



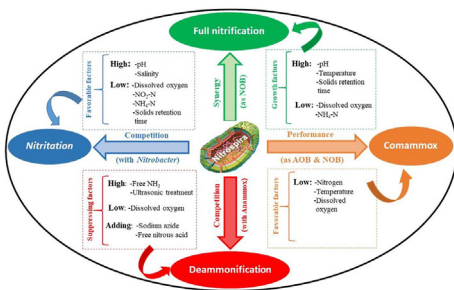
# The occurrence and role of *Nitrospira* in nitrogen removal systems

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## GRAPHICAL ABSTRACT



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## ABSTRACT

Application of the modern microbial techniques changed the paradigm about the microorganisms performing nitrification. Numerous investigations recognized representatives of the genus *Nitrospira* as a key and predominant nitrite-oxidizing bacteria in biological nutrient removal systems, especially under low dissolved oxygen and substrate conditions. The recent discovery of *Nitrospira* capable of performing complete ammonia oxidation (comammox) raised a fundamental question about the actual role of *Nitrospira* in both nitrification steps. This review summarizes the current knowledge about morphological, physiological and genetic characteristics of the canonical and comammox *Nitrospira*. Potential implications of comammox for the functional aspects of nitrogen removal have been highlighted. The complex meta-analysis of literature data was applied to identify specific individual variables and their combined interactions on the *Nitrospira* abundance. In addition to dissolved oxygen and influent nitrogen concentrations, temperature and pH may play an important role in enhancing or suppressing the *Nitrospira* activity.

## 1. Introduction

Nitrification is a central process of the nitrogen (N) cycle in wastewater treatment plants (WWTPs), involving two consecutive steps, i.e. ammonia oxidation ( $\text{NH}_4^+ \rightarrow \text{NO}_2^-$ ) (nitritation) followed by nitrite oxidation ( $\text{NO}_2^- \rightarrow \text{NO}_3^-$ ) (nitratation). The nature of the process was already investigated in the second half of the 19th century (Dworkin

and Gutnick, 2012).

Since nitrite accumulation was not normally observed in WWTPs, for a long time, nitrification research has primarily focused on ammonia-oxidizing bacteria (AOB). This also led to the discovery of new players of nitritation, such as ammonia-oxidizing archaea (AOA). In contrast, nitrite-oxidizing bacteria (NOB) were perceived as obligate chemo-lithoautotrophs with a physiological function strictly limited to

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nitratation (Daims et al., 2016).

The understanding of nitrification and nitrifying microorganisms has improved considerably over the last 20 years. Nitrite has been receiving growing attention as the pivotal component in a variety of novel nitrogen removal processes, including deammonification (known also as partial nitritation/anammox) or a shortened pathway of nitrification-denitrification via nitrite (“nitrite shunt”). As a consequence, the ecological importance of NOB has increased dramatically, but due to the limited knowledge on their biochemistry, NOB still remain a “big unknown of the nitrogen cycle” (Daims et al., 2016). Recent findings have suggested that uncultured members of the genus *Nitrospira*, rather than *Nitrobacter*, are the most diverse and abundant known NOB in municipal WWTPs (Gruber-Dorninger et al., 2014; Cao et al., 2017). Those bacteria (*Nitrospira*) were considered to be canonical NOB with the restricted metabolism (nitratation), however, recent findings identified a broader metabolic activity of *Nitrospira* (Koch et al., 2019).

The complete nitrification process by a single microorganism belonging to the genus *Nitrospira* has recently been discovered independently by two research groups (Daims et al., 2015; van Kessel et al., 2015). The process, known as comammox (complete oxidation of ammonia to nitrate), changes the current understanding of microbiologically mediated nitrogen removal processes involving nitrification (Pinto et al., 2016). The identification of the comammox bacteria overturned “a century-old dogma of nitrification research” (Koch et al., 2019). Metabolism of the members of *Nitrospira* genus is not limited to nitrite oxidation or comammox, but also comprise other functionalities beyond the N cycle, either under aerobic conditions (growth on formate and hydrogen) and anoxic conditions (reduction of nitrate to nitrite) (Koch et al., 2019).

The growing interest in the role of *Nitrospira* in WWTPs is reflected by the number of scientific papers published annually and focused specifically on that microorganism. In the Scopus database, the number of papers with the keywords “*Nitrospira*” and “wastewater” has continuously been increasing over the last decade from 6 (2010) to 89 (2019). Despite those numerous studies, there are still a lot of open questions concerning the importance and actual role of *Nitrospira* in nitrogen removal systems, effective methods of suppression, especially in deammonification systems, comparison of the kinetic parameters of these bacteria and *Nitrobacter*, coexistence of canonical and comammox *Nitrospira*, etc. These issues are addressed in this study by reviewing the physiological and microbial characteristics of *Nitrospira*, their abundance in WWTPs, and factors influencing their growth. The review is supported by meta-analysis of over 100 case studies of different wastewater treatment systems to investigate the *Nitrospira* abundance in terms of the combined effect and interaction of four process variables, such as dissolved oxygen (DO) concentration, influent  $\text{NH}_4\text{-N}$  concentration, pH, and temperature. Moreover, detection methods of the microbial diversity and abundance of *Nitrospira* are also summarized.

## 2. Physiological and morphological characteristics of *Nitrospira*

Currently, there are seven known NOB genera affiliated with four bacterial phyla, including *Proteobacteria* (*Nitrobacter*, *Nitrotoga*,

*Nitrococcus*), *Nitrospinae* (*Nitrospina*, ‘*Candidatus Nitromaritima*’), *Chloroflexi* (*Nitrolancea*) and *Nitrospirae* (*Nitrospira*) (Feng et al., 2017). *Nitrospira* are generally aerobic chemolithoautotrophic bacteria showing extraordinary diversity and plasticity. Members of the genus *Nitrospira* have been found in freshwater, soils, groundwater, geothermal springs and WWTPs. Moreover, *Nitrospira* colonize marine sponges, rhizospheres and leaf surface of plants (Daims and Wagner, 2018). Until now, *Nitrospira* have been divided into six phylogenetic lineages, which show different habitat preferences. In WWTPs, lineages I, II and IV have been detected (Lopez-Vazquez et al., 2014; Nowka et al., 2015), but most of *Nitrospira* were affiliated to the main lineages I or II, which could coexist together and dominate in both full-scale WWTPs and laboratory systems (Gruber-Dorninger et al., 2014). It is suggested that lineage II *Nitrospira* have higher affinity for nitrite and lower affinity for DO in comparison with these organisms in lineage I (Gruber-Dorninger et al., 2014; Park et al., 2017).

*Nitrospira*, like other NOB, is difficult to cultivate and thus growing sufficient amount of biomass for follow-up physiological studies remains challenging (Daims et al., 2016). Since most of *Nitrospira* genus members are uncultivated, and the obtained cultures are difficult to sustain, physiology of these bacteria is still not fully known. *Nitrospira* shows similar morphological properties to other NOB groups, i.e. the cell walls typical for gram-negative bacteria, and a helical to fibroid morphology ( $0.9\text{--}2.2 \times 0.2\text{--}0.4 \mu\text{m}$  in size) or the average characteristic diameter of  $1.3 \pm 0.6 \mu\text{m}$  (Park et al., 2017). Most of *Nitrospira* species prefer to form biofilm structures and grow densely in microcolonies (Cao et al., 2017). In activated sludge, the reported *Nitrospira* enrichment cultures comprised either large cell aggregates in the range approximately  $40\text{--}600 \mu\text{m}$  (Manser et al., 2005; Blackburne et al., 2007), smaller microcolonies ( $1\text{--}12 \mu\text{m}$ ) (Koch et al., 2019) or even planktonic cells with small ( $3\text{--}4 \mu\text{m}$ ) aggregates (Park et al., 2017). It should be noted that smaller floc sizes ( $< 40 \mu\text{m}$ ) would significantly reduce any oxygen mass transfer limitation regardless of the bulk liquid DO concentrations (Blackburne et al., 2007).

Due to the difficulties in cultivation, information about the growth parameters, inhibitory compounds, and influence of environmental conditions on the *Nitrospira* activity are limited. Table 1 summarizes results of the studies on kinetic characterization of *Nitrospira* in terms of such parameters as the maximum specific growth rate ( $\mu_{\text{max}}$ ) and half-saturation (affinity) coefficients for DO ( $K_{\text{O}}$ ) and nitrite ( $K_{\text{S}}$ ). The only complete set of those parameters was reported by Park et al. (2017) in a study on the kinetic characterization of enriched *Nitrospira* from activated sludge.

## 3. Competition of *Nitrospira* with *Nitrobacter*

Two common NOB in WWTPs comprise *Nitrospira* and *Nitrobacter* and prediction of their dominance has commonly been based on the hypothesis that *Nitrospira* is a K-strategist with a high affinity with respect to nitrite and DO concentrations, while *Nitrobacter* is an r-strategist that prevails at higher concentrations of DO and nitrite (Blackburne et al., 2007; Huang et al., 2010; Persson et al., 2014; Wang and Gao, 2016; Cao et al., 2017; Kouba et al., 2017). The K-based selection is

**Table 1**  
Review of most important kinetic parameters of *Nitrospira*.

$\mu_{\text{max}}$ $\text{d}^{-1}$	$K_{\text{S}}$ $\text{mg N/L}$	$K_{\text{O}}$ $\text{mg O}_2/\text{L}$	Remarks	Reference
$0.69 \pm 0.10$	$0.52 \pm 0.14$	$0.33 \pm 0.04$	22 °C, Enriched culture	Park et al., 2017
NA	0.9–1.1	0.54	15–30 °C, Enriched culture	Blackburne et al., 2007
NA	0.11–0.50	0.47	20 °C, Mixed culture	Manser et al., 2005
0.45–0.52	0.13–0.39	NA	28–37 °C, Pure culture	Nowka et al., 2015
NA	0.16	NA	30 °C, Enriched culture	Schramm et al., 1999
0.18	NA	NA	20 °C, Pure culture	Watson et al., 1986

( $\mu_{\text{max}}$  – maximum specific growth rate,  $K_{\text{S}}$  – nitrite half-saturation (affinity) coefficient,  $K_{\text{O}}$  – dissolved oxygen half-saturation (affinity) coefficient).

associated with delayed reproduction, large cell size, and/or stable environments, while the r-selection regime is adopted with an early reproduction, small cell size, and/or variable environments (Andrews and Harris, 1986). Several studies have revealed that the competition between *Nitrospira* and *Nitrobacter* is primarily influenced by nitrite concentrations in the studied system. The growth of *Nitrospira* has been favored under low nitrite conditions, while *Nitrobacter* has been found the dominant NOB at higher nitrite concentrations (> 80 mg N/L) (Nogueira and Melo, 2006; Blackburne et al., 2007; Huang et al., 2010; Park et al., 2017; Wang et al., 2017). On the contrary, results of another study (Blackburne et al., 2007) showed that *Nitrospira* was the dominant NOB when nitrite concentrations were relatively high (> 100 mg N/L).

