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Research Article

COMAMMOX - a new pathway in the nitrogen cycle in wastewater treatment plants

Abstract

The complete nitrification process, i.e. complete oxidation of ammonia to nitrate (COMAMMOX), by only one microorganism was experimentally confirmed only two years ago. That discovery is now considered a real breakthrough in the nitrogen cycle in the environment and it opens new questions regarding the nitrogen metabolism by microorganisms. Moreover, it also brings opportunities to revise the approach to nitrogen management in wastewater treatment systems employing the novel nitrogen removal processes, such as deammonification or shortened nitrification-denitrification. The comammox bacteria may significantly disturb nitrite production in partial nitrification, which is the critical step for the successful operation of both novel processes. The crucial role in identification of "comammox" bacteria is attributed to the latest, advanced molecular techniques (metagenomics and metatranscriptomics).

Discovery of Comammox

Nitrification is an important step in the global nitrogen cycle and plays an essential role in many engineered systems, including wastewater treatment plants (WWTPs). Conventionally, nitrification has been considered a two-step process catabolized by different groups of microorganisms, i.e. the first step (nitritation) - by ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA), and second step (nitratation) - by nitrite oxidizing bacteria (NOB). However, this functional separation has been proven to be energetically less advantageous. A postulate assuming the presence of a single organism with both lower growth rates and higher growth yields than the canonical AOB, capable of performing both nitrification steps, was presented more than 10 years ago by Costa et al. [1]. Those authors modelled the trade-off between the growth rate (favored by short metabolic pathways) and growth yield (favoured by longer pathways). Based on model calculations, they demonstrated that the existence of complete nitrifiers should be favored when the microorganisms grow slowly in clonal colonies (e.g. biofilms that cover surfaces in many natural and engineered systems). The authors concluded that in chemostats and other well-mixed systems, the faster-growing incomplete ammonia oxidizer would outcompete the complete oxidizers. In contrast, in biofilms and other microbial aggregates with low substrate diffusion gradients and low mixing of biomass (clonal clusters), a higher yield of biomass per amount of substrate consumed (which is equivalent to a more economical use of resources) would benefit only the

neighborhood. Therefore, under a broad range of favourable conditions in biofilms, more economical (but slower-growing organisms) would have a higher fitness than resource-wasting, fast-growing competitors.

The hypothesis of Costa et al. [1], was supported by the very recent discoveries of bacteria capable of performing comammox (complete ammonia oxidation) in an aquaculture system (van Kessel et al. 2015) and deep subsurface pipe [2], and subsequently in a bioactive filter at a drinking water treatment plant (Pinto et al. 2015) and a WWTP [3]. In the latter case, Chao et al. (2016) [3], have emphasized that the biofilm in aerobic reactors is exposed to the dissolved oxygen (DO) concentration gradient, which may induce the growth of comammox bacteria. Earlier cross-section studies of biomass distribution in nitrifying biofilm systems (Okabe et al. 1999) found the highest NOB abundance in deeper zones with less DO availability. Moreover, the study of Okabe et al. (1999) showed that these NOB clusters were dominated by *Nitrospira* sp., whereas the faster growing NOB species *Nitrobacter* sp. were almost absent. Therefore, *Nitrospira* sp. implicitly adapt better to DO limited conditions in comparison with *Nitrobacter* sp. This may be related to the presence of "comammox *Nitrospira*" in deeper biofilm layers, which have been recently shown to thrive in substrateinflux, DO limited zones [2]. Modelling studies of a one-dimensional stratified biofilm have revealed that oxygen penetrates to the depth of 20 μm (Mahendran et al. 2012). Moreover, micro-scale studies have demonstrated that there is a correlation between the DO profile and spatial

distribution of different groups of microorganisms (nitrifiers, denitrifiers, phosphorus accumulating organisms) in the biofilm (Gieske et al. 2002; Jabari et al. 2014) [4,5]. Manendran et al. (2012) found that denitrifiers formed both micro-clusters and were dispersed through the biofilm, which provided the evidence of a complex oxygen distribution (not solely depth stratification). The biofilm can assure a niche partitioning as a result of occurrence of different bacteria, which prefer specific nitrite concentrations, have the capability of utilising formate (as an alternative substrate) (Pjevac et al. 2017) and thus be a more favourable environment for the growth of comammox bacteria.

