oxidizing representatives of *Nitrospira* this genus is described as slow-growing K-strategists, which is well adapted to low substrate concentrations (Gruber-Dorninger et al., 2015; Schramm et al., 1999; Maixner et al., 2006; Nowka et al., 2015; Daims et al., 2015; Ushiki et al., 2017). Since 2015 the presence and performance of comammox microorganisms in nitrogen removal wastewater systems have been described (Gonzalez-Martinez et al., 2016; Chao et al., 2016; Bartelme et al., 2017).

Denitrifiers are mostly heterotrophic microorganisms creating a polyphyletic group. This group consists of widely known genera, such as *Bacillus*, *Thiobacillus*, *Propionibacterium*, *Pseudomonas*, *Paracoccus*, *Azoracoccus*, and *Rhodobacter* belonging to α-, β, γ- and ε-*Proteobacteria*. Some representatives of denitrifiers belong also to *Firmicutes*, *Actinomycetes*, *Bacteroidetes*, and *Aquificaceae* (Braker and Conrad, 2011). Representatives of *Archaea*, such as *Haloferax* sp., can also perform the denitrification process (Torregrosa-Crespo et al., 2016). Compared to AOB, NOB, and anammox, this bacterial species multiplies much faster.

Denitrifiers perform the anoxic process of nitrate reduction of gaseous nitrogen according to Eq. 3.3:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \tag{3.3}$$

Particular steps of nitrite to dinitrogen gas conversion are conducted by several enzymes (Fig. 3.2). Nitrite reductase (*nar*), functioning as a membrane bound complex, is responsible for the conversion of NO_3^- to NO_2^-. In some denitrifiers *nar* is replaced by periplasmatic nitrate reductase (*nap*). In the next step of NO_2^- to NO conversion nitrite reductase (copper-based *nirK* and cytochrome cd_1-based *nirS*) are involved. Nitric oxide reductase (*nor*) and nitrous oxide reductase (*nos*) are responsible for NO to N_2O and N_2O to N_2 conversion, respectively (Yang et al., 2020). This biochemical process was regarded to be the only one which can restore free nitrogen in the atmosphere until the discovery of the anaerobic ammonia oxidation–anammox process.

Anaerobic ammonia oxidation–anammox was predicted in the 1970s on the basis of thermodynamic calculations as a missing link for ammonia oxidation to dinitrogen gas in the nitrogen cycle. In 1995 such a process was discovered in a denitrifying bioreactor (Mulder et al., 1995), and a few years later Strous et al. (1999) described the bacteria performing it. The anammox biochemical reaction presents Eq. 3.4:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (3.4)$$

Anammox bacteria (AMX) belong to only one phylum *Planctomycetes*. They are chemolithoautotrophic and they differ from the other representatives of the phylum. Their cell ultrastructure differs from a typical bacterial cell. In AMX the cell cytoplasm is divided into three compartments separated by single bilayer membranes, which is unusual in bacterial world. The first, outer part of anammox cell is called the paryphoplasm, the middle is the riboplasm, and the inner part is called the anammoxosome. In this structure hydrazine oxidoreductase (HZO), which is responsible for the anammox process performance, is located and this enzyme coding gene (*hzo*) is used for AMX research (van Niftrik and Jetten, 2012).

Up to now, five genera of AMX are known: Candidatus *Kuenenia*, *Brocadia*, *Anammoxoglobus*, and *Jettenia* usually present in technological systems, and Candidatus *Scalindua* found in natural ecosystems (van Niftrik and Jetten, 2012). These bacteria grow very slowly. According to Zhang et al. (2017) the maximum specific growth rate of AMX was determined to be 0.33 ± 0.02/d for Candidatus *Brocadia sinica* and

0.18/d for Candidatus *Jettenia caeni* at 37°C. There is still no information about pure culture obtainment at the laboratory which underlines the assumption that despite optimal physiochemical parameters for these bacteria's growth they also required biological interactions with the other microorganisms in the ecosystem in which they live.

3.4 Technological parameters influencing particular bacterial groups

Technological parameters of wastewater treatment strongly influence the functional bacteria groups responsible for processes. DO and temperature are recognized as of great importance along with the organic carbon to total nitrogen ratio, and the sludge age (Metcalf & Eddy, Inc. et al., 2003).