Another critical factor influencing the *Nitrospira* abundance in WWTPs is the operational DO concentration (Ushiki et al., 2017; Chang et al., 2019). Indeed, Park et al. (2017) attributed a high enrichment of *Nitrospira* in a DO- and nitrite-limited SBR to higher affinity for both DO ( $K_O = 0.5\text{--}0.6$  mg O<sub>2</sub>/L vs. 0.2–4.3 mg O<sub>2</sub>/L) and nitrite ( $K_S = 0.1\text{--}1.1$  mg N/L vs. 0.3–7.6 mg N/L) in comparison with *Nitrobacter*. The same authors postulated that there is another feature of *Nitrospira* that is advantageous for competition with *Nitrobacter* and other NOB under nitrite-limited conditions. *Nitrobacter* encodes for a cytoplasmic nitrite oxidoreductase (*nrx*), which requires the transport of nitrite and nitrate across the inner membrane in the reverse directions. In contrast, *Nitrospira* typically encodes for periplasmic *nrx*, which catalyses the second step of nitrification. The latter type of oxidation (periplasmic) is beneficial as a higher specific proton motive force is generated and the transmembrane exchange of nitrite and nitrate does not occur.

Table 2 shows a comparison of these two bacteria in terms of environmental factors which are important for the operation of biological wastewater systems. The maximum activity of *Nitrospira* is much lower than *Nitrobacter* measured as the rates of nitrite oxidation (Kim and Kim, 2006) and oxygen uptake (Blackburne et al., 2007). In addition to low nitrite and DO concentrations, *Nitrospira* may be better adapted to slightly higher pH (8–8.3 vs. 7.6–8.2) and temperatures (29–30 °C vs. 24–25 °C). However, the inhibition thresholds of free ammonia (FA) (0.04–0.08 mg NH<sub>3</sub>-N/L) and free nitrous acid (FNA) (0.03 mg HNO<sub>2</sub>-N/L) are significantly lower in comparison with *Nitrobacter* (10 mg NH<sub>3</sub>-N/L and 0.2–0.4 mg HNO<sub>2</sub>-N/L). This may provide another explanation why *Nitrospira* has been found a dominant NOB in low concentrations of ammonium and nitrite (Blackburne et al., 2007).

#### 4. Comammox *Nitrospira*

The comammox process, shown in Fig. 1, is mediated by some members of the genus *Nitrospira*, including “*Candidatus N. nitrosa*”, “*Candidatus N. nitrificans*”, “*Candidatus N. inopinata*”, and strain Ga0074138 (Daims et al., 2015; van Kessel et al., 2015; Pinto et al., 2016; Camejo et al., 2017). While the canonical NOB possesses the gene *nrx* involved only in nitrite oxidation, the comammox *Nitrospira*

possesses genes involved also in ammonia oxidation, i.e. ammonia monooxygenase (*amo*) and hydroxyloamine dehydrogenase (*hao*) (Santoro, 2016; Camejo et al., 2017; Hu and He, 2017; Annavajhala et al., 2018).

Costa et al. (2006) hypothesized the existence of a single microorganism capable of performing the two nitrification steps. The authors assumed that such a microorganism is slower-growing, but with a higher yield coefficient, in comparison with incomplete ammonia oxidizers (canonical AOB). Theoretical calculations revealed that the favorable conditions for the growth of complete oxidizers are provided in clonal clusters, such as biofilms, with low mixing conditions and low substrate diffusion gradients. In contrast, faster-growing canonical AOB could dominate in chemostats and other well-mixed systems.

Shortly after the discovery of comammox bacteria, Chao et al. (2016) reported the presence of those bacteria in a biofilm grown in aerobic reactors in WWTPs. Earlier studies on biomass distribution in fully nitrifying biofilm systems (Okabe et al., 1999; Schramm et al., 1999) revealed the highest NOB abundance in deeper zones of biofilms under DO limited conditions. Furthermore, Okabe et al. (1999) showed that *Nitrospira* was the dominant NOB, whereas *Nitrobacter* and other faster growing NOB species were hardly detected. This finding implicitly suggests that *Nitrospira* can adapt better to the limited DO availability. It may also explain the presence of “comammox” *Nitrospira* in the environments exposed to the DO concentration gradients, such as deeper zones of biofilms (Chao et al., 2016).

## 5. Methods of *Nitrospira* detection

### 5.1. Classical cultivation-based and biochemical techniques

In order to detect *Nitrospira* related bacteria in complex biomass matrix, such as activated sludge, a set of appropriately sensitive research tools should be applied. In the past, research on biomass samples from WWTPs were carried out using the classical microbiological methods, i.e. cultivation and light microscopy. These methods have allowed to identify many important groups of microorganisms in wastewater treatment processes. For example, the cultivation techniques developed in the early study by Winogradsky enabled to detect bacteria catalyzing both steps of nitrification (Nielsen and McMahon, 2014). Initially, biochemical and physiological studies were focused mainly on *Nitrobacter*, whereas other NOB were studied occasionally (Daims and Wagner, 2011). The progress in detection of the others nitrifying bacteria was linked with the development of a new generation of the biochemical techniques.

The first advanced method used for identification and differentiation of NOB was the whole cell fatty acid methyl esters (FAME) analysis. Lipski et al. (2001) showed that fatty acid profiles of four genera of NOB (*Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira*) were unique. Furthermore, it was proved that these profiles could also be used for single species (Gilbride, 2014) as well the whole microbial communities characterization (Huang et al., 2019).

**Table 2**

Comparison of the prevailing conditions for the competition of *Nitrospira* and *Nitrobacter* in biological wastewater treatment systems.

Factor	Unit	Prevailing range		References
		<i>Nitrospira</i>	<i>Nitrobacter</i>	
Maximum activity (rate):				
Nitrite oxidation	mg N/g NOB h	10.5	93.8	Kim and Kim, 2006
Oxygen uptake	mg O <sub>2</sub> /g VSS h	32	289	Blackburne et al., 2007
pH	–	8–8.3	7.6–8.2	Grunditz and Dalhammar, 2001, Blackburne et al., 2007, Rodrigues et al., 2017
DO concentration	mg O <sub>2</sub> /L	< 1.0	1.0	Huang et al., 2010, Liu and Wang, 2013
Temperature	°C	29–30	24–25	Huang et al., 2010, Courtens et al., 2016a
Inhibition threshold:				
Free ammonia	mg NH <sub>3</sub> -N/L	0.04–0.08	10	Blackburne et al., 2007
Free nitrous acid	mg HNO <sub>2</sub> -N/L	0.03	0.2–0.4	

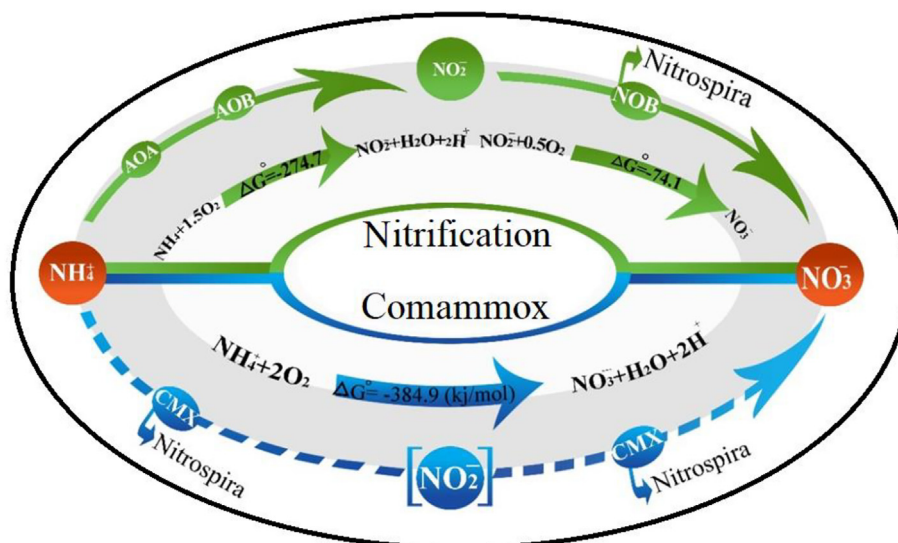


Fig. 1. The role of *Nitrospira* in the two-step nitrification and comammox processes.

The alternative way to identify NOB is based on an immunological approach. In this method, protein extract from enriched cultures is separated in is separated by electrophoresis in gel made of sodium dodecyl sulphate–polyacrylamide (SDS-PAGE) and then blotted onto a cellulose membrane and immune-stained using a protein specific antibody. Bartosch et al. (2002) used Mab 153–3 antibody to determine 13-subunit of the nitrite oxidizing system ( $\beta$ -NOS) of the known NOB. Due to the different mass of this protein, the immunological approach could be useful for differentiation of all major NOB (Bartosch et al., 2002).

The morphological and biochemical characteristics allow to detect individual, well characterized strains. However, in the case of comprehensive analysis of bacterial consortia composed of many different forms of microorganisms, the classical techniques are not practical due their prevailed low resolution and labor-intensity (Nemati et al., 2016). For example, application of the techniques based on the light microscopy is often limited for detection of *Nitrospira* due to the lack of specific phenotypic characters in the structure of their cells. On the contrary, the cultivation-based techniques focus on specific microorganisms, for which the knowledge about environmental parameters required for their growth is available (Salmonová and Bunešová, 2017).

## 5.2. Cultivation-independent techniques

A significant progress in the characterization of microbial communities, including the nitrifying bacteria, took place in the early 1990's along with the development of molecular techniques and their adaptation to the microbial ecology studies. The molecular techniques used in the phylogenetic studies of microorganisms are based on the nucleic acid sequences polymorphism analysis. Especially, polymerase chain reaction (PCR)-based methods, by application of the defined reaction primers, enables selective amplification of the targeted DNA sequences thus provide a fast and sensitive alternative to the biochemical and physiological methods (Gómez-Silván et al., 2014).

In the PCR-based methods, more often the genes incorporated in the operon responsible for the synthesis of ribosomal ribonucleic acids (rRNA) are applied as a molecular marker, due to their ubiquitous presence in the genomes of all types organisms and evolutionary properties, i.e. presence of the regions characterized by both significant degrees of conservation and high variation in the nucleotide composition. Other method that allows for bacteria species detection on site without the need of prior isolation is Fluorescence in Situ Hybridization (FISH). This semi-quantitative technique is used for specific detection of particular bacteria by hybridization of fluorescently labelled probes to

complementary target rRNA sequences within intact cells. After hybridization, samples are analysed by the fluorescence microscopy (Wang et al., 2008). This technique, combined with flow cytometry, allows to enumerate the labelled cells (Lenaerts et al., 2007).