Observation and significances of comammox in wastewater treatment processes

In WWTPs, the most diverse and often predominant known NOB are mainly uncultured members of the genus *Nitrospira* [6,7]. Theoretically, in a fully nitrifying system (i.e. performing ammonia oxidation to nitrate), the ratio of NOB/AOB should equal the ratio of the respective yield coefficients Y_{NOB}/Y_{AOB} (e.g. $Y_{NOB} = 0.09$ and $Y_{AOB} = 0.15$, then $NOB/AOB = 0.6$). This means that AOB should be the dominant bacteria in a nitrifying community. However, the AOB and NOB microbial communities can shift and change along with changes of the environmental and imposed operational conditions (Gao et al. 2017). Indeed, some exceptions have been reported demonstrating much higher ratios of NOB/AOB (0.8-1.5) in municipal WWTPs [8] (Ramdhani et al. 2013) or even 3-4 in an aerobic granular reactor (Mari et al. 2012). High abundances of *Nitrospira* bacteria (exceeding AOB) could be attributed to the fact that some of these *Nitrospira* were actually capable of performing comammox [9]. (Wang et al. 2015). Furthermore, the presence of comammox microorganisms may indeed help in justifying other unexplained observations, such as (i) low nitrite concentrations in the environment if “comammox *Nitrospira*” did not release nitrite as an intermediate product during the complete nitrification [10], and (ii) presence of nitrate during NOB wash-out experiments in the conventional nitrification systems (Sobotka et al. 2017b).

Understanding the role of the comammox process in the nitrogen cycle in wastewater treatment systems could change operational strategies of the novel nitrogen removal processes, such as shortened nitrification-denitrification via nitrite (so-called “nitrite shunt”) or deammonification.

The benefits of the novel nitrogen removal processes result from suppressing nitrite oxidation which has been attributed to the growth of canonical NOB. The comammox process may negatively affect those novel processes due to competition for the same substrate (ammonia) (Figure 1). As a consequence, the comammox bacteria may significantly disturb NO_2 -N production in partial nitrification (nitrification), which is the critical step for the successful operation of both novel processes.

The coexistence of anammox bacteria with “comammox *Nitrospira*” has been observed (Van Kessel et al. 2015), even though the exact role of the latter microorganisms in the deammonification process is yet to be evaluated [7]. The presence of *Nitrospira* genera in deammonification systems has been reported by many authors [11,12]. (Persson et al. 2014;

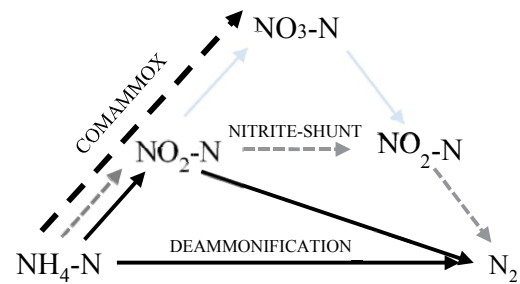


Figure 1: Diagram showing the effect of the comammox process on the novel nitrogen removal processes in wastewater treatment plants

