Occurrence and enrichment of ‘bacterial sherpas’: climb to sustainability in wastewater treatment
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ABSTRACT
The paper presents research on hemoglobin (Hb)-expressing bacteria in biological wastewater treatment systems. The outcome(s) will greatly reduce the aeration needs of wastewater treatment plants (WWTPs) and provide insight into emerging biological nitrogen removal processes using low dissolved oxygen (DO) conditions. In anthropogenic terms, the bacteria that express Hb could be considered as ‘bacterial sherpas’ that can function under low DO conditions. Hitherto, this functionality of bacteria has not been realized due to the initial response of the aerobic treatment stage: namely, morphology change by bacteria to filamentous forms to overcome oxygen mass transfer limitations causing bulking/foaming and nitrification inhibition. There is evidence, however, of the potential expression of Hb proteins by activated sludge (AS) bacteria. First, bacteria known to possess genes coding Hb proteins have been isolated from AS systems. Secondly, there is evidence that WWTPs are able to operate their biological processes at low DO without sludge bulking or incomplete nitrification. Our research has focused on nitrifying systems and has shown that this is due to prolonged operation at low DO conditions (0.1 mg O2/L), which allows sufficient time for bacterial acclimation. Additionally, it has been shown that enhanced Hb expression is linked to acclimation to low DO conditions.

Key words | bacterial hemoglobins, energy efficiency, low DO nitrification, wastewater treatment

INTRODUCTION
Due to the relative intolerance of ammonia oxidizing bacteria (AOB) to low dissolved oxygen (DO) conditions, a commonly accepted design recommendation to achieve complete ammonia oxidation in wastewater treatment plants (WWTPs) is to maintain bulk DO concentrations above 2 mg/L and as high as 6 mg/L (Grady et al. 1999). In order to meet the associated high aeration demand, substantial effort has been put into increasing the supply of oxygen by developing aeration systems that enhance oxygen transfer through the creation of additional air-water surfaces. However, as these transfer capabilities are increased, the energy requirements of aeration devices tend to increase accordingly (Rosso et al. 2008). Since aeration is the most energy-intensive activity in wastewater treatment, amounting to 45–75% of plant energy costs (Rosso et al. 2008), there is great interest in developing wastewater treatment processes that can nitrify at low DO conditions and hence not have such high energy requirements. Additionally, there are several emerging biological nitrogen removal processes that require low DO conditions either for control of process performance (partial nitrification processes), or for sustaining the conditions necessary for adequate operation (anammox and simultaneous nitrification-denitrification processes). Therefore, the promotion of AOB biomass that can function at low DO conditions is of importance in the latter treatment systems.

There is evidence that a great variety of bacteria in nature are capable of expressing hemoglobins (Hbs) (Frey & Kallio 2003). The roles of these molecules have been thoroughly investigated, and they appear to be diverse in nature. An important one of these is to promote oxygen delivery to the cells; molecular oxygen is facilitated both to the respiratory chain and to the enzymes requiring molecular oxygen as substrate (e.g. oxygenases) (Poole et al. 2008). Hitherto, this functionality has not been achieved in activated sludge systems (AS) due to the initial response of the aerobic treatment stage: namely, morphology change by bacteria to filamentous forms causing bulking/foaming (in order to enhance oxygen mass transfer).
and/or nitrification inhibition. In order to avoid this response, aerobic systems are operated at relatively high DO concentrations provided by aeration devices characterized by increasingly high mass transfer coefficient values (kLa). This supply-side approach (aeration device upgrade) to aerobic treatment process operation causes high energy consumption in WWTPs (Rosso et al. 2008). The issues that arise from implementing a supply-side approach by increasing the kLa values has been demonstrated in Figure 1(a). Due to the higher ‘observed affinity’ of floc formers for DO (KDO), the consequence of lowering the DO concentration in the aerobic tank is the out-competition of floc formers, leading to bulking and/or foaming events (point 1). This is why DO setpoints are increased through increases in kLa values (point 2). As can be seen, the steady-state operation point number 2 is placed in the area of the oxygen uptake rate curve where the dependence on DO concentration starts to be of zero order. This is of critical importance, since it means that further upgrading of the aeration system will not lead to a significant increase in process efficiency. Point number 3 demonstrates this principle: a significant increase in kLa from steady-state point number 2 to 3 leads to only a reduced increase in oxygen transfer rate. The loss of mass transfer efficiency with increasing kLa due to higher bulk DO concentration values constitutes a further issue. Summarizing, the supply-side approach to solving increased oxygen transfer requirements leads to moderate increases in treatment performance and significant increases in energy consumption. Conceptually, if a demand-side approach could be deployed, the most effective way would be to decrease the biomass KDO values; the outcome of such an approach has been presented in Figure 1(b). As can be seen, the same increase in oxygen transfer rate achieved in Figure 1(a) by increasing kLa can be realized just by increasing the ‘observed affinity’ of the biological culture towards oxygen. Finally, the demand-side approach described increases the efficiency of oxygen mass transfer due to the lower bulk DO concentrations. Conventionally, it has been thought that this demand-side approach is not feasible. However, there is empirical evidence of full-scale AS wastewater treatment systems operating at low DO conditions without bulking/foaming sludge or incomplete nitrification. This study puts forward the hypothesis that this is due to acclimation of biomass through expression of Hb proteins.