Currently, extensive databases containing 16S rRNA gene sequences from almost all microorganisms known so far are widely available (e.g. SILVA database <https://www.arb-silva.de>, Genomic-based 16S ribosomal RNA Database – GRD <https://metasystems.riken.jp/grd>, Ribosomal Database Project – RDP <https://rdp.cme.msu.edu>) to conduct a comparative analysis to determine the phylogenetic position of isolates derived from the tested environmental samples (Tsukuda et al., 2017). Although the most commonly used gene for examination of microbial populations is the 16S rRNA, its use in the microbial ecology has some drawbacks. The main disadvantage is that it may not be related to the physiology of the target organisms (Kowalchuk and Stephen, 2001). Moreover, since comammox bacteria do not form a unique clade within *Nitrospira* lineage II, comammox and canonical *Nitrospira* NOB cannot be individually detected by 16S rRNA-based methods (Pjevac et al., 2017). Therefore, a preferred approach is based on the genes encoding key enzymes for a specific metabolic pathway (Wang et al., 2018).

The most widely applied molecular marker for *Nitrospira* detection is *nrx* gene which encodes nitrite oxidoreductase (NXR), which a key enzyme of nitritation. The possibility of using *nrx* gene as a functional marker for *Nitrospira* detection has first been described by Pester et al. (2014). This membrane associated enzyme are found in two recognizable forms. One is a cytoplasmic form found in *Nitrobacter*, *Nitrococcus* and *Nitrolanceus*, whereas the second is a periplasmic form found in *Nitrospira* and *Nitrospina*. The gene coding for NXR consists of alpha (*nrxA*), beta (*nrxB*) and gamma (*nrxC*) subunits. The *Nitrospira* NOB was successfully detected using PCR primers specific for *nrxB* gene (Pester et al., 2014).

The *nrx* genes sequences, derived from the canonical *Nitrospira* NOB, show a significant similarity to comammox *Nitrospira* (Daims et al., 2016). In addition, a comparative genomic analysis revealed low numbers of comammox-specific genes which are suitable for detection of comammox *Nitrospira* (Palomo et al., 2018). In order to perform a selective detection of these bacteria, the authors suggested to apply procedures based on the DNA sequences analysis of *amo* and *hao* genes. These genes encode key enzymes of ammonium oxidation step in the comammox pathway. DNA sequences variants of the *amo* and *hao* genes obtained from the currently known comammox *Nitrospira*, are different from the homologs of the other groups. This reflects a high application

potential as a reliable molecular marker (Daims et al., 2015; van Kessel et al., 2015; Daims et al., 2016).

All the known comammox bacteria belong to sublineage II of the genus *Nitrospira* (Lawson and Lückner, 2018). *Amo* orthologs, which are encoded by comammox *Nitrospira*, are dissimilar to both each other and the other betaproteobacterial *amo* (Daims et al., 2015). This suggests that there are two distinct clades (clade A and B) of comammox *Nitrospira* and the pitfall of their detection with PCR results from the uniqueness of comammox-*Nitrospira* gene coding *amo*. Pjevac et al. (2017) and Koch et al. (2019) proposed a pair of PCR primers that would be the best available tool for fast identification of comammox *Nitrospira*.

The recently developed Next Generation Sequencing (NGS) technologies allow for complex analysis of particular bacterial genomes (metagenomics) or complex examination of microbial community genomes (metatranscriptomics) without need of single strains isolation at high resolution level not available for the classical PCR-based methods (He et al., 2018). This approach was used by Annavaiah et al. (2018) to quantify the presence and elucidate the potential functionality of comammox bacteria in 16 full-scale mainstream and sidestream BNR reactors. The sequences specified for those bacteria constituted between 0.28 and 0.64% of the total coding DNA sequences in all the analyzed cases.

NSG and PCR based surveys provide crucial information about abundances and diversity of the key bacterial groups, but do not cover functionality aspects of the complex microbial communities. Therefore, the additional metatranscriptome i.e. profile of the overall gene expression of microorganisms in particular environments, should be implemented. Crovadore et al. (2018) analyzed metagenomes and metatranscriptomes of activated sludge bioreactors, with and without enrichment with aerobic granules. The analysis revealed that the bioaugmentation increased the expression level of genes involved in ammonia removal. Using a similar approach Yu et al. (2018) provided evidence for comammox in an enriched culture of tidal sediments.

Combination of the metagenomes and metatranscriptomes is currently most powerful approach for complex functional analysis of the microbial communities. The advantage of this approach is possible use of it for the measurement of in situ activity of comammox-*Nitrospira* that is extremely important for the understanding the role of this bacteria in wastewater treatment processes.

## 6. Occurrence of *Nitrospira* in nitrogen removal systems

The reported occurrences of *Nitrospira* in the most common nitrogen removal processes in WWTPs, including nitrification-denitrification (N-DN) and deammonification (PN-A), were summarized. *Nitrospira* abundances along with the most important operational parameters, such as pH, temperature, DO, solids retention time (SRT), nitrogen concentrations and removal efficiency/rates, were listed for approximately 100 technological studies (80 for N-DN and 35 PN-A systems). These data have been classified in terms of the scale of the studied system, feed characteristics and reactor types. Fig. 2 shows that the lab-scale studies constituted the majority (approximately 90%) of the analyzed N-DN and PN-A systems. Most of the studied systems were fed with synthetic wastewater (48% – N-DN and 59% – PN-A), while the N-DN systems were also operated with real municipal, domestic and industrial wastewater. Due to the nature of the PN-A process (treatment of high-loaded ammonia streams), the PN-A reactors were primarily operated with reject water from sludge dewatering processes (11%) or synthetic wastewater simulating the composition of reject water (59%). In both cases, the most popular reactor types were SBR/SBBR (42% – N-DN and 44% – PN-A).

In general, the studied systems were laboratory-scale SBRs fed with synthetic wastewater with respect to both N-DN and PN-A. Case studies with the highest observed relative abundances of *Nitrospira* in N-DN and PN-A systems are presented in Table 3. The highest abundance of

*Nitrospira* (53%) was reported in a nitrifying SBR operated for more than one year at low DO concentrations (Roots et al., 2018).

In fully nitrifying systems, the theoretical ratio of NOB/AOB abundances corresponds to the ratio of their yield coefficients ( $Y_{NOB}/Y_{AOB}$ ). When assuming the typical values of  $Y_{NOB}$  (=0.09) and  $Y_{AOB}$  (=0.15), the obtained ratio NOB/AOB = 0.6 suggests that AOB should dominate over NOB. In practice, the AOB and NOB abundances in nitrifying communities can shift and change depending on the local conditions (Cao et al., 2017). Significantly higher ratios of NOB/AOB have indeed been reported in both full-scale municipal WWTPs (0.8–1.5) (Harms et al., 2003; Ramdhani et al., 2013) and a lab-scale aerobic granular reactor (3–4) (Winkler et al., 2012). These deviations from the theoretical ratio NOB/AOB could be explained by the comammox process (Wang and Li, 2015; Daims et al., 2016).

In deammonification systems, the presence of *Nitrospira* has been observed in numerous studies (e.g. Malovany et al., 2015; Persson et al., 2014; Varas et al., 2015; Wang and Gao, 2016; Soliman and Eldyasti, 2016; Poot et al., 2016; Zhang et al., 2016). The presence of comammox *Nitrospira* seems to be undesirable in all anammox-based systems due to disturbance of nitrite production. However, the actual role of comammox *Nitrospira* in deammonification still needs to be evaluated (Cao et al., 2017), even though their coexistence with anammox bacteria has been reported (Van Kessel et al., 2015).

The out-selection of NOB is a critical factor for the efficient and stable deammonification (Zhang et al., 2016). The literature data (Varas et al., 2015; Poot et al., 2016; Soliman and Eldyasti, 2016; Wang and Gao, 2016) explicitly indicate that it is possible to suppress NOB activity, but without removing completely those bacteria from the system. For example, Wang and Gao (2016) observed this in a granular deammonification reactor, which had been deteriorated due to high abundances of *Nitrospira* and *Nitrobacter*. In the course of the experiment, the NOB activity was successfully suppressed by keeping low DO concentrations (< 0.13 mg/L) and high FA levels (5–40 mg N/L). After 2 months of the reactor operation, the ratio of produced nitrate/consumed ammonia decreased from 37% to 7%. However, the investigation of 16S rRNA gene copy numbers revealed that NOB were still highly abundant in the studied system. Only the copy numbers for *Nitrospira* increased approximately 50 times (from  $2.63 \times 10^6$  to  $1.06 \times 10^8$  copies/mg), while the copy numbers of *Nitrobacter* decreased approximately 5 times (from  $4.52 \times 10^7$  to  $2.17 \times 10^6$  copies/mg).

## 7. Factors influencing the *Nitrospira* activity and abundance in nitrification-denitrification and deammonification systems

A list of factors affecting the *Nitrospira* abundance (e.g. DO, temperature, pH, TNL, FA, SRT/HRT, time of reactor operation or salinity) in nitrification-denitrification and anammox-based systems, and their effect on the *Nitrospira* abundance are summarized Table 4. The effects of those factors on the *Nitrospira* activity and abundance are discussed in the following sub-sections.

### 7.1. Dissolved oxygen

Abundant amounts of *Nitrospira* have been maintained or even increased in low-DO nitrifying reactors (with DO concentration below 1.0 mg O<sub>2</sub>/L) as reported by Huang et al. (2010), Liu and Wang (2013), Wang and Gao (2016), Zhou et al. (2018) and Roots et al. (2018). At the extreme case, *Nitrospira* reached 53% of the overall microbial relative during the operation at low DO concentration (0.2–1 mg O<sub>2</sub>/L) in the nitrifying SBR (Roots et al., 2018). During the long term operation of the lab-scale SBR (DO = 0.5–1.0 mg O<sub>2</sub>/L), Park et al. (2017) observed increased of the *Nitrospira* concentration from  $7.0 \times 10^7 \pm 1.2 \times 10^6$  gen copies/mL to  $7.7 \times 10^8 \pm 7.5 \times 10^7$  gen copies/mL. Zhou et al. (2018) gradually decreased DO concentration in a SBR from 3 to 0.5 mg O<sub>2</sub>/L, which resulted in an increase in the *Nitrospira* abundance from