Equations (3.1) and (3.2) allow one to calculate stoichiometric oxygen demand for ammonia oxidation to nitrite and then to nitrate by nitrifying bacteria as 4.57 g O_2/g N-NH_4^+. In fact, oxygen uptake for full nitrification is a bit lower, equal to 4.25 g O_2/g N-NH_4^+, because of the partial nitrogen uptake for bacteria growth. As oxygen is a substrate for nitrifying bacteria, its concentration in the bioreactors influences a rate of nitrifying bacteria growth according to the modified Monod equation presented as Eq. 3.5 (Metcalf & Eddy, Inc. et al., 2003):

$$\mu = \mu_{\max} \frac{S_{NH_4}}{S_{NH_4} + K_{NH_4}} \cdot \frac{S_{O_2}}{S_{O_2} + K_{O_2}} \tag{3.5}$$

where μ is bacteria specific growth rate 1/d; $\mu_{\max}$ is maximum bacteria specific growth rate, 1/d; S_{NH_4} is ammonia concentration, mg/L; S_{O_2} is DO concentration, mg/L; K_{NH_4} is half-saturation constant for ammonia, mg/L; and K_{O_2} is half-saturation constant for oxygen, mg/L.

Influence of the DO concentration in bioreactors on nitrifying bacteria activity depends on their form of growth and the affinity to oxygen. AOB affinity to oxygen is recognized as better then NOB (Brockmann and Morgenroth, 2010; Henze, 2000) but there are also opposite conclusions reporting better NOB affinity to oxygen than AOB (Regmi et al., 2014; Sliekers et al., 2005). Oxygen half-saturation constant values given in the literature vary from 0.4 to 2.0 mg O_2/L (Henze et al., 1997; Brockmann et al., 2008). When nitrifying bacteria grow in suspension culture as activated sludge flocs they have easy access to substrates present in bulk liquid and both AOB and NOB have their oxygen half-saturation constants lower than 1.5 mg O_2/L. Therefore DO

equal to 1.5−2.0 mg O_2/L are reported as sufficient for full nitrification (Henze et al., 1997). Higher DO concentration in bioreactors is required when nitrifying bacteria grow in biofilm or granules. Their access to oxygen depends not only on DO concentration in bulk liquid and their affinity to oxygen but also on oxygen distribution in biofilm or granule. The oxygen transport in biofilm or granules is limited by diffusion and bacterial uptake. The higher DO concentration in bulk liquid, the deeper the oxygen penetration in the biofilm and granules. DO equal to 1 mg/L at the depth of 1 mm of the biofilm was observed when DO in bulk liquid was equal to 3.5−4.0 mg/L ensuring effective (>90%) ammonia oxidation in synthetic municipal wastewater (Wang et al., 2018). But DO in bulk liquid equal to 2 mg O_2/L was sufficient to ensure aerobic condition in the whole granules of 2 mm thickness. Only larger granules presented in deeper layers an anoxic condition (Layer et al., 2020).

Oxygen has a different effect on denitrifiers and AMX. The oxygen for these two groups of bacteria acts as an inhibitor inactivating their enzymes involved in nitrogen removal and its concentration in bioreactors should not exceed 0.5 mg O_2/L. The oxygen inhibition is reversible when exposition time is limited. It allows these bacteria coexistence with nitrifying bacteria in microbial communities at many wastewater treatment technologies (Metcalf & Eddy, Inc. et al., 2003).

Generally nitrogen cycle bacteria prefer mesophilic temperatures but growth rates of particular functional groups differ and change with temperature fluctuation. Bacterial growth rate is directly related with sludge retention time (SRT) in bioreactors. SRT can be calculated as an approximate reversal of maximum bacteria specific growth rate ($1/\mu_{max}$). Therefore temperature influence on growth rate is important from technological point of view, especially in case of nitrifiers and AMX.

