Considering microbial and aggregate heterogeneity in biofilm reactor models: how far do we need to go?

Thomas P. W. Vannecke, George Wells, Nathalie Hubaux, Eberhard Morgenroth and Eveline I. P. Volcke

ABSTRACT

A model describing a given system should be as simple as possible – but not simpler. The appropriate level of complexity depends both on the type of system and on the intended use of the model. This paper addresses the critical question of which purposes justify increased complexity of biofilm (reactor) models. Additional model features compared to conventional models considered are: (1) the inclusion of microbial diversity, distinguishing between different species performing the same function; and (2) the distinction between flocs and granules in putatively granular sludge reactors. With a multispecies model considering interspecies diversity, it was demonstrated that a given macroscopic reactor performance does not necessarily reflect steady state conditions on the microscale. In a second case study, it was shown that the addition of a small level of flocs can have a significant impact on macroscale process performance and on microbial population and activity distributions in granular sludge reactors. It was concluded that increased complexity in biofilm models, concerning microbial diversity or mesoscale aggregate architecture, is likely more useful when the focus is on understanding fundamental microscale outputs, but under specific conditions, these additional model features can be critically informative for bulk reactor behavior prediction and general understanding.

Key words | biofilm reactors, biological nitrogen conversion, flocs, granules, microbial coexistence, population dynamics

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INTRODUCTION

Mathematical biofilm (reactor) models are excellent tools for predicting overall process performance (macroscale outputs) as well as for understanding underlying phenomena such as microbial interactions, segregation, or competition (microscale outputs). Deciding which features to include in biofilm (reactor) models is a critical component of model structure selection. Eberl *et al.* (2006) emphasize the value of identifying model features that can be omitted without decreasing the utility of the model for its intended purpose, as summarized in their 'golden rule' of modeling: 'a model should be as simple as possible, and only as complex as needed.' In essence, decreasing model complexity via simplifying assumptions can greatly ease computational requirements and interpretation of model outputs. The level of complexity to include in a model depends in large part on its intended use, but determining this level is not always straightforward.

One example of the utility of increasing biofilm model complexity in certain circumstances is the use of multidimensional (2D, 3D) simulations instead of the simpler, and more common, 1D models. Even within numerical 1D biofilm models, a range of complexity exists. In this contribution, the focus is on numerical 1D biofilm models with stratification of biomass, multiple substrates, and multiple functional guilds. Two common simplifying assumptions in such biofilm models are: (1) to neglect microbial diversity and resulting internal microbial competition within function guilds; and (2) to ignore mesoscale heterogeneity in aggregate structure (e.g., size distribution of granules, or variation in transport properties of a hybrid mixture of aggregate types, such as flocs and granules). However, experimental observations have demonstrated diverse assemblages of microbial populations within individual

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functional guilds in, for example, nitrifying biofilm reactors, where several genetically different populations of ammonium-oxidizers (Schramm *et al.* 2000; Bernet *et al.* 2004; Lydmark *et al.* 2006; Volcke *et al.* 2008; Terada *et al.* 2010; Almstrand *et al.* 2013) or nitrite-oxidizers (Schramm *et al.* 1998; Schramm *et al.* 2000; Downing & Nerenberg 2008) coexisted in the biofilm. Experimental observations have also highlighted routine coexistence of multiple types of biomass aggregate types in a single biofilm reactor. In putative granular sludge reactors, these two types of biomass aggregates probably coexist more often than supposed *a priori* (Innerebner *et al.* 2007; Vlaeminck *et al.* 2009; Vlaeminck *et al.* 2010; Winkler *et al.* 2012).

Moreover, and of critical importance to this paper, both diversity within functional guilds and heterogeneous aggregate characteristics have been proposed to influence macroscale reactor performance and process stability (Wittebolle *et al.* 2005; Siripong & Rittmann 2007; Wett 2007; Ramirez *et al.* 2009). Indeed, mathematical models including microbial community information have proven useful in investigating the link between the microbial community and the macroscopic reactor behavior, e.g., Vannecke *et al.* (2014) and Vannecke & Volcke (in press). Also, the possible role of suspended biomass in influencing performance in biofilm reactors, and the general lack of consideration of this fraction in biofilm models, was noted as a possible oversimplification by Morgenroth *et al.* (2000).