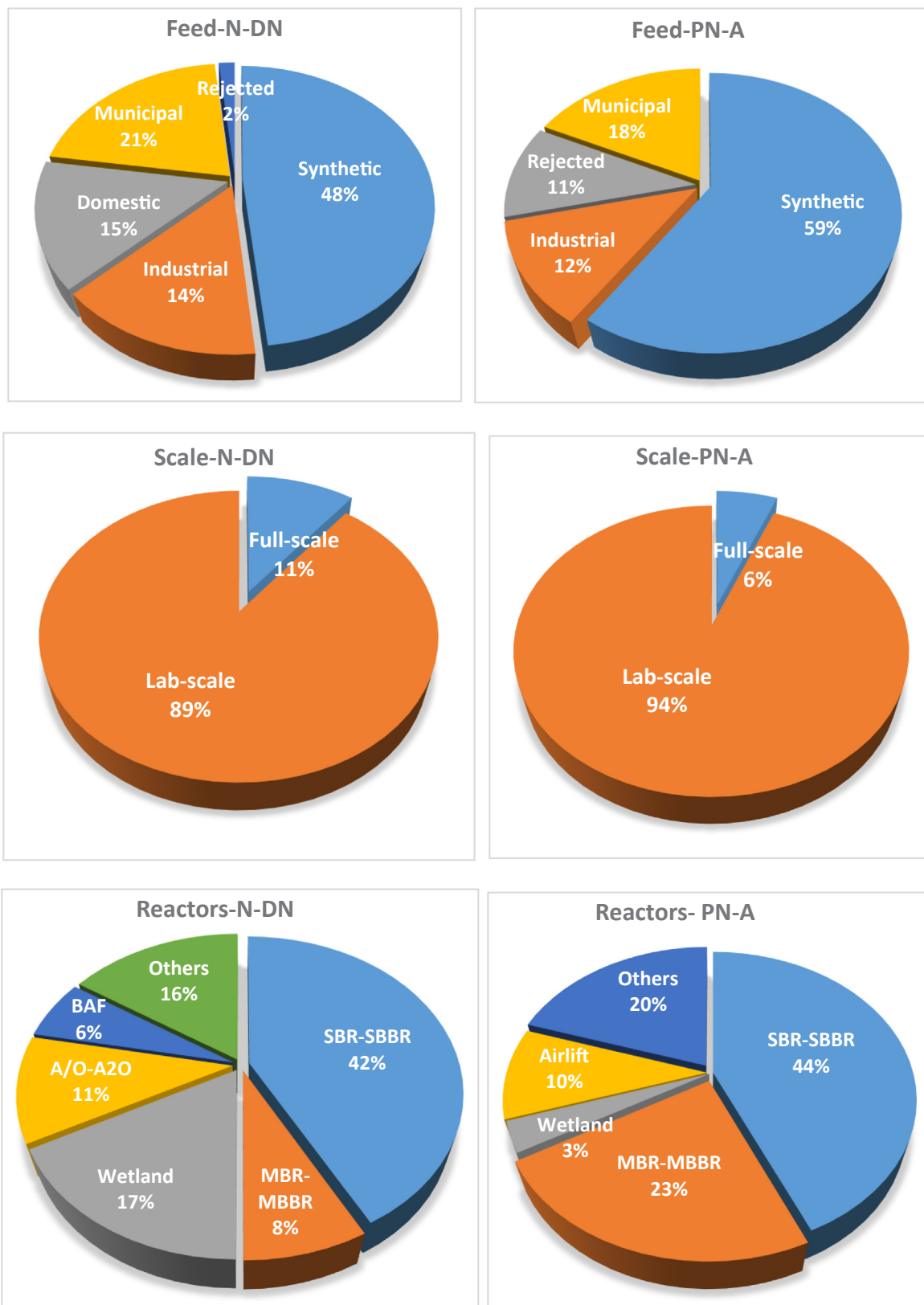


Fig. 2. Classifications of approximately 80N-DN systems and 35 PN-A systems in which *Nitrospira* were detected.

$2.07 \times 10^9$  to  $9.19 \times 10^{10}$  copies/g MLSS during 114 days of operation. Moreover, Bao et al. (2016), Fitzgerald et al. (2015), and How et al. (2018) showed that efficient nitrification was possible also at even lower DO concentrations (0.3–0.5 mg O<sub>2</sub>/L). Fitzgerald et al. (2015) divided *Nitrospira* into two groups: low-DO *Nitrospira* (represented by

*Nitrospira moscoviensis*) and high-DO *Nitrospira* (represented by *Candidatus Nitrospira defluvii*). Experimental results confirmed an increase of the relative abundance of low-DO *Nitrospira* in a reactor with a very low DO concentration (0.13 mg O<sub>2</sub>/L) and a significant decrease in the reactor with a high DO concentration (8.7 mg O<sub>2</sub>/L). On the contrary, the

**Table 3**  
Maximum relative abundances of *Nitrospira* reported for N-DN and PN-A systems.

System	Maximum relative abundance, %	Main operational conditions	References
N-DN	53	See Table 4 for details	Roots et al., 2018
N-DN	22	DO: 0.8–2.2 mg O <sub>2</sub> /L, T: 30 °C, pH: 7.0–8.0, FA: 22 mg/L	Yang et al., 2018
N-DN	20	See Table 4 for details	Bhatia et al., 2017
N-DN	16	SRT: 18 d, NH <sub>4</sub> -N: 30–40 mg/L,	Liu et al., 2018
N-DN	12	See Table 4 for details	Jia et al., 2017
N-DN	10	See Table 4 for details	Quartaroli et al., 2017
N-DN	9	See Table 4 for details	Song et al., 2017
N-DN	5	DO: 0.8 mg O <sub>2</sub> /L, T: 22.54 °C, pH: 6.0–8.0, NH <sub>4</sub> -N: 17 mg/L	Ouyang et al., 2017
N-DN	4.6	See Table 4 for details	Gao et al., 2017
N-DN	3.3	T: 25 °C, pH: 10, NH <sub>4</sub> -N: 63 mg/L	Yuan et al., 2016
N-DN	3	See Table 4 for details	Dong et al., 2017
N-DN	2	See Table 4 for details	Tian et al., 2017
N-DN	2	See Table 4 for details	Luo et al., 2017
N-DN	2	DO: 2.5 mg O <sub>2</sub> /L, T: 20 °C, SRT: 25 d, pH: 7.0–8.0, NH <sub>4</sub> -N: 20 mg/L	Ma et al., 2017
PN-A	27.9	DO: 0.2 – 8.0 mg O <sub>2</sub> /L, T: 16 °C, pH: 6.3–8.0, NH <sub>4</sub> -N: 50 mg/L	Pedrouso et al., 2017
PN-A	10.5	See Table 4 for details	Wang et al., 2017
PN-A	7.5	DO: 1 mg O <sub>2</sub> /L, T: 22 °C, pH: 7.2, NH <sub>4</sub> -N: 45–68 mg/L,	Du et al., 2019
PN-A	5.35	See Table 4 for details	Mardanov et al., 2016
PN-A	4	See Table 4 for details	Liu et al., 2017

(N-DN – nitrification-denitrification, PN-A – partial nitrification/anammox).

relative abundance of high-DO *Nitrospira* increased in a reactor with a high DO concentration (8.5 mg O<sub>2</sub>/L) and decreased in a reactor with a low DO concentration (0.12–0.24 mg O<sub>2</sub>/L). The influence of DO concentration on the *Nitrospira* abundance in the partial nitrification SBR was investigated by Bao et al. (2016). A stable and complete nitrification was achieved at the DO concentration of  $0.3 \pm 0.14$  mgO<sub>2</sub>/L. The *Nitrospira*-like bacteria were the dominant NOB and their abundance increased from  $1.03 \times 10^6$  to  $2.64 \times 10^6$  cells/mL. When a higher DO concentration ( $1.8 \pm 0.32$  mgO<sub>2</sub>/L) was applied, the *Nitrospira* abundance gradually decreased from  $2.64 \times 10^6$  to  $8.85 \times 10^5$  cells/mL. This explicitly suggests that high DO conditions may lead to continuous suppression of the *Nitrospira* activity.

Low DO concentrations are also the most preferred strategy in the wastewater treatment systems with partial nitrification process, as a one of the factors that selectively suppress NOB growth (Wang et al., 2017; Peng and Zhu, 2006; Ma et al., 2009, 2011). The lowest reported DO concentration ( $0.17 \pm 0.08$  mg O<sub>2</sub>/L) was used in a PN-A SBR by Miao (2016). In that DO-limited system, *Nitrospira* was detected as a dominant NOB. The authors reported an increase in the *Nitrospira* gene copy number from  $2.61 \times 10^8$  to  $1.67 \times 10^{10}$  copies/g MLSS. A slightly higher DO concentration (0.3 mg O<sub>2</sub>/L) was used in the CANON process by Wang et al. (2017) and *Nitrospira* was detected as a dominant NOB in that system. Cao et al. (2018) observed that reduction of DO from 1.7 to 1.0 mg O<sub>2</sub>/L in the aeration phases caused a shift of the dominant NOB from *Nitrobacter* to *Nitrospira*. Even more case studies for *Nitrospira* dominance (due to low DO) were reported for biofilm systems (Kindaichi et al., 2007; De Clippeleir et al., 2011; De Clippeleir et al., 2013; Gilbert et al., 2015) in comparison with the *Nitrobacter*-dominant cases (Isanta et al., 2015).

Opposite observations were made by Mardanov et al. (2016) and Qian et al. (2017). Mardanov et al. (2016) noted a decreased abundance of *Nitrospira* (from 5.35 to 3.34%) in the PN-A SBR operated with DO = 0.5 mg O<sub>2</sub>/L. In the PN-A continuous reactor operated at DO in the range 0.8–1.5 mg O<sub>2</sub>/L, the *Nitrospira* abundance was effectively inhibited (from 0.44 to 0.04%) with the increase of nitrogen removal efficiency (Qian et al., 2017).

In the partial nitrification systems, not only DO concentration but also aeration mode (continuous or intermittent) has a significant importance for the growth of NOB. The concept of intermittent aeration has recently been applied to effectively suppress nitrite oxidation primarily in lab-scale systems (Sun et al., 2018; Zhou et al., 2018; Roots

et al., 2018; Bao et al., 2016; Ma et al., 2017; Park et al., 2017; Regmi et al., 2014; Zubrowska-Sudol et al., 2011; Li et al., 2011), but also in full-scale systems (Miao et al., 2018; Joss et al., 2009). Park (2008) found that DO substantially influenced a shift within *Nitrospira* between lineage I and II. Bao et al. (2016) found that a sudden switch to high DO conditions from a low DO level caused inhibition and gradually decreased the *Nitrospira* abundance from  $2.64 \times 10^6$  to  $8.85 \times 10^5$  cells/mL. The authors concluded that *Candidatus Nitrospira defluvii*-like bacteria favor limited DO conditions and cannot adapt to rapid transition to the high DO concentration. Sun et al. (2018) carried out four intermittent aerated reactors, two SBR operated under high DO = 2 mg O<sub>2</sub>/L (SBR-H) and low DO = 1 mg O<sub>2</sub>/L (SBR-L) and two continuous-flow multiple reactors (CMR) operated at the same conditions, i.e. DO = 2 mg O<sub>2</sub>/L (CMR-H), and DO = 1 mg O<sub>2</sub>/L (CMR-L). The authors observed (1) a higher abundance of *Nitrospira* in SBR-H (2.99%) compared to SBR-L (1.81%), and (2) higher abundance of *Nitrospira* in the SBR compared to the CMR (0.66% – CMR-H and 1.38% – CMR-L). Higher abundances of *Nitrospira* in the high-DO system is in contradiction to previously presented data. In deammonification systems, the successful NOB suppression was achieved with either short (Katsogiannis et al., 2003) or long aerobic periods (Mota et al., 2005; Zubrowska-Sudol et al., 2011; Li et al., 2011; Miao et al., 2016). During the SBR operation with intermittent aeration and low DO concentration (0.2–1.0 mg O<sub>2</sub>/L), the *Nitrospira* abundance observed in 16S rRNA sequencing datasets increased from 3.1% (day 3) to 53% (day 407) (Roots et al., 2018). An appropriate configuration of the intermittent aeration system is challenging, and the recent discovery of the comammox *Nitrospira* might bring additionally challenges in implementation of that aeration mode.

