Bacterial composition of activated sludge – importance for floc and sludge properties

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Abstract. Activated sludge flocs consist of numerous constituents which, together with other factors, are responsible for floc structure and floc properties. These properties largely determine the sludge properties such as flocculation, settling and dewaterability. In this paper we briefly review the present knowledge about the role of bacteria in relation to floc and sludge properties, and we present a new approach to investigate the identity and function of the bacteria in the activated sludge flocs. The approach includes identification of the important bacteria and a characterization of their physiological and functional properties. It is carried out by use of culture-independent molecular biological methods linked with other methods to study the physiology and function maintaining a single cell resolution. Using this approach it was found that floc-forming properties differed among the various bacterial groups, e.g. that different microcolony-forming bacteria had very different sensitivities to shear and that some of them deflocculated under anaerobic conditions. In our opinion, the approach to combine identity with functional analysis of the dominant bacteria in activated sludge by in situ methods is a very promising way to investigate correlations between presence of specific bacteria, and floc and sludge properties that are of interest.
Keywords: Activated sludge, dewatering, FISH, flocculation, floc strength, settling

Introduction

Activated sludge flocs consist of numerous constituents such as bacteria, extracelluar polymeric substances (EPS), organic and inorganic particles. Together with other factors, e.g. the physical conditions such as shear, these constituents are responsible for floc structure and floc properties. These properties, on the other hand, largely determine the sludge properties such as flocculation, settling and dewaterability.

When we want to assess the direct role of the bacteria on floc and sludge properties, several questions appear: What are the bacterial number and total cell biomass in the floc (e.g. as fraction of all organic matter), how many are alive and/or active and how many are dead and inactive, what kinds of bacteria are dominating in terms of function and taxonomic affiliation, which factors determine the bacterial composition in different treatment plants, how do different species affect floc and sludge properties, and how can microbial activities affect the properties? Answers to these and several other questions are important if we want to understand the behavior of activated sludge flocs, and if we want to control and optimize sludge properties.

In this paper we briefly review the present knowledge about the role of bacteria in relation to floc and sludge properties, and we present a new approach to investigate the identity and the function of the bacteria present in the activated sludge flocs. The approach includes an identification of the important bacteria, combined with a characterization of their physiological and functional properties.

Activated sludge floc structure and composition

Bacterial composition and function.

The number of bacteria in activated sludge is in the range of 1-10 x10^{12}/g VSS (Nielsen, 2002). Of these typically 80% are active or alive. The total number has been assessed by DAPI staining and the living fraction as positive by either fluorescence in situ hybridization (FISH) with oligonucleotide gene probes staining all bacteria with a significant content of ribosomes, or the fraction able to take up radioactive substrates as measured by microautoradiography (MAR) (Nielsen and Nielsen, 2002a). The bacterial cells make up only a minor part of the organic material in the flocs. Different methods based either on respiration rates (Lavallée *et al.*, 2002) or cell count

and average content of carbon or nitrogen (Frølund *et al.*, 1996) have been used to estimate this fraction, and values of 5-20% have been recorded. This means that the remaining 80-95% of the organic matter can be regarded as dead material responsible for many of the colloidal-chemical properties of the floc. The bacteria are present either as single cells, microcolonies or as filamentous bacteria.

Many different functional bacterial groups are present in activated sludge systems. An example from a nutrient removal plant is shown in Table 1. This clearly shows that many groups are present – and also groups, which are usually considered of less importance in the normal activated sludge processes (e.g. iron reducers, sulfate reducers and methanogens). Under some conditions (e.g. when bulking problems appear), many sulfide oxidizing bacteria can also be present. It is also important to note that the majority of the bacteria are just known as aerobic heterotrophs or denitrifiers, and that we know very little about their main functions in the sludge. It is our experience that these functional groups can be detected in most types of treatment plants, although e.g. nitrifiers and phosphorus accumulating organisms (PAOs) are only present if the right conditions prevail. The direct taxonomical identity on a species level is presently almost unknown except for a few functional groups. Many dominant filamentous bacteria are identified (e.g. Wagner and Loy, 2002), the ammonium oxidizers (Purkhold *et al.*, 2000) and the nitrite oxidizers (Daims *et al.*, 2002), some PAOs (Crocetti *et al.*, 2000; Hesselmann *et al.*, 1999) and some sulfate reducers (Manz *et al.*, 1998). Most of these and other bacterial species are still uncultured and can only be detected by cultivation-independent molecular methods (Wagner *et al.*, 1993; Wagner and Loy, 2002).