Nitrifying bacteria growth rate is limited when temperature is lower than 8°C−10°C (Gnida et al., 2016). With increasing temperature from 10°C−35°C their growth rate accelerates but differences between AOB and NOB are observed. In the temperature range 10°C−20°C, which is typical for municipal wastewater in temperate climate, AOB and NOB have comparable maximum specific growth rates and SRT longer than 10 days is sufficient to fulfill complete nitrification (Hellinga et al., 1998; Metcalf & Eddy, Inc. et al., 2003). However, at temperature higher than 20°C the maximum specific growth rate of AOB is faster than NOB. Therefore the required SRT for AOB is shorter than for NOB (Hellinga et al., 1998). The difference in required SRT for AOB and NOB in temperatures above 20°C depends also on

ammonia and nitrite concentration and pH in the bulk liquid. These three parameters shape free ammonia (FA) and free nitrous acid (FNA) concentrations responsible for nitrifying bacteria inhibition. In the bulk liquid FA is in equilibrium with the ammonium ion. Anthonisen et al. (1976) presented the formula for FA (NH_3) as Eq. 3.6:

$$NH_3 = \frac{17}{14} \cdot \frac{NH_4 \cdot 10^{pH}}{K_b/K_w + 10^{pH}} \tag{3.6}$$

where K_b/K_w are ionization constants, respectively, for ammonia and water. Their values depend on temperature according to Eq. 3.7:

$$K_b/K_w = e^{6344/(273+T)} \tag{3.7}$$

where T is temperature in °C.

Similar balance is observed between nitrite and FNA (HNO_2). FNA value is calculated with Eq. 3.8 (Anthonisen et al., 1976):

$$HNO_2 = \frac{46}{14} \cdot \frac{NO_2}{K_a + 10^{pH}} \tag{3.8}$$

where K_a is ionization constant for nitrous acid. Its value depends on temperature according to Eq. 3.9:

$$K_a = e^{-2300/(273+T)} \tag{3.9}$$

Anthonisen et al. (1976) have set wide ranges of FA and FNA, dependent on pH, responsible for AOB and NOB inhibition. Their results have been confirmed by many works reporting that NOB are more susceptible to FA and FNA than AOB (Raszka et al., 2011; Sin et al., 2008), therefore the buildup of FA in the bulk liquid increases the difference between SRTs required for AOB and NOB. This phenomenon is used in technologies of nitrogen removal based on partial nitrification.

AMX are characterized by approximately 10 times lower growth rate than AOB (Sin et al., 2008). Their doubling time is in the range of 4–15 days (Strous et al., 1998; van der Star et al., 2008). For suspended biomass growing at a temperature of 30°C, a doubling time equal to 3.3 days, corresponding to a maximum specific growth rate of 0.21 1/d, was reported by Lotti et al. (2014). However, this parameter depends heavily on taxonomic affiliation (Zhang et al., 2017). As AMX are mesophilic their metabolic activity and growth rate at temperatures below 10°C–15°C are very low, especially when bacteria are cultivated at temperatures close to optimal and then exposed to

temporal temperature decreases (Lotti et al., 2015). With temperature increasing from 15°C to 31°C the maximum growth rate increases from below 0.05 1/d to 0.29 1/d and with a further temperature increase above 36°C it drastically decreases (Straka et al., 2019). The specific growth rate of AMX dependence on temperature and the long doubling time result in required long SRT. The lower expected temperature in the bioreactor, the longer SRT or biomass concentration is required. Straka et al. (2019) reported that the decrease of the temperature from 30°C to 21°C has to be compensated by doubled SRT or doubled biomass concentration to reach the same efficiency of AMX at the same hydraulic retention time of wastewater. A further temperature decrease to 18°C requires four times longer SRT or biomass concentration. The biomass retention in the bioreactor and adoption of proper SRT fitting to temperature fluctuation is essential when anammox is considered as a key process of nitrogen removal in mainstream WWTP, which is characterized by much lower temperatures than those registered in the anaerobic sludge liquor sidestream.

Therefore anammox technologies are based on systems ensuring long SRT. Generally, there are systems with biomass growing as biofilms or granules because they guarantee high biomass concentration and limited uncontrolled biomass washout (Cema et al., 2007; Lotti et al., 2015; Straka et al., 2019). Similar effects are accomplished at membrane-assisted bioreactors because even if bacteria grow in suspension it is easy to control their growth and there is a lack of biomass washout (Hoekstra et al., 2018; Lotti et al., 2014, Lotti et al., 2015). Among the different reactors applied for anammox technologies, moving bed bioreactors (MBBRs) are very popular (Cema et al., 2011; Laureni et al., 2016). They give an opportunity to merge both types of bacteria growth—in suspension and as a biofilm. They are often called integrated fixed film activated sludge or hybrid-MBBR and applied as full-scale partial nitrification/anammox systems for waste sludge liquor treatment (Laureni et al., 2016; Laureni et al., 2019). Research on nitrifiers and AMX behavior in hybrid-MBBR showed that relatively faster growing nitrifiers prefer growth as flocs while AMX grow in biofilm. It allows easy control of nitrifiers SRT for maintaining longer SRT of AMX (Laureni et al., 2019). All these systems ensure SRT longer than 50 days is required when the temperature is equal to 20°C or lower (Hoekstra et al., 2018; Laureni et al., 2016; Lotti et al., 2014).