In this paper, we focus on the critical question of what modeling questions justify an increase in complexity in biofilm (reactor) models. We base our discussion on two examples with increased complexity that provide new insights at both the macroscale and microscale. The two examples deal with: (1) the influence of microbial diversity on biofilm development and microscale microbial population dynamics in a nitrifying biofilm (this contribution); and (2) the influence of small levels of floccular biomass in a granular sludge combined nitritation-anammox reactor.

METHODS

Modeling microbial diversity

To model microbial competition in a nitrifying biofilm and to verify the importance of various microbial parameters in determining the competition outcome, a two-step nitrification biofilm model including the growth and endogenous respiration of 10 ammonium-oxidizing species (AOO) and 10 nitrite-oxidizing species (NOO) was set up and implemented in Aquasim (Reichert *et al.* 1995). The grid number was set to 20, allowing capture of the required level of detail and at the same time keeping a reasonable computation time. Temperature and pH were kept constant at 30 °C and pH = 7.5, respectively. The influent contained only ammonium (250 g $N.m^{-3}$), resulting in a nitrogen loading rate of 0.9 g $N.m^{-3}.d^{-1}$. The general stoichiometric matrix and kinetics for this case study were based on Vannecke *et al.* (2014).

Possible ranges of values for maximum growth rate (μ_{max}), yield (Y), affinity for the nitrogen substrate (K_{NH}^{AOO} and K_{NO2}^{NOO}) and the affinity for oxygen (K_{O2}) were determined based on an extensive literature review (Vannecke & Volcke in press). For each microbial parameter, a normal bimodal distribution was constructed as in Ramirez *et al.* (2009). The eight bimodal distributions were each typified by two means ($\mu_1 = 0.6 \times k$; $\mu_2 = 1.4 \times k$) and standard deviations of $\sigma_{1,2} = 0.125 \times k$, with k the average value of the range of values found in the literature for the corresponding parameter. Ten species per type were then constructed by picking 10 random numbers from each bimodal distribution. Parameter values taken up in the final model are given in Table 1. The endogenous respiration rate for each species was assumed to be 5% of its corresponding maximum growth rate. The initial concentration of each AOO and NOO species was equal for all species of the same type (AOO: 7,000 and NOO: 2,333 g $COD.m^{-3}$) (COD: chemical oxygen demand). As heterotrophic growth on biomass decay products can be neglected (Mozumder *et al.* 2014), and the influent did not contain an organic carbon source, heterotrophic growth was not considered in this model. The initial biofilm thickness was 1 μm. At the steady state biofilm thickness of 1 mm, it was assumed that the biofilm growth rate and the detachment rate kept each other in balance. The initial concentration of ammonium in the bulk liquid was set equal to the influent ammonium concentration (250 g $N.m^{-3}$) while the initial concentrations of nitrite and nitrate were negligible (1 g $N.m^{-3}$). The bulk liquid oxygen concentration was kept constant at 3 g $O_2.m^{-3}$ during the simulations. The simulations were run during a sufficient period of time to ensure steady state reactor conditions, both at micro- and macroscale.

Modeling heterogeneity in aggregate structure

The importance of small levels of flocs in putatively granular sludge combined nitritation-anammox reactors was assessed using two one-dimensional multispecies biofilm models implemented in Aquasim using 30 grid points, as described

Table 1 | Microbial parameters characterizing the AOO and NOO species in the multispecies nitrification biofilm model