Table 1. Functional groups of bacteria in a nutrient removal plant (partly after Nielsen and Nielsen, 2002a and Nielsen *et al.*, 2002a).

Functional group	Percentage of total bacteria %
Bacteria detectable with FISH	80
Aerobic heterotrophic bacteria	74
Nitrate reducing bacteria	71
Phosphorus accumulating organisms (PAO)	4
Glycogen accumulating organisms (GAO)	?
Fe(III)-reducing bacteria	4
Sulfate-reducing bacteria	3-4
Methane-producing bacteria	0-1
Ammonia-oxidizing bacteria	2-3
Nitrite-oxidizing bacteria	2-3
Fe(II)-oxidizing bacteria	0-1

Physico-chemical composition.

Apart from the bacterial cells, 80-95% of the organic matter in the activated sludge floc consists of various types of organic material. The EPS fraction is the largest fraction and it consists of polysaccharides, proteins, lipids, nucleic acids, humic substances and various heteropolymers (summarized by Nielsen, 2002). Protein is usually considered as the largest fraction (Frølund *et al.*, 1996; Higgins and Novak, 1997), although in some studies, the polysaccharides have been reported to dominate the EPS fraction (e.g. Liu and Tay, 2002). Whether this is due to different types of flocs or the use of different analytical tools is not clear. The EPS are produced by the bacteria, they are remains from lysed bacteria or adsorbed matter from the wastewater. Also organic and inorganic particles may be attached to the flocs. These dead organic and inorganic compounds are responsible for many of the colloidal-chemical properties of the floc and particularly the gel-forming properties, which are of key importance to the floc properties (Keiding *et al.*, 2001). Apart from the properties

of the organic part of the EPS matrix, also presence of cations, particularly the ration of monovalent to divalent cations (e.g. Higgins and Novak, 1997) and the presence of trivalent ions (Nielsen and Keiding, 1998) are important for the gel properties.

Effect of bacteria on floc and sludge properties

There are several relatively well-established correlations between floc properties and sludge properties (summarized by Nielsen, 2002). Related to settling these are: small porous flocs settle poorly while dense large flocs settle well. Related to dewatering it is known that weak flocs may deflocculate during the dewatering process and are thus difficult to dewater. Furthermore, it is known that a large EPS pool with a high charge density has a high water-binding capacity making it difficult to obtain a high dry matter content (Mikkelsen and Keiding, 2002). The formation of good flocs is important and it is well known that poor flocculation (or deflocculation) strongly affects the sludge properties and may leave many colloids in the effluent. Despite the minor importance in mass, the living bacterial cells are however, still key players in relation to floc properties and thus the sludge properties. Their growth pattern affect the floc as some bacteria can move around and either stay in the floc or swim away, others can form stable microcolonies, and some grow as filamentous bacteria and thus form a backbone in the floc or in cases of excessive growth, also grow in the bulk water and cause bulking. Furthermore, the bacteria produce various EPS components, which make up a significant part of the EPS matrix. The EPS composition and amount are important, but we know very little about EPS production of the different species present in activated sludge. Bacteria belonging to the genus *Zoogloea* are, however, known to produce copious amounts of highly water-containing gels which strongly affects the dewaterability (Lajoie *et al.*, 2000).