Denitrifying bacteria involved in nitrogen removal in a classical path of nitrification/denitrification belong to heterotrophs.

They are able to respire both with oxygen or nitrates/nitrites as terminal electron acceptors. As electron donors they use organic compounds. Their growth rate is much faster than nitrifiers and AMX that belong to autotrophs, therefore they require a much lower SRT. Henze (2000) recommend to calculate their maximum anoxic specific growth rate as a maximum aerobic specific growth rate with a factor of 0.8. The value of the maximum specific growth rate depends on temperature and COD (chemical oxygen demand) to NO_3-N/NO_2-N ratio, as both organic compounds represented by COD and nitrates/nitrites are substrates regulating denitrifiers growth (Eq. 3.10):

$$\mu = \mu_{\max} \cdot \frac{S_{NO_3/NO_2}}{S_{NO_3/NO_2} + K_{NO_3/NO_2}} \cdot \frac{S}{S + K_s} \tag{3.10}$$

where S_{NO_3/NO_2} is respectively, nitrate or nitrite concentration, mg/L; S is organic substrate concentration, mg/L; k_{NO_3/NO_2} is half-saturation constant for nitrate or nitrite respectively, mg/L; and K_S is half-saturation constant for organic substrate, mg/L.

As denitrifiers are mesophilic bacteria their growth at low temperatures below 10°C is slow and with increasing temperature up to 30°C–40°C the growth rate increase is observed. For wastewater treatment design and SRT calculation in a temperate climate, the maximum anoxic specific growth rates of 3–6 1/d and 5–10 1/d are recommended respectively, when municipal wastewater and methanol are electron donors (Henze et al., 1997). For temperatures 30°C–40°C Vandekerckhove et al. (2018) reported maximum anoxic specific growth rate increase up to 9.6 1/d, but for thermophilic conditions (50°C–60°C) it decreased to 4 1/d. Consequently, for mesophilic temperatures (up to 40°C) SRT is in the range 3–5 days and for thermophilic ones (50°C–60°C) 9–11 days is required.

Activity of denitrifying bacteria depends on substrates—biodegradable organic carbon (represented often by COD) and nitrates/nitrites. Denitrifiers have high affinity to nitrates. Their half-saturation constant for nitrates is in the range 0.2–0.5 mg N/L. The half-saturation constant for organic carbon is higher and depends on organic compounds. It varies in a wide range from 5 to 20 mg COD/L (Henze et al., 1997). In mesophilic temperatures denitrifiers require 5–6 g COD for 1 g NO_3-N removed and 2–3.5 g COD for 1 g NO_2-N removed. A shift to thermophilic temperatures required COD for nitrates reduction to decrease by around 23% while for nitrites the reduction remained unchanged Vandekerckhove et al. (2018).

3.5 Examples of nitrogen removal community structure in various wastewater systems

Many studies have been performed on nitrogen removal communities' characteristics in order to present their biodiversity, dynamics, and to draw general trends in these biocenoses changeability according to physiochemical and biological parameters during the wastewater treatment they perform.