	μ_{max}^{AOO} [d^{-1}]	K_{NH}^{AOO}[g N·m^{-3}]	K_{O2}^{AOO}[g O_2·m^{-3}]	Y^{AOO}[g COD/g N]		μ_{max}^{NOO} [d^{-1}]	K_{NO2}^{NOO}[g N·m^{-3}]	K_{O2}^{NOO}[g O_2·m^{-3}]	Y^{NOO}[g COD/g N]
AOO1	1.10	2.84	0.95	0.23	NOO1	1.77	4.31	0.99	0.10
AOO2	2.41	6.51	0.37	0.11	NOO2	0.74	1.91	1.69	0.11
AOO3	1.91	12.97	0.35	0.07	NOO3	0.74	4.45	0.84	0.10
AOO4	0.79	4.82	0.47	0.08	NOO4	0.87	3.84	0.66	0.09
AOO5	2.08	10.54	0.33	0.24	NOO5	0.66	1.98	1.75	0.04
AOO6	2.22	5.96	0.36	0.10	NOO6	1.67	2.73	1.58	0.09
AOO7	0.71	4.62	0.82	0.25	NOO7	0.71	5.07	0.67	0.04
AOO8	1.77	4.71	0.83	0.21	NOO8	0.50	5.16	0.99	0.08
AOO9	0.59	12.10	0.91	0.08	NOO9	1.54	4.45	2.05	0.06
AOO10	0.68	12.27	0.27	0.13	NOO10	0.63	4.26	0.73	0.10

in Hubaux *et al.* (2015). Briefly, the first model included only granular biomass in a continuous flow bioreactor, while the second model structure included both granular and flocculent biomass. In addition to AOO and NOO, growth and decay (death-regeneration) of anammox (AMO) and ordinary heterotrophic organisms (OHO) were also included in both models. Granular biomass was modeled considering mass transfer limitations for soluble substrate and stratified biomass corresponding to local growth conditions. It was assumed that flocs are not mass transport limited.

RESULTS AND DISCUSSION

Case study 1: Modeling microbial diversity

Using the two-step nitrification biofilm model implementing the growth and endogenous respiration of 10 AOO and 10 NOO species, it was observed that the macroscopic reactor behavior, in terms of nitrifying performance, was already at steady state within 10 days after start-up (Figure 1(a)). At first, nitrite accumulated to a maximum concentration of 185 g NO_2^--N.m^{-3} on day 1, but was completely converted after four days. At steady state, ammonium was almost completely converted to nitrate, resulting in a nitrate effluent concentration of 241 g NO_3^--N.m^{-3}.

In contrast to the macroscopic reactor behavior, the steady state biofilm thickness of 1 mm was only reached after about 2.5 years (Figure 1(b)), indicating that constant reactor performance does not necessarily imply that the steady state biofilm thickness is already reached. The biofilm thickness increased linearly due to the formation of active biomass by microbial growth and the formation of inert particulate components by endogenous respiration. Inert particulate components made up more than 90% of the total particulate mass in the biofilm at steady state.

The steady state conditions of the microbial community were only reached after 140 months (Table 2). A major microbial community shift was even observed after 100 months of operation. Initially, all AOO species made up

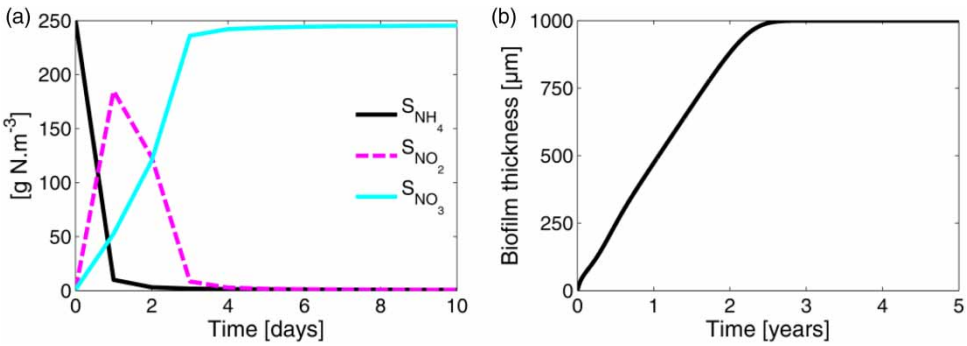

Figure 1 | Bulk liquid concentration of nitrogen components (a) and the biofilm thickness (b) in function of time. Note the different scale and units (days versus years) of the x-axis in both figures.