The study of AOB adaptation to low DO conditions is of particular importance given their high oxygen requirements as compared with heterotrophic bacteria. Some studies have provided evidence that AOB can adapt to low DO conditions. Most of these studies have hypothesized that low DO operation selects for certain groups within AOB characterized by high ‘oxygen affinities’ (Park & Noguera 2007; Park et al. 2008; Bellucci et al. 2011). The results of these studies have provided conflicting evidence on the AOB lineages enriched under oxygen deprivation conditions. Alternatively, some studies have linked ammonia-oxidizing Archaea (AOA) to low DO nitrification (Park et al. 2006). These contradictions suggest that the effect of low DO conditions on nitrifying bacteria is not simply the selection of those lineages that have a higher affinity for oxygen. This

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**Figure 1** Conceptual analysis of low DO process for sustainable wastewater treatment.
conclusion is supported by other studies carried out on the adaptation of nitrifiers to low DO in root oxygenated sediments; these have proven that low DO conditions cause a physiological adaptation of a generalist nitrifying community (Kowalchuk et al. 1998).

The ultimate objectives of this study are to demonstrate ammonia oxidation at very low DO levels (0.1 mg O₂/L), and to investigate the mechanisms of AOB adaptation to such an oxygen-limited environment. We hypothesize first that Hb-protein expression plays a role in the acclimation of the generalist AOB community to low DO conditions. In order to test this hypothesis, two sequencing batch reactors (SBRs), one at low DO conditions (0.1 mg/L O₂) and the other one at or near oxygen saturation (control reactor), have been operated for more than 220 days. Nitrification performance, protein expression and microbial population studies have been carried out to confirm this hypothesis. Additionally, modelling studies of the low DO process have been carried out to calculate the aeration savings achieved by operating AS processes with acclimated biomass.

MATERIAL AND METHODS

We have used a set of experimental methods to show the occurrence and enrichment of Hb-expressing bacteria in nitrifying AS. The experimental work involved nitrification process performance studies (in addition to general process performance studies), microbial population studies, protein expression studies and modelling studies.

General performance studies

Traditional water and biomass analysis were conducted for N species, total suspended solids (TSS)/volatile suspended solids (VSS), and specific oxygen uptake rate using Standard Methods (APHA AWWA & WEF 2005).