In the research of Chen et al. (2018) the community of low DO (0.1–0.4 mg/L for 60 days and 1 mg/L for 60 days) is described in a membrane bioreactor with domination of *Proteobacteria*. The system of wastewater treatment was based on the performance of nitrifiers and denitrifiers. The inoculum used for the experiment was a mixture of full-scale and lab-scale activated sludges. The dominant phyla in the community created in the activated sludge in this bioreactor were *Proteobacteria* (81.7%), *Bacterioidetes* (6.5%), *Acidobacteria* (2.7%), and *Planctomycetes* (1.2%). The results of qPCR (quantitative Polymerase Chain Reaction) analysis of functional genes of particular nitrogen removal bacteria indicated that the relative abundance of AOB (*amoA* genes) and denitrifiers increased, while NOB group performance (*nxrB* genes) was inhibited in the first 60 days of the experiment. While in conditions of higher DO both *nxrB* and *amoA* genes increased, and *nirS, nirK*, and *nosZ* genes decreased. These results clearly show the coexistence of AOB, NOB, and denitrifiers in the same bioreactor in which the dominance of the particular group changed according to aeration parameters. Interestingly, NOB bacteria mainly belonged to *Nitrospiraceae* which could point at commamox microorganisms presence in this bioreactor as well.

Systems with partial nitrification and full nitrification vary in microbial proportions of the nitrogen removal groups which are coexisting in these systems. Technological parameters settings create an environment favoring particular groups of microorganisms regarded to be the most important (and effective) for a particular wastewater treatment. Zhao et al. (2018) reported phyla of *Proteobacteria* in both full and partial nitrification as the dominant group under low C/N ratio in wastewater. Nonetheless, all nitrogen removal bacteria were present in such systems but in different proportions. Groups of *Chloroflexi*, *Sphingobacteria*, and *Planctomycetes* were coexisting in this community as groups with relative high abundance with *Proteobacteria* representatives in the partial nitrification system. While in case of a nitrification/denitrification system the same groups were accompanying

Proteobacteria in a far lower proportion. In partial nitrification *Sphingobacteria*, known as involved in nitrogen removal processes, was estimated at 22.9%, while in full nitrification it was only 4.2%. Also in case of *Chloroflexi* abundance changed from 28.1% in partial nitrification to 0.38% in complete nitrification and *Planctomycetes* proportion changed from 10.8% to 2.3% for partial and full nitrification, respectively (Zhao et al., 2018). The same authors researched the influence of sludge circulating ratio on community structure. Their studies based on qPCR revealed that proportions of AOB and NOB are comparable when the circulating ratio is 25% and 100%. The proportion of 75% of circulating ratio supported AOB and their number was much higher than NOB. Thus it could be stated that such a circulating ratio would be useful in obtaining AOB domination during partial nitrification under low oxygen conditions (Zhao et al., 2018). Similar results were obtained by Akaboci et al. (2018) who introduced partial nitrification-anammox in sequencing batch reactor (SBR). *Proteobacteria* together with *Planctomycetes* were the most abundant phyla with proportions of $36.29\% \pm 3.3\%$ and $26.75\% \pm 4.7\%$ abundancy, respectively. *Chloroflexi* and *Bacterioidetes* were also observed in a proportion larger than 7.5%. The authors underlined the role of *Chloroflexi* and *Bacterioidetes* as microorganisms responsible for soluble microbial products decomposition and underlined their role as important players in total effluent nitrate decrease. *Bacterioidetes* play an important role in a nitrite loop facilitation with AMX or where complete denitrification support was also suspected. The coexistence od Candidatus *Kuenenia* with *Nitrosomonas* and *Nitrospira* as representatives of AOB and NOB, respectively, was also confirmed. In the case of bacterial community response to a medium shift in SBR system with anammox, Banach-Wiśniewska et al. (2020) found that feeding medium change has the strongest impact on the relative abundance of denitrifiers and representatives of *Planctomycetes*. Again, in the case of anammox technology all the nitrogen removal bacteria were present in the system. but varied in proportion.

Previous research of process startup in the same SBR system was performed by Ziembińska-Buczyńska et al. (2019b). In this work the results of fluorescent in situ hybridization (FISH) enabled the conclusion that particular nitrogen removal bacteria stay in a close relationship with each other and they cooperate in the bacterial community of activated sludge in the nitrogen removal process. Moreover, these results suggested AOB's protective role for AMX in activated sludge flocs. Despite the fluctuations in AMX number their performance was at a high level. Also denitrifiers and NOB were detected in this SBR system.