Table 2 | Evolution of the percentage of the total particulate matter (100 g COD) made up by each species in the biofilm through time

Time (months) → Fraction (%) ↓	0	10	20	30	40	50	60	70	80	90	100	110	120	130	140
AOO1	7.5	24.63	13.98	7.83	5.55	5.52	5.51	5.51	5.50	5.48	5.40	5.31	5.26	5.25	5.25
AOO2	7.5	0.002	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO3	7.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO4	7.5	0.002	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO5	7.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO6	7.5	0.029	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO7	7.5	0.002	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO8	7.5	0.069	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO9	7.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AOO10	7.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total AOO	**75**	**24.73**	**13.98**	**7.82**	**5.55**	**5.52**	**5.51**	**5.51**	**5.50**	**5.48**	**5.40**	**5.31**	**5.26**	**5.25**	**5.25**
NOO1	2.5	0.003	0	0	0	0	0	0	0	0	0	0	0	0	0
NOO2	2.5	0.10	0.002	0	0	0	0.007	0.042	0.17	0.63	1.60	2.69	3.25	3.44	3.49
NOO3	2.5	0.001	0	0	0	0	0	0	0	0	0	0	0	0	0
NOO4	2.5	0.005	0	0	0	0	0	0	0	0	0	0	0	0	0
NOO5	2.5	0.037	0	0	0	0	0	0.001	0.001	0	0	0	0	0	0
NOO6	2.5	7.06	3.90	2.35	1.85	1.84	1.83	1.82	1.77	1.59	1.19	0.70	0.43	0.34	0.31
NOO7	2.5	0.001	0	0	0	0	0	0	0	0	0	0	0	0	0
NOO8	2.5	0.001	0	0	0	0	0	0	0	0	0	0	0	0	0
NOO9	2.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NOO10	2.5	0.002	0	0	0	0	0	0	0	0	0	0	0	0	0
Total NOO	**25**	**7.22**	**3.90**	**2.35**	**1.85**	**1.84**	**1.84**	**1.86**	**1.94**	**2.22**	**2.79**	**3.38**	**3.68**	**3.77**	**3.80**
Total XI	**0**	**68.05**	**82.12**	**89.82**	**92.61**	**92.64**	**92.64**	**92.63**	**92.55**	**92.30**	**91.80**	**91.31**	**91.06**	**90.98**	**90.96**

Percentages of individual AOO and NOO species are visualized by color codes from 0% (white) to 25% (dark grey). At steady state (>140 months), three species coexisted: AOO1, NOO2 and NOO6.

7.5% and all NOO species 2.5% of the total particulate matter mass (100 g COD) in the biofilm. Due to microbial competition, the initial fraction of each species changed in time to its steady state value. In the AOO community, species AOO1 became dominant. In the NOO community, NOO6 remained dominant for 90 months (7.5 years). However, after 60 months, species NOO2, which was virtually absent in the biofilm for 40 months, reappeared in the biofilm. This species became dominant after 100 months and remained the dominant NOO species at steady state. At steady state, three dominant species coexisted in the biofilm: AOO1, NOO6 and NOO2. All the non-dominant species could be considered absent and not contributing to the microbial conversions. However, it is assumed that when the operation conditions change, these species could re-emerge when the new conditions are favorable for them, as their concentrations were negligible, but non-zero.

The steady state substrate and biomass concentration gradients are displayed in Figure 2. One could note that the number of species coexisting at steady state might be influenced by the number of grid points, an effect which may be more pronounced as more species are taken up in the model. This was not investigated in detail; the number of grid points applied in this study was found sufficient to capture the required level of detail concerning microbial coexistence at steady state.

In this study, ammonium and nitrite were limiting, as the concentrations of these substrates prevailing in the biofilm, 0.28 g NH_4^+-N.m^{-3} and 0.26 g NO_2^--N.m^{-3} respectively, were much lower than the affinity constants considered (Figure 2(a)–2(b)). Indeed, species with a rather high affinity for ammonium (AOO1: $K_{NH}^{AOO1} = 2.84$ NH_4^+-N.m^{-3}) and nitrite (NOO2: $K_{NO2}^{NOO2} = 1.91$ g NO_2^--N.m^{-3} and NOO6: $K_{NO2}^{NOO6} = 2.73$ g NO_2^--N.m^{-3}) were selected for. Oxygen was not as limiting, since its concentration (Figure 2(c)) prevailing in the biofilm was much closer to the considered oxygen affinity constants.