## 7.2. Temperature

Several authors have reported that the optimum temperature for the growth of *Nitrospira* is in the range of 30–35 °C (Yao and Peng, 2017; Huang et al., 2010; Blackburne et al., 2007). Huang et al. (2010) analyzed the effect of temperature on the main representatives of NOB (including *Nitrospira*) in a biological reactor at a municipal WWTP. During one-year study, the temperature in the reactor was in the range 24–30 °C, depending on the season. The authors observed a strong effect of the temperature ( $r = 0.59$ ,  $P < 0.0001$ ) on the *Nitrospira* abundance. In their study, the peak concentrations were achieved

**Table 4**  
Summary of influencing factors on growing of *Nitrospira* in nitrogen removal systems.

Main Factor	Value	Process	<i>Nitrospira</i> abundance	Other factors	Reference
DO	0.2 ÷ 1.0 mg O <sub>2</sub> /L	PN-A	Increase from 3.1 to 53%	T: 20.3 ± 1.1 °C, SRT: 99 d, NH <sub>4</sub> <sup>+</sup> : 0–14 mg/L	Roots et al., 2018
	2.0 mg O <sub>2</sub> /L	N	2.99%	T: 24 ± 0.5 °C, SRT: 15 d, HRT: 12 h,	Sun et al., 2018
	1.0 mg O <sub>2</sub> /L	N	1.81%	NH <sub>4</sub> -N: 43 ± 2.0 mg/L	
	Reduction from 1.7 to 1.0 mg O <sub>2</sub> /L	PN-A	“Dominant” NOB	pH: 6.9 ± 0.2, SRT: 3–7 d	Cao et al., 2018
DO	0.8 ÷ 1.5 mg O <sub>2</sub> /L	PN-A	Decrease from 0.44 to 0.04%	SRT: 33–56 d, NH <sub>4</sub> <sup>+</sup> : 105 mg/L	Qian et al., 2017
	0.5 mg O <sub>2</sub> /L	PN-A	Decrease from 5.35 to 3.34%	SRT: 25 d, NH <sub>4</sub> <sup>+</sup> : 11 mg/L	Mardanov et al., 2016
Aeration to mixing ratio (aer:mix)	1 h : 1 h	N	1.6%	pH: 7.6–7.8, HRT: 3 d,	Mota et al., 2005
	0.5 h: 1.5 h		2.9%		
	2.5 h : 0.5 h	N	20%	DO: 3 mg O <sub>2</sub> L <sup>-1</sup> , HRT: 11.1 h	Bhatia et al., 2017
Temperature	1.5 h : 1 h		8%		
	30 – 35 °C	PN	“Dominant” NOB	DO: < 1 mg O <sub>2</sub> /L, SRT: 4.27 d, Long operational time (> 1 year), pH: 6.5–7.5, SRT: ~92 days	Huang et al., 2010 Courtens et al., 2016a
Temperature	38 – 50 °C	N	“Dominant” NOB		
	Increase from 25 to 40 °C	N	Decrease from 2.02% to 0.09%	DO: 0.7 mg O <sub>2</sub> /L, FA: 2.7 mg/L	Luo et al., 2017
	10, 17 and 28 °C	N	“Dominant” NOB in 17 °C	NH <sub>4</sub> -N: 39.1 mg/L	Alawi et al., 2009
	10, 13, 16 and 19 °C	PN-A	“Dominant” NOB in 16 °C	DO: 0.5 mg O <sub>2</sub> /L, HRT: 1.3 h, NH <sub>4</sub> -N: 400 ± 8 mg/L,	Liu et al., 2016
Temperature	15–17 °C	A	4.19%	DO: 0 mg O <sub>2</sub> /L, pH: 7.5–7.8, HRT: 6–2 d, NH <sub>4</sub> -N: 20–30 mg/L, NO <sub>2</sub> -N: 22–30 mg/L	Liu et al., 2017
	Reduction from 26 to 20 °C	PN-A	Decrease from 6.2% to 5.2%	DO: 0.2–1.5 mg O <sub>2</sub> /L, pH: 7.4–8.5, NLR: 0.5–2.2 g N (m <sup>2</sup> d)	Zekker et al., 2017
	Reduction from 25 to 15 °C	PN-A	Increased from 4.63 to 7.23%	DO: 0.2 μmol O <sub>2</sub> /L, pH: 7.54 ± 0.20 ÷ 8.45 ± 0.20, NLR: 612.5 ± 25.4 mg N/(L·d)	Akaboci et al., 2018
				NH <sub>4</sub> -N: 220–550 mg/L	Jia et al., 2017
Weather seasonality	Winter (9.2 °C)	N	4.14%		
	Summer (25.6 °C)		12.02%		
pH	Winter (24–25 °C)	N	“Nondominant” NOB	DO: 1.0 mg O <sub>2</sub> /L, SRT: 4.27 ± 0.4 d, HRT: 4.38 ± 0.19 h,	Huang et al., 2010
	Summer (29–30 °C)	N	“Dominant” NOB	DO: 1.5 mg O <sub>2</sub> /L, T: 22 ± 1 °C	Blackburne et al., 2007
	6–9	N	The highest activity in range from 8.0 to 8.3	T: 22.1 °C, pH: 7.2–8.8, SRT: 7d, NH <sub>4</sub> -N: 30 mg/L	Rodrigues et al., 2017
pH	> 9	PN		T: 17 °C	Wegen et al., 2019
	6.4	N	“Dominant” NOB		
Low NLR	0.095 ÷ 0.238 kg/(m <sup>3</sup> ·d)	N	Increased from 1.5 to 2.0%	DO: 2–3 mg O <sub>2</sub> /L, T: 25 ± 3 °C, SRT: 30 d, NH <sub>4</sub> -N: 22.41–34.24 mg/L	Tian et al., 2017
High FA	49 mg/L	PN-A	< 0.5%	DO: < 1.0 mg O <sub>2</sub> /L, pH: 8.2, T: 32 ± 1 °C, HRT: 0.83–2.5 h	Wang and Gao, 2018
	85.7 ± 15.35 mg/L	PN	Wash out from the reactor	DO: 5.42 ± 0.72 mg O <sub>2</sub> /L, T: 24.1–26.9 °C, HRT: 11.70 ± 1.72 h, NH <sub>4</sub> <sup>+</sup> : 800 mg/L	Liang et al., 2014, 2015
FNA	18.08–24.95 mg/L	N	4.78%	DO: 1–1.5 mg O <sub>2</sub> /L, T: 25 °C,	Zhang et al., 2018
	36.06 – 50.66 mg/L	N	12.08%	pH: 8.1–8.2, HRT: 5 h	
	3.64 mg N/L	PN-A	Decrease from 15.7 ± 3.9 to 0.4 ± 0.1%	T: 22 ± 1 °C, pH: 7.5–5.7, SRT: 12 d, HRT: 13.2 h, NH <sub>4</sub> -N: 15–28 mg/L	Wang et al., 2016
HRT	30 – 15 h	N	“Dominant” NOB	DO: 4 mg O <sub>2</sub> /L, T: 20 °C, pH: 7.5–8.0, NH <sub>4</sub> -N: 500 mg/L	Li et al., 2013
	15 – 5 h	N	“Nondominant” NOB	DO: 4 mg O <sub>2</sub> /L, T: 20 °C, pH: 7.5–8.0, NH <sub>4</sub> -N: 90 mg/L	
Organic compounds	1.7 – 2.3 h	N	“Dominant” NOB	DO: 7 mg O <sub>2</sub> /L, T: 28 °C,	Winkler et al., 2017
	1.5 h		Wash out from the reactor	NO <sub>2</sub> -N: 230 mg/(L·d)	
	Increase from 0.97 to 3.20 kg COD/(m <sup>3</sup> ·d)	PN-A	Decrease from 0.4% to undetected level	T: 26.1–32.0 °C, HRT: 3–4.8 h, NLR: 0.57–1.5 mg N/(L·d)	Watari et al., 2016
	Increase C/N ratio from 10:1 to 30:1	N	Decrease from 9 to 4%	DO: 3.0–4.0 mg O <sub>2</sub> /L, T: 25 °C, HRT: 10 h, NH <sub>4</sub> -N: 20 mg/L	Song et al., 2017
Salinity	C/N	N	Increase from 0.58 to 4.6%	HRT: 1–3 d	Gao et al., 2017
	C/N = 1	N	3%	T: 30.0 °C, HRT: 20–44 h	Dong et al., 2017
	C/N = 2		Undetected level		
Tetracycline	25 mg Cl/L	N	10%	DO: 2 mg O <sub>2</sub> /L, T: 30.0 °C, pH: 6.5–7.5,	Quartaroli et al., 2017
	125 mg Cl/L		Undetected level		
Tetracycline	0 mg Cl/L	PN-A	2.5%	DO: 0.3 mg O <sub>2</sub> /L, pH: 8.0 ± 0.2,	Wang et al., 2017
	15 mg Cl/L		10.5%	HRT: 16 h, NH <sub>4</sub> -N: 200 mg/L	
	10 mg/L	N	1.6%	DO: 2 mg O <sub>2</sub> /L	Zheng et al., 2016a
	35 mg/		1.2%		
	20 μg/L	N	5–7%	SRT: 18 d, HRT: 16.5 h,	Liu et al., 2018
2 mg/L		3.5–4.9%			
50 μg/L	N	15–16%			
5 mg/		10.5–11.2%			