Wang et al. (2019) presented the results of the community shift in partial nitrification-anammox bioreactor with low DO (0.1–0.8 mg/L). During the startup period the *Planctomycetes* abundance decreased, which was caused by the environmental change from full-scale activated sludge into a lab-scale bioreactor with a lower number of ecological niches available. The dominant anammox genus shifted from Candidatus *Kuenenia* to Candidatus *Brocadia* which stands in contradiction with previous studies of van der Star et al. (2008) who reported a Candidatus *Brocadia* to Candidatus *Kuenenia* shift caused by limiting substrate concertation. These results of Wang et al. (2019) underline the importance of the seeding sludge usage and feeding parameters used. In Wang et al. (2019) the sludge derived from a continuously operating partial nitrification–anammox bioreactor with a sequencing feeding strategy, which probably enriched the AMX. AOB was the second most active group in this bioreactor. After the start up period the NOB group was almost undetectable (0.06%) which pointed at the successful partial nitrification–anammox obtainment (Wang et al., 2019).

Nitrospirae was reported to be the phylum becoming more abundant in a SBR system during the experiment in which the nitrogen removal bacteria community was treating synthetic mineral medium (Zhang et al., 2019). This study compared the SBR and biofilter. The seeding sludge for both systems was obtained from secondary settling tank of a full-scale wastewater treatment plant. The two other dominant phyla in both bioreactors were *Proteobacteria* and *Planctomycetes*. In case of SBR the proportions of *Planctomycetes*, *Proteobacteria*, and *Nitrospirae* were 31.08%, 23.72%, and 6.91%, respectively. In the case of the biofilter the proportion of the aforementioned phyla were 33.79%, 36.42%, and 0.39%, respectively, with decreasing Shannon biodiversity index from 6.75 for seeding sludge to 5.10 and 5.18 for SBR and biofilter, respectively. As mentioned previously, descaling of the technological system decreases the number of ecological niches available, thus the biodiversity of such a community usually decreases. AOB were present in seeding sludge also long after the acclimation time, thus it was stated that ammonia removal was performed by both, AOB and AMX. *Chloroflexi* were also present in all the samples supporting nitrogen removal systems by preventing organics accumulation with effective biodegradation of dead biomass (Zhang et al., 2019). Interestingly, the dominant anammox species were Candidatus *Anammoxoglobus* and Candidatus *Kuenenia*, while Candidatus *Brocadia* was present only in biofilter. *Denitamisoma*, a representative of denitrifiers, was also present in the biofilter community in a small proportion. Such community

composition in both bioreactors points at the importance of seeding sludge composition in shaping community structure and performance.

Also the communities in SBR systems in which additional substances to enhance the nitrogen removal processes were used presented the community with coexisting groups of AOB, NOB, denitrifiers, and anammox. Tomaszewski et al. (2019) presented the usage of graphene oxide and reduced graphene oxide to accelerate cold anammox process. Their research revealed that the most abundant phyla of bacteria present in all samples were *Planctomycetes, Nitrospirae,* and *Firmicutes,* in fluctuating proportions. Interestingly, *Planctomycetes* genera, the most often present in WWTPs systems, such as Candidatus *Brocadia*, *Jettenia*, or *Scalindua* were absent in the bioreactor.

Real nitrogen-rich wastewater is regarded as a challenge for efficient nitrogen removal mainly due to the organic matter concentration. It has been already stated that high organic content negatively influences the anammox community which occurs mainly because of the competition between heterotrophic denitrifiers and AMX (Gonzalez-Martinez et al., 2018). However, high organic load influence can be neutralized by high organic concentration in the effluent and the technology used. These conclusions were draw on the basis of AOB, NOB, denitrifiers, and AMX coexistence observations in the community treating synthetic wastewater with organic matter concertation of 100 mg COD/L with no visible influence on bioreactor performance (Gonzalez-Martinez et al., 2018). Moreover, in their earlier research García-Ruiz et al. (2018) reported the influence of the various concentrations of organic matter addition (as acetate) on the community structure of nitrogen removal bacteria. In a bioreactor with no organic carbon addition *Planctomycetes* was the predominant phylum (45.37%), supported with *Proteobacteria* (38.22%) and *Ignavibacteria* (6.45%). In bioreactors with organic matter addition *Proteobacteria*, *Chloroflexi*, and *Chlorobi* were the main identified phyla. Interestingly, *Ignavibacteria* was identified only in bioreactors without organics addition, while *Chlorobi* was only in organic matter-supported bioreactors. Nonetheless, the heterotrophic microorganisms coexisted with AOB and AMX in all the reactors studied (García-Ruiz et al., 2018).