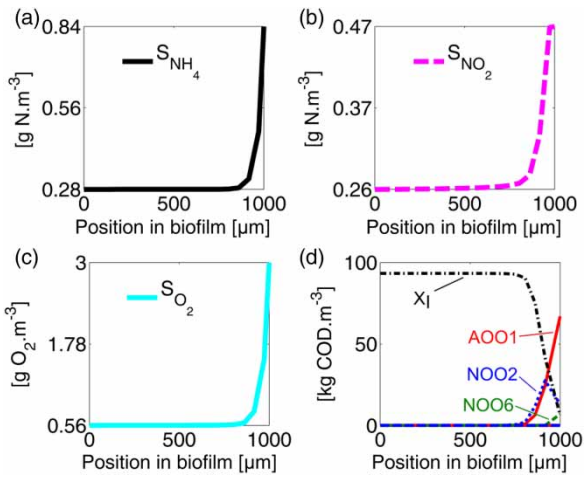

Figure 2 | Steady state concentration profiles for ammonium (a), nitrite (b), oxygen (c) and particulate matter (d) in function of the position of the biofilm (0 μm = bottom, 1,000 μm = surface of the biofilm). Note the different scale of the y-axis of the substrate concentration profiles.

From the biomass concentration profile (Figure 2(d)), it is observed that at steady state, NOO6 was present in a small concentration at the surface of the biofilm while NOO2 had the highest concentration 83 μm below the surface of the biofilm. The coexistence of two genetically and morphologically different populations of NOO with different distribution patterns in a biofilm was observed experimentally by Schramm *et al.* (1998). When coexistence of species performing the same function is observed, a distinction is typically made between slow growing species with a high substrate affinity (K-strategists) and fast growing species with a low substrate affinity (r-strategists). The r- and K-selection strategy (Andrews & Harris 1986) could explain experimentally observed population shifts and microbial coexistence in nitrifying biofilms, e.g. by Schramm *et al.* (2000) and Almstrand *et al.* (2013). In the NOO community considered in this study, NOO6 was an r-strategist with a relatively high growth rate ($\mu_{max}^{NOO6} = 1.67$ d^{-1}) and NOO2 was a K-strategist with a relatively high affinity for nitrite, corresponding with a low affinity constant ($K_{NO2}^{NOO2} = 1.91$ g NO_2^--N.m^{-3}). The r-strategist NOO6 was able to survive close to the surface due to the higher substrate concentrations prevailing there, in combination with its high maximum growth rate. As a K-strategist, NOO2 was able to cope with the limiting substrate concentrations deeper in the biofilm.

Considering the development of the NOO community in time, it was observed that the r-strategist NOO6 was able to cope rapidly with the prevailing conditions and grew at a high rate due to its relatively high maximum growth rate. After 100 months, the slow growing K-strategist NOO2 became dominant over NOO6 due to its higher affinity for nitrite. It can thus be concluded that the r- and K-selection strategy not only can be used here to explain the steady state microbial distribution profile but also the development of the microbial community composition over time.

Concerning microbial diversity, further simulation studies based on multispecies nitrification biofilm models are required to investigate the individual role of various microbial characteristics and operation conditions on microbial competition. Besides, there is increasing interest in explicitly incorporating our rapidly expanding understanding of microbial community structure and dynamics

via molecular tools into predictive process models. Seshan *et al.* (2014) present an example of this via a support vector regression model using microbial community diversity indices derived from DNA fingerprinting (T-RFLP) to predict reactor removal performance for COD, ammonia, nitrate, and 3-chloroanaline. Wastewater treatment modelers would also be well served by adapting emerging techniques in this direction in biogeochemical modeling. For example, Reed *et al.* (2014) provide a gene-based framework for incorporating environmental genomics data into a model of nitrogen cycling in the Arabian Sea oxygen minimum zone. A similar approach may be possible in bioprocess modeling to refine our understanding of the role of microbial diversity and community dynamics on both microscale and macroscale outputs in biofilm reactors.