Varas et al. 2015; Wang and Gao, 2016; Soliman and Eldyasti, 2016; Poot et al. 2016; Zhang et al. 2016). Soliman and Eldyasti (2016) observed the presence of anammox bacteria, AOB and *Nitrospira* genera in the biomass from nitrification-anammox SBR, whereas *Nitrobacter* genera of NOB were not detected. Poot et al. (2016) reported similar results, however, after 75 days of the reactor operation, *Nitrobacter* were still abundant indicating the insufficient NOB suppression. Stable and efficient deammonification is dependent on balancing the growth rates of aerobic AOB and anoxic anammox bacteria, and out-selection of NOB (Zhang et al. 2016). NOB tend to proliferate in long-term partial nitrification operations, especially under mainstream conditions, affecting the process by oxidizing nitrite to nitrate and therefore making the effluent unsuitable for further treatment by the anammox process. The NOB suppression in the deammonification process has been widely studied and the reported literature data indicate the possibility to repress NOB activity, but it is not possible to completely remove them from the system. There are different proven technologies based on the process that consider several strategies, such as controls of sludge retention time (SRT), pH, dissolved oxygen limitation, aeration intensity, redox potential and concentrations of free ammonia. For example, Varas et al. (2015) reported approximately 75% removal efficiency of total nitrogen with the oxygen-limitation strategy. However, molecular analyses demonstrated that the NOB group remained the most abundant bacteria in the system. Wang and Gao (2016) presented an “in-situ” strategy for suppressing the NOB activity in a one-stage granular deammonification system, which had been deteriorated by overgrowth of NOB. The results showed that the NOB activity was successfully suppressed after 56 days and the ratio of produced nitrate/consumed ammonia was reduced from 37% to ultimately 7%. In the course of the experiment, large copy numbers of NOB (*Nitrospira*, *Nitrobacter*) were found in the studied system. After 2 months of restoration and wash-out attempts, high amounts of NOB were still persistent in the system. 16S rRNA gene copy numbers of *Nitrospira* increased from 2.63×10^6 copies/mg to 1.06×10^8 copies/mg and copy numbers of *Nitrobacter* declined from 4.52×10^7 copies/mg to 2.17×10^6 copies/mg. Wang and Gao (2016) and Persson et al. (2014) reported that *Nitrospira*-like NOB behaved as K-strategists with a high substrate affinity to adapt to low nitrite and DO concentrations. In contrast, *Nitrobacter*-like NOB behaved as r-strategists with a low substrate affinity to adapt to high nitrite and DO concentrations. Therefore, *Nitrospira* may outcompete *Nitrobacter* under DO-limited conditions in deammonification systems.

Microbiology of Comammox

The recent discovery of the complete ammonia oxidizers in *Nitrospira* genera (Daims et al. 2015; van Kessel et al. 2015) [2], has dramatically changed the understanding of microbiologically mediated nitrogen transformations. Until the discovery of comammox, members of genus *Nitrospira* were regarded as the canonical NOB, i.e. which could grow only on nitrite. Seven genera within four bacterial phyla have been identified to reflect such the activity. Despite genetic distances between the particular NOB groups, all representatives show similar morphological properties i.e. gram-negative cell structure and thick cell wall. About 60% of all the characterized NOB belong to highly diverse genera *Nitrospira*, from which certain members are now considered “comammox” bacteria. In contrast to the canonical NOB, the genomes of “comammox *Nitrospira*” harbor the full set of genomes necessary for both ammonia oxidation (i.e. ammonia monooxygenase – AMO and hydroxyloamine dehydrogenase – HAO) and nitrite oxidation (molybdoprotein nitrite oxidoreductase – NXR) [13], (Santoro, 2016) [13]. All comammox organisms identified to date belong to “*Candidatus Nitrospira nitrosa*”, “*Candidatus Nitrospira nitrificans*”, “*Candidatus Nitrospira inopinata*”, and *Nitrospira* sp. strain Ga0074138 [2,13]. (van Kessel et al. 2015).

Sensitive and reliable microbiological methods are needed for comammox bacteria identification and assessing of their role in biological nitrogen removal processes. Due to the morphological similarity of *Nitrospira* members to other NOB, only DNA based methods are able to identify “comammox” bacteria (Novka et al. 2015). Currently, the most useful and powerful approach is based on so-called the next generation sequencing (NGS). The NGS analysis examines not only a selected marker gene (such as 16S rRNA) but also the entire genetic information stored in all the bacterial genomes derived from the examined samples (metagenomes). Metagenomic analysis of comammox bacteria have been used by Pinto et al. (2016), Chao et al., Palomo et al. (2016), Pievac et al. (2017) and Camejo et al. [3,13]. However, the metagenomics analysis does not provide information about the activity of those bacteria. Thus, the metagenomic approach could be integrated with metatranscriptomics, which allows for analysis of mRNA extracted from the living cells of microbial communities. So far, this procedure has not been applied to comammox bacteria.

Conclusion

The recent discovery of the complete nitrification process (COMAMMOX) adds a new dimension to the current understanding of the nitrogen cycle (especially nitrification). Moreover, it also brings opportunities to revise the approach to nitrogen management in wastewater treatment systems employing the novel nitrogen removal processes, such as deammonification or shortened nitrification-denitrification. The comammox bacteria may significantly disturb nitrite production in partial nitrification, which is the critical step for the successful operation of both novel processes.

Modern tools of molecular microbiology (metagenomics) enable to detect, identify and estimate of the comammox bacteria content in biomass samples. When combined with metatranscriptomics, the influence of comammox bacteria activity on nitrogen removal processes may be evaluated.

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