For difficult real wastewater types various and mixed technologies are applied in order to obtain the effluent of high quality. In the case of piggery wastewater, Huang et al. (2018) used a combined system of UASB + SHARON + anammox (upflow anaerobic sludge bed + single reactor for high activity of ammonium removal over nitrite + anammox) for sufficient elimination of nitrogen compounds as well as high organic load present in this

sort of wastewater. Huang et al. (2018) started three separated bioreactors integrated after 229 days of the experiment. Depending on the technology used different compositions of the bacteria community were obtained. For the anammox bioreactor the dominant phyla were *Proteobacteria*, *Chloroflexi*, *Chlorobi*, *Planctomycetes*, and *Bacterioidetes* with relative abundance above 5% each. The SHARON bioreactor was dominated at 63.6%–77.3% by *Bacterioidetes* and *Proteobacteria*, but interestingly, when SHARON effluent was directed to the anammox bioreactor it had a minute effect on the anammox community present there. Dominant *Plactomycetes* genera (ca. 98.7% of *Planctomycetes* community) in this bioreactor were the representatives of Candidatus *Brocadia* and bacteria related to Candidatus *Kuenenia*. As expected, the biodiversity of the bacteria community decreased gradually after the start up process, but in the stage of stable effective performance (after 233 day of experiment) the biodiversity slightly declined and remained stable (Huang et al., 2018). Here, as in the case of any microbial system, the community aimed to establish a new equilibrium in which unsuitable microorganisms were mostly eliminated, with the community structure supporting effective biocenosis performance. In this case the increase of *Chloroflexi* after the acclimation period may indicate the enhancement of the nitrite loop supporting *Planctomycetes* in effective nitrogen removal. These microorganisms are together with *Acidobacteria* and *Bacterioidetes* the important heterotrophic players in nitrogen removal communities in anammox systems (Lawson et al., 2017). In this study also AOB and NOB were detected, but the AMX were the key factor for nitrogen removal performance here and they were heavily influenced by nitrogen load, despite the gradual acclimation to increasing nitrogen concertation with nitrite inhibitory effect being much higher than ammonia as previously stated (van Hulle et al., 2010).

Also the rotating biological contactor is regarded to be a good tool for real wastewater treatment, especially in the case of industrial wastewater, as a system based on biofilm. In research of Ziembińska-Buczyńska et al. (2019a) the nitrogen removal performance at community level was analyzed while changing the feeding medium from synthetic to real coke wastewater. This analysis revealed that the community was dominated with phylum *Proteobacteria*, while *Planctomycetes* was represented at a low proportion after real wastewater application. Despite these facts, the anammox process was performed relatively effectively. These results were coherent with those presented by Banach-Wiśniewska et al. (2020) for an SBR system with anammox

technology, where *Planctomycetes* number decreased after medium shift from synthetic into real wastewater. Also groups of *Flavobacteriia* and *Actinobacteria* were present in the biofilm community coexisting with nitrogen removal bacteria.

Interesting results in biodiversity of the nitrogen removal community was obtained by Chen et al. (2019). In this study, UASB was seeded with municipal wastewater plant and operated over 200 days with synthetic inorganic wastewater. According to the Shannon biodiversity index, Ace richness estimator and Heip evenness index values obtained, the total bacterial community and *Planctomycetes* biodiversity, richness, and evenness decreased during the experiment, while the same parameters of AOA and AOA increased. These results are supported with taxonomic changes in bacterial and archaeal community. At the phylum level *Proteobacteria* relative abundance increased from 26.4% to 90.7% together with relative abundance of AOB genera *Nitrosomonas*, *Nitrosococcus* and *Nitrosospira*. A similar situation was observed in the case of the AOA community with the increase of *Nitrosopumilis* and *Nitrososphaera*. Anammox community diversity decreased because of the dominant genera abundance change. Candidatus *Kuenenia*, Candidatus *Brocadia*, and Candidatus *Scalindua* were abundant at levels of 11.5%, 77.1%, and 10.6%, respectively, in seeding sludge, while after startup their relative proportion was 82% and 18% for the first two bacteria and Candidatus *Scalindua* disappeared from the system. NOB was represented by only one phylum *Nitrospirae* which were suppressed by aeration conditions from 2.2% to 0.1%. The environmental factors also caused the decrease of denitrifying bacteria number at genus level from 12.9% to 2.1% (Chen et al., 2019). These results support the thesis of the nitrogen removal bacteria coexisting in wastewater treatment systems with particular nitrogen removal bacteria group dominance according to physiochemical parameters of the technology used.