(DO – dissolved oxygen, FA – free ammonia, FNA – free nitrous acid, HRT – hydraulic retention time, N – nitrification, NLR – nitrogen loading rate, PN-A – partial nitrification-anammox, PN – partial nitrification, A – Anammox, SRT – solids retention time, T – temperature)



during the periods of the highest process temperatures. Moreover, during pure culture studies on *Nitrospira*, the authors showed that these bacteria thrived between 30 and 35 °C. The impact of seasonality on the growth of *Nitrospira* was also observed by Jia et al. (2017). In a lab-scale wetland system, *Nitrospira* (the most dominant genus) was always higher in summer (12.0%) than in winter (4.1%). The average temperatures in these seasons were 25.6 and 9.2 °C, respectively. Blackburne et al. (2007) investigated short-term effects of temperature in the range 14–40 °C on the oxygen uptake rate (OUR) of *Nitrospira*. In the temperature range from 14 to 35 °C, the OUR increased from 11 to 32 mg O<sub>2</sub>/(g VSS · h), while between 35 and 40 °C, the activity of *Nitrospira* decreased almost twice. Based on these results, Blackburne et al. (2007) determined the optimum temperature range for *Nitrospira* as 30–35 °C, whereas the inhibitory effect at 40 °C was either reversible or irreversible, depending on the exposure period. The negative effect of high temperatures (above 40 °C) was also found by Luo et al. (2017). The increase in the process temperature from 25 to 40 °C resulted in a decreasing ammonia utilization rate (AUR). A high FA concentration (about 2.7 mg NH<sub>3</sub>-N/L) at T = 40 °C, combined with a low DO concentration (0.07 mg O<sub>2</sub>/L), inhibited the growth of *Nitrospira* which resulted in the decrease of its abundance from 2.02 to 0.09%. Zekker et al. (2017) observed that after a reduction of the process temperature from 26 to 20 °C in a PN-A moving bed biological reactor (MBBR), a relative abundance of *Nitrospira* (dominant NOB) slightly decreased from 6.2 to 5.2%. The positive effect of high temperature on the *Nitrospira* was also observed in a deammonification system by Miao et al. (2016). The authors observed that in the high temperature (32 ± 1 °C) even a very low DO concentration was not able to suppress NOB (represented by *Nitrospira*) activity.

Courtens et al. (2016b), Edwards et al. (2013), and Lebedeva et al. (2008, 2011) showed that *Nitrospira* was the dominant NOB also in the thermophilic conditions (38–50 °C). Edwards et al. (2013) successfully enriched *Nitrospira calida* and *Nitrospira moscoviensis* with similar physiological properties, temperature optimum of 45–50 °C and an upper-temperature limit between 60 and 65 °C. Lebedeva et al. (2011) and Lebedeva et al. (2008) isolated *Nitrospira calida* and *Candidatus Nitrospira bockiana* with the growth temperature ranges of 46–58 °C and 28–44 °C, respectively.

Ambiguous results regarding the optimum temperature for *Nitrospira* growth were presented by Chen et al. (2018) and Alawi et al. (2009). Chen et al. (2018) observed that *Nitrospira* were more preferable at low-temperature conditions (10–20 °C). Alawi et al. (2009) compared the NOB communities grown at different temperatures (10, 17 and 28 °C). *Nitrospira defluvii* genus was detected in all samples, dominating at T = 17 °C. After the temperature decrease from 25 to 15 °C, the relative abundance of *Nitrospira* increased from 4.6 to 7.2% (Akaboci et al., 2018). Persson et al. (2014) decreased the process temperature in a PN-A MBBR from 19 to 10 °C. Although *Nitrospira* was not the dominant NOB, the authors reported a significantly higher abundance at 16 °C than at 19, 13 or 10 °C. Moreover, during the 300 days of the reactor operation at a temperature of 13 °C, there was no significant change in the abundance of *Nitrospira*. *Nitrospira* was also the dominant NOB in the anoxic anammox reactor operated at low temperatures (15–17 °C), with the maximum relative abundance 16.34% in the biomass fraction of 200–400 μm (Liu et al., 2018).

The relationship between microbial growth and temperature in the entire physiological range can be described by the modified Ratkowsky equation (Ratkowsky et al., 1983):

$$\sqrt{r} = b(T - T_{MIN}) \left(1 - e^{c(T - T_{MAX})}\right) \quad (1)$$

where T is the absolute temperature in K, r is the growth rate constant, T<sub>MIN</sub> and T<sub>MAX</sub> are the minimum and maximum temperatures, respectively, at which the growth rate is zero, and 'b' and 'c' are the fitting parameters. For *Nitrospira*, the effect of temperature on the normalized reaction rate could be accurately described by that equation (Fig. 3).

The data in the temperature range 15–30 °C were developed based on the exponential equation of Blackburne et al. (0.44e<sup>0.055(T-15)</sup>), while the actual experimental data were used for the temperatures > 30 °C.

### 7.3. pH

*Nitrospira*-like bacteria are sensitive to the high pH (> 9.0) because of growing the FA content and inhibiting their activity in both nitrification (Grunditz and Dalhammar, 2001; Blackburne et al., 2007) and anammox based systems (Rodriguez et al., 2017). According to Blackburne et al. (2007), the optimum pH for *Nitrospira* is in the range 8.0–8.3. A similar optimum pH (8.1 ± 0.1) was found by Zhang et al. (2018) in a nitrifying reactor. A lower range (7.6–8.0) was found for isolated pure cultures of *Nitrospira moscoviensis* sp. (Ehrlich et al., 1995). Similar pH values (7.6–7.8) were kept by in five nitrifying intermittently aerated reactors (Mota et al., 2005). In all the reactors *Nitrospira* was the dominant NOB and accounted for > 73% of the total NOB population. Lower pH values (7.0–7.6) were selected in the studies of Park et al. (2017) and Blackburne et al. (2008), and *Nitrospira* was found to be the dominant NOB at pH 6.4 at T = 17 °C (Wegen et al., 2019).

### 7.4. Nitrogen concentration

The concentration of inorganic forms of nitrogen, such as ammonium, nitrite, nitrate as well as FA in the reactor, have a significant impact on the activated sludge composition. A positive influence of low ammonia loading rate (ALR) on the *Nitrospira* growth was found by Roots et al. (2018) and Camejo et al. (2017). The ALR were 0.0401 and 0.024 kg NH<sub>4</sub>-N/(m<sup>3</sup>·d), respectively. In both systems, nitrogen concentrations in the reactors were in the range of 0–12 mg N/L of NH<sub>4</sub>, NO<sub>3</sub>, and NO<sub>2</sub> (Camejo et al., 2017), and 0–14 mg N/L of NH<sub>4</sub> and NO<sub>3</sub>, 0–0.2 mg N/L of NO<sub>2</sub> (Roots et al., 2018). The combination of those nitrogen concentrations, a low DO concentration and sufficiently long SRT, allowed *Nitrospira* to reach 53% of the overall microbial population. The opposite approach was proposed by Tian et al. (2017) who conducted research in a highly loaded and aerated reactor. The *Nitrospira* abundance increased (from 1.5 to 2%) with the increase of the ALR from 0.095 to 0.238 kg NH<sub>4</sub>-N/(m<sup>3</sup>·d) in a short operational time (30 d), DO of 2–3 mg O<sub>2</sub>/L, temperature of 25 ± 3 °C, and influent NH<sub>4</sub>-N of 22.4–34.2 mg N/L.

It well known that FA inhibits the activity of NOB (Ushiki et al., 2017). Recently, the influence of FA specifically on *Nitrospira* was investigated by Blackburne et al. (2007), Simm et al. (2006) and Ushiki et al. (2017). Simm et al. (2006) carried out two kinds of inhibitory tests, first with mixed microbial culture from a bench scale reactor and second with a pure culture of *Nitrospira moscoviensis*. The tests conducted with the mixed microbial population did not show classical FA inhibition of NOB at FA concentrations as high as 14.8 mg N/L. FA concentrations up to 10 mg N/L did not inhibit the activity of pure cultures of *Nitrospira moscoviensis* growth in batch cultures. Blackburne et al. (2007) estimated the inhibition thresholds of *Nitrospira* by FA at 0.04–0.08 mg N/L. For pure cultures, Ushiki et al. (2017) found that *Nitrospira* sp. Strain ND1 and *Nitrospira japonica* strain NJ1 were inhibited by FA 0.85 and 4.3 mg N/L, respectively. Zhang et al. (2018) concluded that the low levels of FA (18–25 mg N/L) had a limited effect on *Nitrospira*, while the higher levels of FA (36–50 mg N/L) had a evidently negative effect on *Nitrospira*. Wang and Gao (2018) suppressed the activity of *Nitrospira* (< 0.5%) in lab-scale anammox reactor by high FA of 49 mg N/L and limited DO (< 0.6 mg O<sub>2</sub>/L). Liang et al. (2015b) observed successful suppression of *Nitrospira* in the CANON process with FA of 85.7 mg N/L.

*Nitrospira* is also sensitive to high nitrite levels. Wagner et al. (2002) observed suppression of the growth of *Nitrospira* at nitrite concentrations above 80 mg N/L. Kinnunen et al. (2017) analyzed the influence of nitrite on the NOB guild composition in a biofilm. They observed a

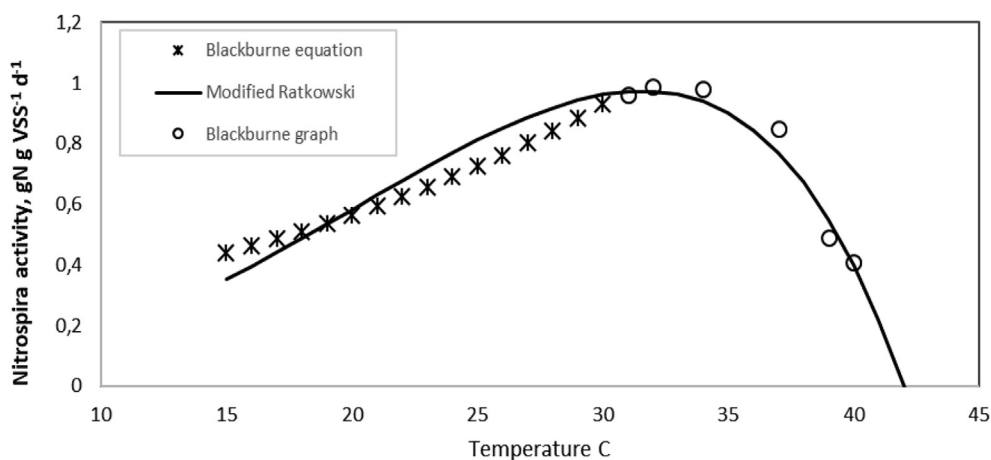


Fig. 3. Effect of temperature on the activity of *Nitrospira* described by the modified Ratkowski equation (Eq. (1)) based on the data from the study of Blackburne et al. (2007) ( $R^2 = 0.93$ ,  $c = 0.07457$ ,  $b = 0.04252$ ,  $T_{\text{MIN}} = 0$ ,  $T_{\text{MAX}} = 42$ ).