In an indirect way, the bacteria can also affect the floc properties by their metabolic activity. They can change the local environment in a way such that it affects the EPS matrix and thus the floc properties. Examples are processes that affect the charges in the EPS matrix such as a decrease in pH (nitrification), an increase in pH (denitrification), and a change in multivalent cation concentration (e.g. reduction of Fe(III) to Fe(II) by iron reducers or sulfide produced by sulfate reducers, Nielsen and Keiding, (1998)). Another example is excretion of exoenzymes that degrade the EPS matrix.

The interesting thing here is that the various functional groups and the different species can affect the floc structure in completely different ways. This means that the relative amount of the different bacterial groups both determine the general structure and the properties of flocs in a certain treatment plant, and the response on different external conditions on a short term basis (minutes or few hours). Short term anaerobic conditions, for example, can cause activated sludge floc to disintegrate, and it can only to some extent reflocculate when oxygen is subsequently added (Wilén *et al.*, 2000b).

In situ investigation of identity and function of dominant bacteria

Identification.

It is now well known that it is impossible to isolate and grow most of the bacteria present in activated sludge by using the cultivation methods currently available (e.g. Wagner *et al.*, 1993). Therefore, the only way to reveal their identity is by using molecular biological methods that are independent of cultivation. These methods have been applied in wastewater microbiology for the last 10-15 years and are described in detail elsewhere so here only a few aspects are mentioned. The basic concept is extraction of nucleic acids from the sludge, amplification of the 16S-rDNA gene by PCR, construction of a clone library, sequencing of the clones and identification by using databases. This gives an overview of the species present in the sample, but is only qualitative or semi quantitative. Therefore, it is important to use the full-cycle rRNA-approach, which also includes design and application of gene probes by FISH so the growth form, number and location of dominant bacteria can be viewed directly in a fluorescence microscope. The entire full-cycle rRNA-

approach is very time-consuming and requires expertise that is still limited to a few research groups dealing with wastewater treatment research, so no comprehensive identification of dominant bacteria in plants treating municipal wastewater exist. Only one single complete study on activated sludge from a plant treating a specific type of industrial wastewater has been conducted (Juretscko *et al.*, 2002).

Some new methods are, however, appearing that can reduce the work in elucidating the identity of dominant bacteria in activated sludge. Micromanipulation has been used to take out specific filamentous bacteria (Snaidr *et al.*, 2002) or dominant floc-forming bacteria (Thomsen *et al.*, 2003) for further molecular analysis and gene probe design.

Linking function with identity.

The function of the bacteria in the sludge flocs can be studied by various in situ methods that allow the study of different aspects of physiology and other properties. The basic approach is shown in Figure 1. The identity is determined by FISH, and other properties are determined by other means – usually on the very same cells in the same microscopic field allowing a direct link between identity and property. The physiology can be studied by using a number of methods: substrate uptake can be determined by the use of radioactive isotopes, microautoradiography, MAR (e.g. Nielsen *et al.*, 1999; Lee *et al.*, 1999); intracellular storage products such as lipids, P-granules and S-granules can also be observed (e.g. Nielsen *et al.*, 2000; Serafilm *et al.* 2002); exoenzymatic activity can be assessed by enzyme-linked fluorescent substrates, ELF, (Kloeke *et al.*, 1998; Nielsen *et al.*, 2002b). Some surface components can be determined by the binding of fluorescent lectins (Neu *et al.*, 2001), while surface properties can be assessed by microspheres adhesion to cells, MAC, (Zita and Hermansson, 1999; Nielsen *et al.*, 2001), where hydrophobic or hydrophilic microbeads may attach to the various bacteria in the floc. In this way it has been observed that both filamentous bacteria and the microcolony-forming bacteria exhibit a very large heterogeneity in physiology, exoenzyme activity and surface hydrophobicity in activated sludge flocs. However, these functions are still at the very beginning to be linked to bacterial species.