Nitrification performance studies

Under controlled laboratory conditions, two SBRs seeded with activated sludge from the same source were operated at high DO (near saturation) and low DO (0.1 mg O₂/L) concentrations for a period of more than 220 days. The working volume of each SBR was 3 L with a full sequence period of 12 hours (fill-react-settle-draw) (Table 1). Oxygen concentrations in the low DO reactor were gradually lowered from 2 mg O₂/L to 0.5 mg O₂/L to a final 0.1 mg O₂/L. The DO setpoint was changed when the nitrifying biomass was capable of achieving an effluent ammonium concentration of 5 mg N/L (Figure 2). The DO concentration in the low DO reactor was controlled using a 5200A Continuous Controller (YSI, Yellow Springs, OH) connected to a YSI 5561 DO and temperature probe. The flow of air delivered to each reactor was measured using DFM mass flow meters (Aalborg, Orangeburg, NY). Further details of the SBR can be found in Arnaldos et al. (2013a).

Microbial population studies

Microbial population studies were carried out using the clone library method, fluorescent in situ hybridization (FISH) and quantitative polymerase chain reaction (qPCR) of eubacteria, AOB, nitrite oxidizing bacteria (NOB), AOA and anammox (Webster et al. 2001; Nielsen et al. 2009).

Protein expression studies

Identification and quantification of heme proteins were achieved by CO-difference spectra (Dikshit & Webster 1988). In order to rule out the expression of heme proteins
other than Hb proteins at low DO conditions, protein activity assays were carried out for the soluble peroxidase (Anderson et al. 1968) and soluble oxidase (Miller & Nicholas 1985) known to be present in AOB metabolism (Hooper et al. 2004).

Modelling studies

Firstly, the kinetic parameters related to DO of both reactors were calculated by obtaining the kinetic curves as a function of DO concentration for both reactors. The concentrations of oxygen assayed were 0.1, 0.5, 1, 2, 4, and 7 mg O2/L; half of the DO values were lower than or equal to 1 mg O2/L in order to capture growth dynamics at low oxygen levels accurately. The rate of ammonium consumption under substrate saturation conditions was measured for each DO concentration. These results were fitted to a Monod growth model to obtain the KDO and specific maximum growth rates. The mass transfer coefficients for the different reactors were obtained following the standard procedure by the American Society of Civil Engineers (ASCE 1991). This information was transformed into oxygen uptake and transfer rates in order to assess the effects of changing DO concentrations on process performance and the achievable aeration savings in terms of kLa.

RESULTS AND DISCUSSION

The results showed that the low DO process nitrified successfully after prolonged DO pressure at 0.1 mg O2/L. Figure 2 shows the progressive adaptation of the low DO nitrifying biomass after each successive lowering of DO setpoint compared with the performance of the high DO biomass. As can be seen, the high DO biomass was able to oxidize completely the 80 mg N/L ammonium influent within some days of operation. In contrast, the low DO reactor reached complete ammonia oxidation after approximately 140 days of controlled operation. After this acclimation period, the performances of both reactors within the operation cycle were comparable. The steady-state biomass concentration in both processes was about 2,100 ± 100 mg TSS/L when operated at a biomass solids retention time (SRT) of 50 days. The key differences in various parameters measured between low DO and high DO biomass are shown in Table 2 and explained further below.

Table 2 shows that the low DO biomass has higher specific oxygen uptake rates and lower half saturation coefficient than the high DO biomass for similar maximum specific growth rates. Hence, prolonged operation at low DO conditions of nitrifying biomass brings about higher ‘observed oxygen affinities’ in oxygen deprivation conditions. This is a significant advantage in full scale systems; for a given overall oxygen transfer coefficient in an activated sludge system, the oxygen transfer rate will be much higher at lower operating DO than at higher DO due to a higher concentration gradient. In order to calculate the associated aeration energy savings, modelling of the low DO and high DO reactors using Monod kinetics and the determination of kLa coefficients at different airflow rates was conducted. The low DO reactor was shown to achieve approximately 20% savings in aeration requirements and 20% improvement in mass transfer capabilities when compared with a process operated at the design concentration of 2 mg O2/L (Arnaldos & Pagilla 2014). Therefore, this study proves that there is potential for substantial savings in energy and operational costs by acclimating nitrifying communities to low DO conditions.