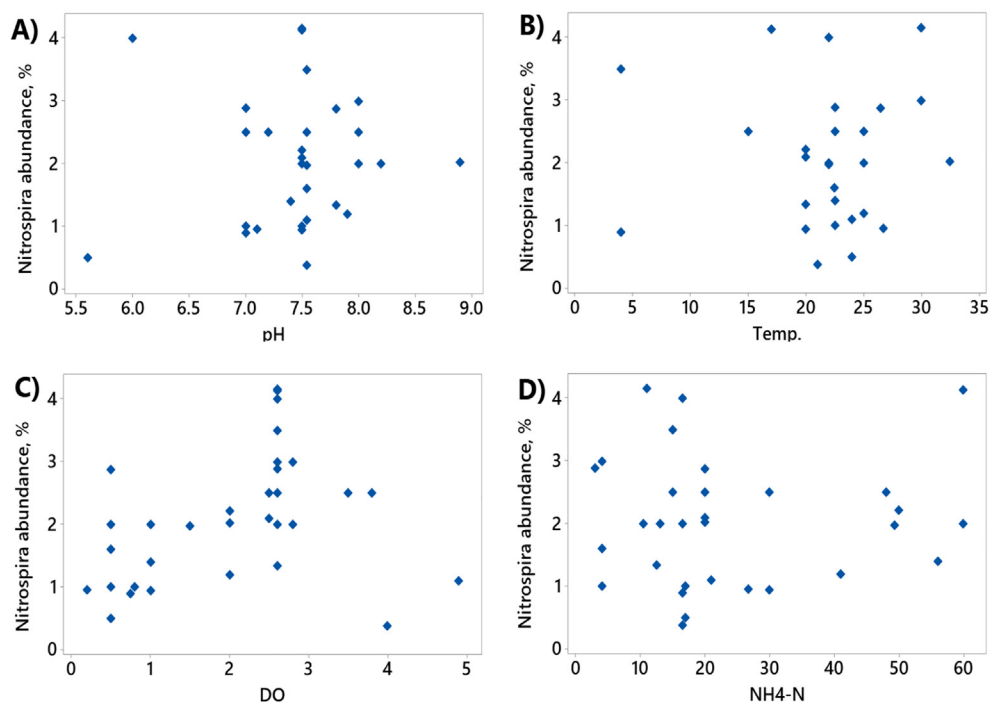


Fig. 4. Scatter plot of variables versus the *Nitrospira* abundance.

low abundance of *Nitrospira* in the source community and its dominance in the low nitrite loading biofilm (18.7%). In the high nitrite loading biofilm, the guild composition was dominated by *Nitrotoga* genus. With half-saturation constants ( $K_s$ ) between 1.4 and 4.1 mg N/L *Nitrospira* bacteria are adapted to substrate limited conditions (Nowka et al., 2015). While *Nitrobacter* prevails in high-strength systems, *Nitrospira* predominates under mainstream conditions due to a higher affinity for nitrite (and DO) (Law et al., 2019). According to Park et al. (2017), *Nitrospira* could be enriched from the activated sludge through a long-term cultivation in a continuous-flow reactor operated under nitrite- and DO-limited conditions. The authors noted that the increased *Nitrospira* abundance resulted from the increased influent nitrite concentration. The enriched *Nitrospira* reflected 97% similarity of 16S rRNA sequence to *Candidatus Nitrospira defluvii*, which belongs to *Nitrospira* lineage I. Furthermore, *Nitrospira defluvii* (lineage I) displayed a higher resistance to nitrite inhibition than the members of lineage II, which may suggest that elevated nitrite concentrations influence the niche differentiation between the lineages of *Nitrospira* genus (Nowka

et al., 2015).

#### 7.5. Solids and hydraulic retention times

The SRT and HRT are important operating parameters influencing the diversity of the microbial community in biological reactors, especially in membrane reactors (Silva et al., 2016). As the literature data show, there is a very wide range of SRT (10–99 d), allowing for an increase of the *Nitrospira* abundance (Roots et al., 2018; Bao et al., 2016; Park et al., 2017; Courtens et al., 2016a; Fitzgerald et al., 2015; Regmi et al., 2014; Liu and Wang, 2013). Pongsak et al. (2017) found *Nitrospira* at four WWTP with SRT  $\geq 6$  days. On the other hand, Liu and Wang (2013) observed that *Nitrobacter* and *Nitrospira* were the superior competitors at short SRTs (5 d) and long SRT (10–40 d), respectively. The authors suggested that nitrite concentration was a more important factor than SRT for the competition between *Nitrobacter* and *Nitrospira*.

Based on the maximum growth rate of *Nitrospira defluvii*, Winkler et al. (2017) determined the minimum HRT between 0.6 and 0.67 d.

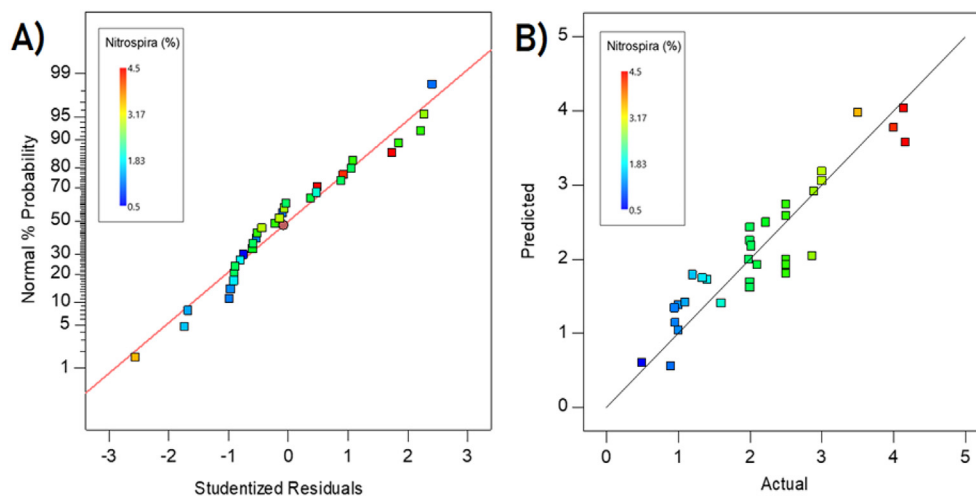


Fig. 5. A) Data normality, B) validation of the regression equation (predicted versus actual data).

Table 5

Validation of the regression equation results.

pH	Temp. (°C)	DO (mg/L)	NH <sub>4</sub> -N (mg/L)	Nitrospira abundance (%)		References
				Predicted	Actual	
7.5*	22.2*	0.5	4.06	1.26	1.0	Gao et al., 2018
7.5*	25	2.07*	13	2.24	2.0	
7.2	22*	3.6	19	2.13	2.6	Courtens et al., 2016a
7	30	0.6	23	2.59	2.0	Yang et al., 2018
8.2	30	2	4.1	3.16	3.0	Dong et al., 2017

The authors observed that at high HRTs (> 3 days), *Nitrospira* out-competed *Nitrobacter* instantaneously, while at the HRT higher than 0.64 day, *Nitrospira* was washed out of the reactor. According to Li et al. (2013), in the HRT from 15 to 30 h, *Nitrospira* was a dominant NOB in the conventional activated sludge system, and its abundance increased from 2.6% to 10.3%. Along with a decreasing HRT from 15 to 5 h, *Nitrobacter* began to dominate over *Nitrospira*.

## 7.6. Others factors

Roots et al. (2018), Park et al. (2017), Ouyang et al. (2017), Courtens et al. (2016a), Regmi et al. (2014) and Huang et al. (2010) observed a positive correlation between abundance of *Nitrospira* and long operational time. In their studies, the operational times were 407, 220, 200, 200, 560, 340 and 370 days, respectively. A similar observation, however, for much shorter operation time was made by Zhang et al. (2018). The authors observed that during 62 days of continuous-flow operation, HRT of 6.3 h, temperature of 25 °C and DO of about 0.15 mg/L, the *Nitrospira* abundances improved up to ratios of 2.1% and 12.1%.

Liu et al. (2018) and Zheng et al. (2016b) analyzed the impact of tetracycline (typical antibiotic, frequently detected in municipal wastewater) on the *Nitrospira* in the N-DN process. Liu et al. (2018) observed the positive impact of trace concentrations of tetracycline on the *Nitrospira* growth. The addition of 20 and 50 µg/L of tetracycline, caused in *Nitrospira* increase from 5–7% to 15–16% of the overall microbial community. Yim et al. (2006) found a positive effect of dosing trace-level tetracycline on the enrichment of *Nitrospira*. The authors related that effect to the fact that trace antibiotics could play a role of the surrogate auto-inducer and activate the transcription from quorum-sensing promoters. However, in both cases, a negative impact of higher concentration of tetracycline on the *Nitrospira* growth rate was observed. The *Nitrospira* abundance dropped from 1.6 to 1.2% with the growth of chlortetracycline concentration from 10 to 35 mg/L in a low

DO (0.5 mg/L) lab-scale SBR (Zheng et al., 2016a).

Many former observations indicate that salt is an important factor influencing growth of *Nitrospira* in nitrifying and anammox based reactors (Quartaroli et al., 2017; Wang et al., 2017; Moussa et al., 2006; Dionisi et al., 2002; Daims et al., 2001; Gieseke et al., 2001). The salinity effect on *Nitrospira* can be twofold – in nitrifying reactors is negative, whereas in anammox reactors could be positive. Quartaroli et al. (2017) noticed a significant decrease, from 10 to 0%, of the *Nitrospira* abundance in a nitrifying reactor. The decrease was caused by a small addition of sodium chloride to the reactor (125 mg NaCl/L). The higher salt concentration (10 g NaCl/L) was obtained by Moussa et al. (2006) after one year of adaptation of nitrifiers to higher salinity. *Nitrospira* was a dominant NOB at the salt concentration lower than 10 g NaCl/L. Wang et al. (2017) showed that salinity influenced the microbial population dynamics of the functional bacteria in a CANON system. The authors observed an increasing trend of *Nitrospira* abundance from 2% to 10.5%, when the salinity was increased from 5 to 15 g NaCl/L. The overgrowth of *Nitrospira*, despite an extreme sensitivity of the nitrate oxidation rate (NOR) to elevated salinity, should be an operational concern at salt levels up to 20 g NaCl/L, especially in substrate-limited (low DO and nitrite) environments.

A negative effect of high light intensity on *Nitrospira* was shown in algal-bacterial reactors (Merbt et al., 2012; Zhang et al. 2019). Merbt et al. (2012) showed that the irradiance level of 500 µmol m<sup>-2</sup>s<sup>-1</sup> caused the complete inhibition of *Nitrospira multiformis*. Zhang et al. (2019) studied three systems with different light intensities, including no light (0 µmol/(m<sup>2</sup> s)), low intensity (142 ± 10 µmol/(m<sup>2</sup> s)), and high intensity (316 ± 12 µmol/(m<sup>2</sup> s)). The highest relative abundance of *Nitrospira*, which reached 3.2% of the total microbial community, was found at the low light intensity. In contrast, the lowest abundance (0.85%) was observed at the high light intensity. The beneficial effect of the lack of light was also shown by Marks et al. (2012). The authors observed that *Nitrospira* was a dominant member of a geothermal ecosystem isolated from light.