Case study 2: Modeling heterogeneity in aggregate structure

The results of Hubaux *et al.* (2015) suggest that small levels of floccular biomass in granular sludge reactors can influence reactor performance and optimization under certain operating conditions, and can lead to microscale segregation of linked microbial populations and processes between aggregate fractions. Maximum predicted N-removal efficiencies were similar for granular sludge nitritation-anammox model structures with and without floccular biomass. However, floc addition led to a lower optimal dissolved oxygen (DO) concentration and a narrower maximum N-removal peak. This suggests that even small levels of floccular material may decrease process robustness to shifts in oxygen supply or concentration. Shifts in macroscale process performance were paralleled by microscale segregation in microbial populations between aggregate types (Figure 3). AOO predominated in flocs, resulting in relatively AMO-rich granules. Similarly, OHO populations were concentrated in the floccular fraction under low DO conditions when both flocs and granules were considered. In the combined granular and floccular sludge reactor, NOO comprised ~ 30% of the total active biomass at DO = 0.3 mg/l (Figure 3, right). In contrast, NOO reached this fraction only at DO = 0.5 mg/l in the exclusively granular sludge reactor (Figure 3, left). These patterns in population segregation were accompanied by associated shifts in microbial activity, and can be explained in part by the lack of diffusive mass transfer limitation in floccular biomass, such that organisms in this fraction were directly exposed to oxygen.

Volcke *et al.* (2012) assessed the influence of a non-uniform granule size distribution on granular nitritation-anammox reactor macroscale and microscale characteristics, and concluded that size distribution influences microscale solute transport due to the increased relative abundance of AOO in smaller granules and AMO in larger granules. Hubaux *et al.* (2015) indicated a similar segregation of microbial populations and activities due to the inclusion of a second biomass fraction without mass transport limitations (flocs), with AMO concentrated in (uniform size distribution) granules and AOO predominating in floccular biomass. Overall, neglecting small levels of heterogeneity in aggregate structure (e.g., small levels of floccular biomass in granular sludge reactors) in biofilm models may lead to erroneous patterns or results at the microscale (e.g., because of the balance between aerobic and anoxic metabolisms), and also for macroscale performance (e.g., concerning the optimal bulk DO concentration) in some cases.

It should be noted that the model presented by Hubaux *et al.* (2015) evaluated only two aggregate fractions (flocs and granules), whereas in reality there is a continuum of structures between the two. A similar approach is followed when modeling granular sludge reactors, typically characterizing the granule size distribution by a single diameter, a methodology which was found sufficient in case only the overall reactor behavior needs to be assessed, but which neglects solute exchange between particles of different sizes (Volcke *et al.* 2012). Further modeling efforts are warranted to test to which extent this conclusion can be generalized, i.e., in which cases structural complexities can be neglected when evaluating macroscale outputs and to assess their influence on microscale microbial distribution and solute exchange. Evaluating the combined influence of granule size distribution with small levels of floccular material in both granular nitritation-anammox reactors and in other biofilm systems, particularly those that involve cross-feeding between multiple functional groups, seems a logical next step.

CONCLUSIONS

Two case studies were highlighted in which additional model complexity was included beyond the conventional formulation for numerical one-dimensional biofilm models. In case study 1, it was shown that multispecies models are a useful tool to investigate the individual influence of various microbial characteristics on microbial population dynamics, and that coexistence of several species performing the same function is linked to the ecological niches created by the substrate concentration gradients in the biofilm. Nitrifying biofilm models including the growth of several species performing the same function not only demonstrate that a