Membrane reactors (MBRs) are known to be excellent tools for enriching slow-growing microorganisms by preventing bacterial cells removal from the technological systems. In case of nitrogen removal bacteria, this technology supports mainly AMX. Ren et al. (2015) analyzed MBR bacterial community with Illumina sequencing. Their research revealed that the most abundant bacterial groups were: *Proteobacteria* and *Planctomycetes* accompanying with *Actinobacteria*, *Armatimonadetes*, *Bacterioidetes*, *Chlorobi*, *Chloroflexi*, and *Nitrospirae* representing all nitrogen removal microorganisms groups. At the genus level the research revealed that from the *Planctomycetes* group only two out of 102 OTUs (operational taxonomic units) were recognized as anammox

species, belonging to genera Candidiatus *Kuenenia* and Candidatus *Jettenia*. Most of the *Planctomycetes* present in the bioreactor were not identified as AMX. Also research of Tao et al. (2012) supported these results. In their study AMX together with AOB, NOB, and denitrifiers were present in seeding sludge derived from aerobic granular sludge. During bioreactor operation with mineral medium under conditions supporting AMX the other nitrogen removal bacteria were still present in the system after 242 days of the experiment, although in a relative low number.

Although efficient, MBRs are regarded to be expensive technological solutions. In the case of nitrogen removal systems, since anammox is found to be cost-efficient and eco-friendly, various technologies tending to shorten start up and prevent the biomass loss are proposed. One example is the upflow porous-plate anaerobic reactor (UPPAR) used by Zhang et al. (2019) to immobilize anammox biomass and accelerate this process startup for rare-earth mining nitrogen-rich wastewater treatment. The bacterial community established in UPPAR system consisted of *Planctomycetes*, *Proteobacteria* and *Chloroflexi* at 43.52%, 26.63%, and 5.87%, respectively. The most abundant *Planctomycetes* (87.9%) of the phylum were Candidatus *Brocadia* and Candidatus *Kuenenia*. AOB and NOB were also present, but in a relatively low proportion (<2.5%) together with *Ignavibacterium* and *Thermomonas* known to take part in denitrification. Interestingly, in case of these studies the authors underlined the presence of filamentous bacteria, which can create a net-like structure and together with representatives of *Proteobacteria* support anammox enrichment. A similar structure of community was obtained in the studies of Gonzalez-Martinez et al. (2015) with the presence of *Planctomycetes* and *Proteobacteria* at a level of 75.25% and 5.14%, respectively, in MBR. Such high values of *Planctomycetes* presence supports the assumption that MBR systems are highly efficient in anammox biomass retainment in the bioreactor. However, despite the technology used, which supports a particular bacteria group, the other nitrogen removal microorganisms are still present in the system, such as the AOB representative *Nitrosospira*, or heterotrophs able to undertake anaerobic nitrate reduction such as genus *Flexibacter*, as found by Gonzalez-Martinez et al. (2015).

3.6 Summary

As it is observed in nature, also in wastewater treatment bioreactors various microorganisms coexist and cooperate to

maintain ecological equilibrium. Wastewater treatment technologies are designed on the basis of microbial ecology and physiology research to create environments favoring a group of microorganisms performing a particular biochemical process in a highly efficient manner. In case of nitrogen removal bacteria this research is of utmost importance due to the fact that nitrogen is a factor responsible for water eutrophication and treated wastewater is required to meet strict regulations in this field.

However, despite the usage of the technological parameters supporting efficient performance of a specific bacteria group process, it is impossible to remove completely the other nitrogen removal bacteria from the bioreactor. The final composition of the nitrogen removal bacteria community relies not only on the physiochemical characteristics of the process, but also on the seeding sludge used in the treatment as well as the wastewater used. Molecular tools, such as high-throughput sequencing or qPCR enable to present the complexity and dynamics of nitrogen removal communities in various systems. This research helps to develop reliable ecophysiological models of the bioreactors communities and enable one to draw conclusions on the basics of which new, efficient technologies can be design and operated.

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