The results from protein expression studies using the CO-difference spectral method showed that heme protein expression was higher in the low DO reactor biomass (Arnaldos et al. 2013a). As can be seen in Figure 3, the increase of heme protein expression in the low DO reactor coincided in time with an increase in the specific oxygen uptake rate after approximately 140 days of operation under oxygen deprived conditions. The fact that both heme protein expression and increase in the oxygen uptake rate took place simultaneously when the low DO reactor achieved complete ammonia oxidation (Figure 3) constitutes a clear indication that acclimation of the reactor was linked to heme protein expression. To the authors’ best knowledge, this is the first time that AOB acclimation has
been linked to expression of heme proteins; the consequences in terms of process operation and evaluation are significant and will be further discussed below.

The clone library method and microbial measurements using qPCR showed that the variety and quantity of AOB population in the low DO reactor are similar to those in the high DO reactor (mainly *Nitrosomonas*, *Nitrospira* and *Nitrobacter* spp.), suggesting that the generalist population had undergone physiological changes rather than being selected for a certain bacterial group (Arnaldos et al. 2013a). FISH experiments ruled out the presence of anammox and AOA, and confirmed the dominance of AOB and NOB. Our findings are consistent with the fact that AOB need oxygen as a substrate for the ammonia monooxygenase reaction to convert ammonia to hydroxylamine; HB protein expression at low DO might be enhancing the DO transport to this reaction. The expression of HB proteins by AOB is also consistent with the observation that heme proteins of unknown functions have been found in *Nitrosomonas europaea* (Dispirito et al. 1985; Hooper et al. 2004). Furthermore, peroxidase and oxidase assays of the protein from the low DO biomass confirmed that the heme proteins expressed by the low DO biomass were neither peroxidases nor oxidases (Arnaldos et al. 2013b). Since these are the only two soluble heme proteins in AOB metabolism, these results further confirm that the heme proteins detected in the low DO reactor are in fact HB proteins.

It is important to note that the conclusions achieved through this study are highly relevant not only for achieving energy savings in conventional nitrifying systems, but also in more novel nitrogen removal processes such as simultaneous nitrification-denitrification, mainstream anammox and partial nitrification/denitritation. A critical operation parameter in the latter processes is the operational DO concentration, which has to be controlled at low levels for adequate performance and/or process control. In the case of simultaneous nitrification-denitrification, low oxygen operation has been found to cause insufficient nitrification and nitrous oxide production (Jia et al. 2013). Mainstream anammox is a very promising novel process with multiple

Figure 3 | Expression of heme proteins and specific oxygen uptake rate values in the low DO reactor.
operational issues pending to be addressed, one of them being the need to maintain low DO concentrations in order for anammox bacteria not to be outcompeted by faster-growing organisms (Wett et al. 2013). There exist different types of control systems that try to regulate oxygen levels in partial nitrification systems that are coupled with anammox or in nitrification-denitrification systems. The systems that are proving to be more promising rely on simultaneous online measurement of dissolved nitrogen species as well as oxygen levels. The operation and troubleshooting of these sensors are proving to be challenging at the pilot plant scale, given the fact that accurate online measurement of dissolved nitrogen species (e.g. ammonia) is difficult (Regmi et al. 2014). In this framework, directed acclimation of the nitrifying biomass to low DO conditions would address the presented operational issues without having to rely on complex control systems and simultaneously help achieve additional energy savings.

CONCLUSIONS

The present paper presents research carried out on the acclimation of nitrifying systems to low DO conditions. The results presented have shown that prolonged operation at low DO conditions brings about acclimation of nitrifying biomass. After the adaptation period, the process performance achieved is similar to that obtained at high DO conditions; additionally, no bulking or foaming events are observed throughout the operational period. Additionally, it has been proven that Hb expression is linked to biomass acclimation. Process modelling has shown that the process using nitrifying biomass acclimated to low DO conditions can achieve a minimum of 20% savings in kLa requirements and 20% improvement in oxygen mass transfer efficiencies as compared with processes operated at 2 mg O₂/L. Furthermore, directed acclimation of nitrifying communities to low DO conditions could address some of the operational issues encountered in novel nitrification processes such as mainstream anammox, simultaneous nitrification-denitrification or partial nitrification/denitrification.

REFERENCES


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