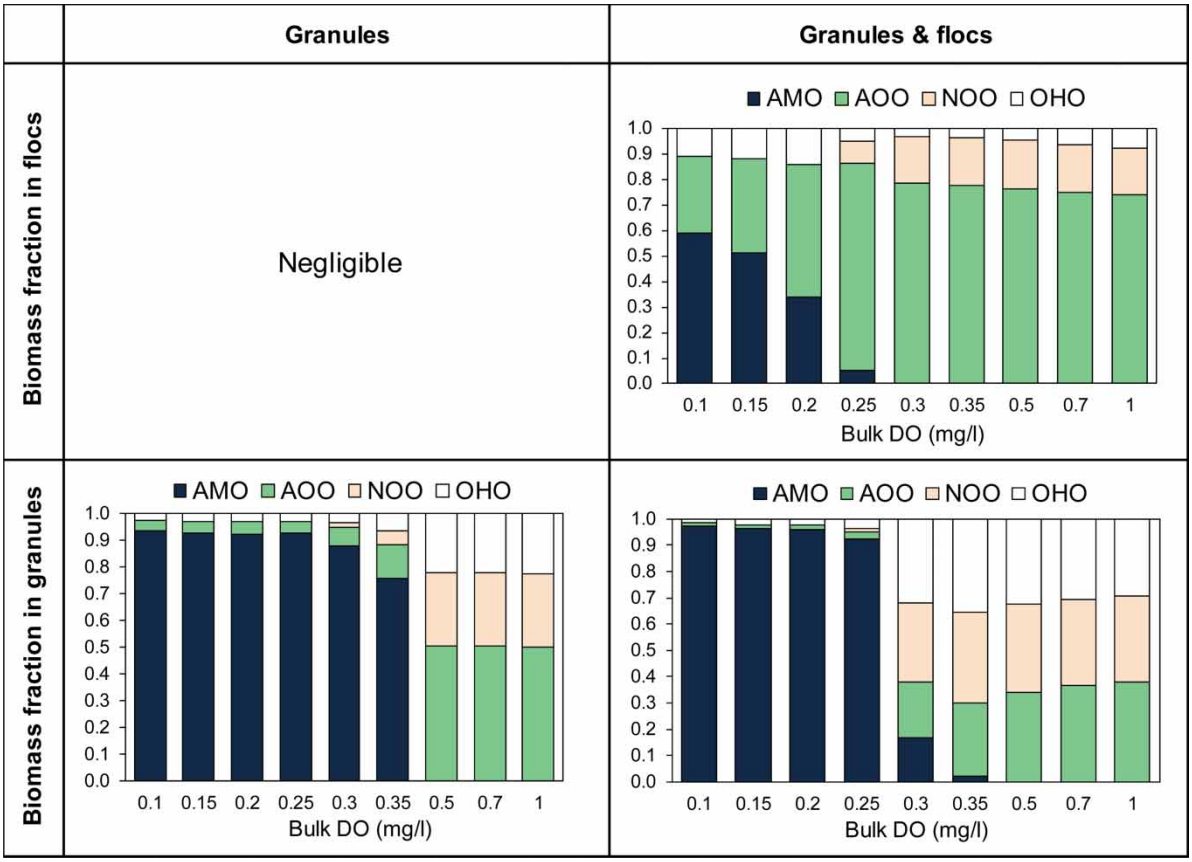

Figure 3 | Steady state active biomass partitioning between flocs (5% total biomass, above) and granules (below) in a granular sludge reactor (left) and in a mixed granules-flocs reactor (right) for different bulk oxygen concentrations. N surface load: 0.45 g $N \cdot m^{-2} \cdot d^{-1}$, COD surface load: 0.015 g $COD \cdot m^{-2} \cdot d^{-1}$ (COD:N = 0.1:3). Reprinted with kind permission from Elsevier (Hubaux *et al.* 2015).

constant macroscopic reactor behavior may be hiding major microbial community shifts, but can also be used to investigate major microbial population shifts resulting in a different nitrifying performance (Vannecke *et al.* 2014). In case study 2, our model suggests that even low levels of flocs can have a significant impact on process performance, optimal operating ranges, and microbial population and activity distributions in combined nitritation-anammox granular sludge reactors (Hubaux *et al.* 2015). The implication is that a better characterization of size distribution, mass transfer properties, and microbial population segregation of microbial aggregates – including flocs and granules – could improve operation of these reactors and contribute to better understanding of unexpected reactor behaviors. A worthwhile goal would be to assess the combined influence of both multiple taxa within the same functional group and heterogeneity in mesoscale aggregate architecture on microscale and macroscale biofilm/ biofilm reactor behavior.

The additional model complexity considered in this study had a substantial impact on macroscale outputs in some specific conditions, and on microscale outputs (namely, spatial distribution of dissolved and particulate components) under all conditions. It is likely a general rule that increased complexity concerning microbial diversity and/or mesoscale aggregate architecture will be more useful when the focus is on understanding fundamental microscale outputs. When the focus is on macroscale outputs (e.g., substrate removal rates, optimal bulk conditions), this complexity is clearly not always necessary. However, under some conditions, such additional model features can be critically informative for bulk reactor behavior prediction or understanding.

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