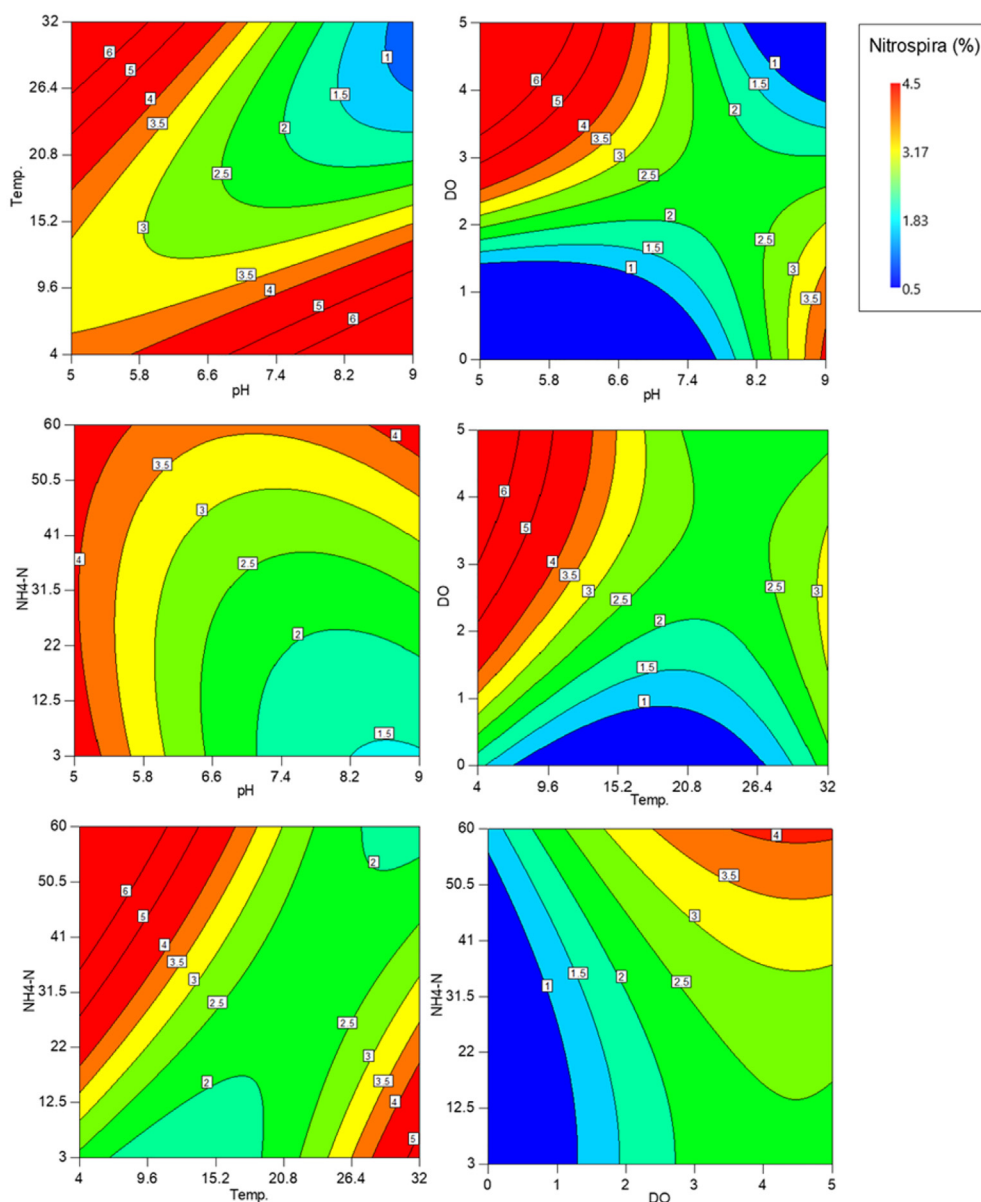


Fig. 6. Contour plot of the effect of the four factors on the response (*Nitrospira* abundance).

The factors that have a negative effect on the growth of *Nitrospira* also comprise high organic loads (Song et al., 2017), addition of sludge fermentation products (Yuan et al., 2016) and hydraulic loading rates (HLR) (Liang et al., 2017)). Along with an increase of COD concentration in the influent for membrane bioreactors (MBRs), from 200 to 600 mg COD/L, the *Nitrospira* abundance decreased from 9 to 4%. The C/N ratios were 10:1 and 30:1, respectively (Song et al., 2017). Yuan et al. (2016) observed that after addition of the sludge fermentation products, *Nitrospira* depicted a higher diversity (3.3%) in a SBR without sludge fermentation products than in a SBR with sludge fermentation products as (0.11%) operated at the same operational conditions. The negative effect of high HLR on *Nitrospira* has been reported in both activated sludge and constructed wetland systems. Liang et al. (2017) observed that the maximum number of *Nitrospira* genera sequences was significantly higher in a wetland with the HLR of 125 mm/d (92) in comparison with the HLR of 375 mm/d (34). The suppression of *Nitrospira* was obtained also by adding sodium azide (Pedrouso et al., 2017) or using ultrasound (Zheng et al., 2016a). In a lab-scale SBR with the PN-A process, by adding 5 mg/L of sodium azide, the *Nitrospira* abundance decreased sharply from 27.9 to 3.5%. Zheng et al. (2016a)

obtained *Nitrospira* suppressing (from 3% to nearly zero) using ultrasonic treatment (frequency > 20 kHz).

## 8. Meta-analysis of the literature data

The Response Surface Methodology (RSM) is a method to investigate a relationship between one or more responses with multiple variables (factors). The RSM is useful where statistical data play a key role, and the effect of specific individual variables and their combined interaction on each response can be determined (Anwar et al., 2015). In this study, a standard RSM model, implemented in Minitab (19.1) and DX (10.1) software (Stat-Ease, USA), was applied to determine the effects and interactions of four process variables (factors) influencing the *Nitrospira* abundance (response) in nitrogen removal systems. Based on the results of previous studies, four process parameters were used as input independent variables, including DO concentration, influent  $\text{NH}_4\text{-N}$  concentration, pH, and temperature. The mean values of those variables were determined based on the reported range in literature.

Actual values are the response data and the model predictions are generated by using the approximation functions. Fig. 4 presents

scattered data on the matrix plot of distribution of the four factors vs. *Nitrospira* abundance. The majority of evaluated data varied in the range 7–8 for pH, 20–25 °C for temperature, 0.2–4.0 mg O<sub>2</sub>/L for DO concentration, and 5–60 mg N/L for influent NH<sub>4</sub>-N concentration. The independent variables were coded according to Eq. (2) for factor appraisals:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i} \quad i = 1, 2, 3, \dots, k \quad (2)$$

where,  $x_i$  is a dimensionless variable;  $X_i$  is the actual value of each independent variable;  $X_{cp}$  is the actual value of each independent variable at the focal point, and  $\Delta X_i$  is the step change of the actual value of variable  $i$ .

A mathematical relationship between the predicted response, i.e. percentage of *Nitrospira* abundance ( $Y$ ) and the four independent variables, i.e. pH ( $X_1$ ), temperature ( $X_2$ ), DO concentration ( $X_3$ ) and influent NH<sub>4</sub>-N concentration ( $X_4$ ) can be described by the following empirical polynomial (second-order) model (Eq. (3)):

$$Y = \beta_0 + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \beta_{ii} X_i^2 + \sum_{i < j} \beta_{ij} X_i X_j + \epsilon \quad (3)$$

where  $\beta_0$  is a constant coefficient,  $\beta_i$  are the linear coefficients,  $\beta_{ii}$  are the quadratic coefficients,  $\beta_{ij}$  are the interplay coefficients,  $X_i$  and  $X_j$  are each independent process variable (coded values), and  $\epsilon$  is the residual error.

The data were normalized and fitted to the quadratic model. The overall prediction equation, resulted from the regression analysis, can be written in the following form:

$$\begin{aligned} \text{Nitrospira abundance (\%)} &= -20.1 - 0.52 \text{ pH} + 1.216 \text{ Temp.} + 8.88 \text{ DO} - 0.002 \\ &\text{NH}_4 - \text{N} + 0.450 \text{ pH}^2 + 0.01215 \text{ Temp}^2 - 0.1620 \text{ DO}^2 + \\ &0.000544 \text{ NH}_4 - \text{N}^2 - 0.200 \text{ pH} \times \text{Temp.} - 1.027 \text{ pH} \times \text{DO} + \\ &0.0167 \text{ pH} \times \text{NH}_4 - \text{N} - 0.0159 \text{ Temp.} \times \text{DO} - 0.00690 \\ &\text{Temp.} \times \text{NH}_4 - \text{N} + 0.00217 \text{ DO} \times \text{NH}_4 - \text{N} \end{aligned} \quad (4)$$

The residual versus normal probability plot (Fig. 5a) verified the assumption of the normality of residuals, whereas Fig. 5b illustrates the high accuracy of model predictions. A low value of the standard deviation ( $\sigma = 0.5$ ) and a high value of the determination coefficient ( $R^2 = 0.86$ ) confirm the acceptable goodness-of-fit. Specifically, the factors explain 86% of the variation in the response, while the standard deviation between the data points and the model predictions is approximately 0.5 unit. The ANOVA results with low p-values indicate suitable evidence against the null hypothesis. The level of importance (sensitive analysis) of each input factor and interaction between them were evaluated using the Pareto analysis. In addition, the regression equation was accurately validated with other data, which had not been used for the statistical analysis (Table 5).

The combined effect of each pair of the independent variables on the response (*Nitrospira* abundance) are shown in Fig. 6 (contour plots). From the figures, it can be seen that the highest *Nitrospira* abundances (red areas) can be expected under the following conditions (occurring simultaneously): high DO (> 3.0 mg O<sub>2</sub>/L) and influent NH<sub>4</sub>-N (> 20 mg N/L) as well as low temperature (< 15 °C) and pH (< 7). On the contrary, the simultaneous conditions for the lowest *Nitrospira* abundances cannot be specified unambiguously.

## 9. Conclusions

The latest genetic and experimental surveys revealed extraordinary versatility, adaptive capabilities and significant role of *Nitrospira* in catalyzing metabolic pathways during nitrification. Despite the canonical role in nitrification, the discovery of comammox, performed by selected *Nitrospira* representatives, reconsiders the current

understanding of nitrification as a strict interaction between AOB and NOB. However, the actual role and significance of *Nitrospira* in nitrogen removal process still needs to be validated by application of the latest approaches, such as a combination of genomic and transcriptomic data. The meta-analysis of literature data identified specific individual variables and their combined interactions on the *Nitrospira* abundance.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2020.122936>.

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