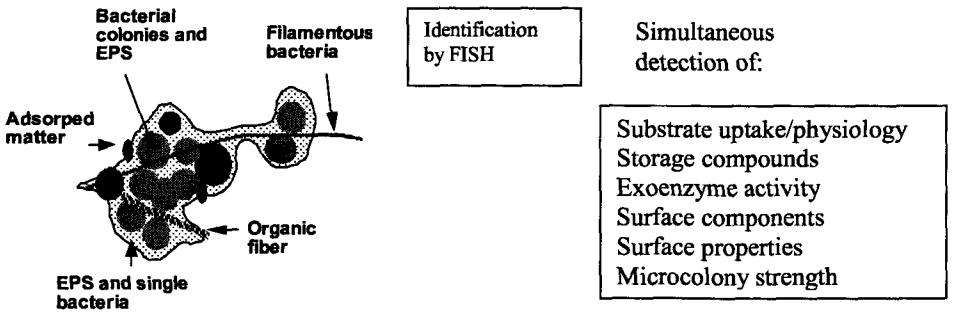

Figure 1. Approach to link identification of dominant bacteria in activated sludge with their function as studied by in situ methods.

All these methods can be used to gain information about the dominant bacteria in the sludge flocs. It does not give a complete description of the function of a specific bacterial species, but it can give some key properties that may be of significance for a specific sludge.

It is also possible to conduct various manipulations of the flocs, e.g. to expose them for settling, shear, variations in pH etc. and then identify the dominant bacteria that respond to the treatment (Klausen *et al.*, 2003). It can give important information about the properties of certain bacterial groups. In another study is has been found that poor and good settling flocs in a specific treatment plant are different in size and physical structure, and they are also composed of different bacterial

populations (Schmid *et al.*, 2003). Thus, all flocs do not have an identical microbial composition within the same plant, and this observation is important for our understanding of an "average" sludge floc.

Floc strength and deflocculation – an example

The strength and stability of activated sludge flocs are as mentioned above important for the flocculation, settling, dewaterability and effluent quality. Many factors determine the floc stability, and one key factor could be the presence or absence of oxygen as described by Wilén *et al.*, (2000a). They observed that removal of oxygen caused an immediate decrease in floc strength as could be measured as a deflocculation when shear was applied. We wondered whether this was due to specific bacterial groups or whether it was a deflocculation of an "average" part of the floc. Figure 2 shows the bacterial composition of microcolonies above 6 μm in diameter in the sludge before exposure to oxygen limitation and the deflocculation of sludge under anaerobic conditions. It shows that bacteria belonging to the *Beta-proteobacteria* were most abundant in the sludge investigated as is often found in activated sludge plants. The identification was carried out by FISH analysis with group-specific (and not species-specific) gene probes (Klausen *et al.*, 2003). Figure 3a shows the reduction in microcolony size of the individual groups after shear under aerobic conditions, and Figure 3b shows the same after shear under anaerobic conditions. We could clearly see that different microcolony-forming bacteria had a different sensitivity to shear. In particular, bacteria belonging to the *Alpha-proteobacteria*, *Bacteroides* and *Firmicutes* were very sensitive, while most microcolonies belonging to the *Beta-proteobacteria* were hardly affected by the shear applied. When the shear was applied under anaerobic conditions, other bacterial groups were affected as well. It was mainly some of the *Beta- and Delta-proteobacteria.* Whether they needed oxygen in order to stay in the floc or whether the anaerobic conditions stimulated their activity and release is not known at this stage. The very different responses from the different phylogenetic groups were surprising because each probe-defined group may contain many bacterial species. Thus, the results indicate that only few species were present in each group, or that all bacterial species within each group had very similar properties.

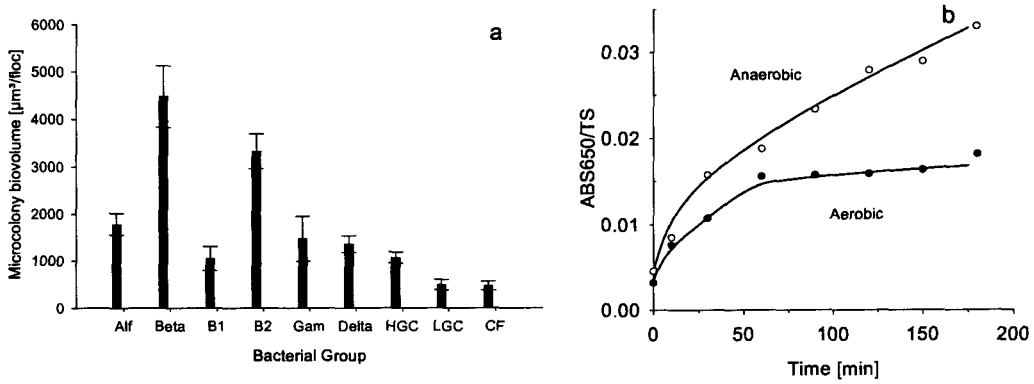

Figure 2. Bacterial composition of microcolonies above 6 μm in diameter in an average activated sludge floc from Aalborg East WWTP (a). Deflocculation of activated sludge under aerobic and anaerobic conditions as measured by change in turbidity after a strong centrifugation of the sludge leaving single bacteria in the supernatant (b) (Klausen, 2001).

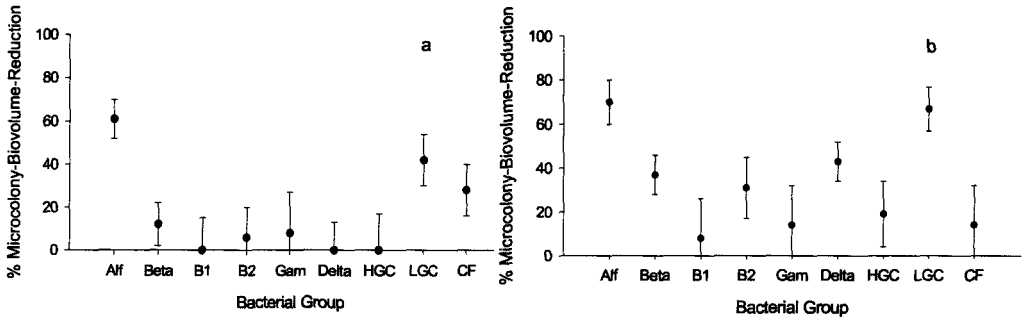

Figure 3. Reduction in biovolume of microcolonies in various bacterial groups after shear at aerobic conditions (a) and under anaerobic conditions (b). The results are given in % of an unsheared control (Klausen, 2001).

It is not known which species and how many species the applied group-specific gene probes covered in the sludge investigated, but the study clearly showed that different groups or species had different properties that could affect both floc and sludge properties in different ways. This means that the variations in the relative number of the different bacteria can give differences in floc strength and shear sensitivity. This was also observed in two industrial treatment plants, where the bacterial composition was quite different. The sludge with most bacterial groups forming strong microcolonies (related to the *Beta-proteobacteria*) also had the strongest flocs and thus appeared to reflect the differences in bacterial population composition (Klausen, Thomsen and Nielsen, unpublished results).

Conclusion and perspectives

The approach to combine identity with functional analysis of the dominant bacteria in activated sludge by in situ methods is in our opinion a very promising way to investigate correlations between presence of specific bacteria and floc and sludge properties that are of interest. Today there is only limited knowledge about the identity and physiology of the most important functional groups in the activated sludge process (e.g. nitrifiers and PAOs), and literally nothing is known about their floc-forming properties. In this study it could be observed that the various bacterial groups possess very different properties in terms of floc-formation, which determine floc structure and stability and thus sludge properties (e.g. settling and dewaterability). Future studies linking such knowledge together with treatment plant design and operation can provide important knowledge about key factors controlling floc and sludge properties in different types of wastewater treatment plants.

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