Polyphosphate Accumulating Organisms – recent advances in the microbiology of enhanced biological phosphorus removal

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Introduction

Eutrophication is a major problem that is characterized by excessive growth of algae, and consequently depletion of oxygen and fish death in water bodies. To prevent eutrophication in water bodies, nutrients such as phosphorus and nitrogen needs to be removed from the wastewater. Especially phosphorus is shown to be a limiting nutrient in freshwater bodies, therefore technologies that remove phosphorus from wastewater are of great interest. One such technology is called the enhanced biological phosphorus removal (EBPR) (Tchobanoglous et al. 2003).

Advantages by using EBPR, compared to chemical phosphorus removal, are mainly reduced chemical cost, lower sludge production and more reusable sludge product for agriculture as the phosphorus is more available for the plants (Janssen et al. 2002).

EBPR has been used in many wastewater treatment plants around the world with great success, but a lot questions regarding the microbiology of the process remain unanswered. Therefore EBPR has been intensely studied the last two decades.

Sometimes the plants are known to experience deterioration of the process. Sometimes the deterioration can be explained by external events, such that intensive rainfall, loss of nutrients, and similar events. In other cases, the deterioration could not be explained and a better understanding of the microbiology of the process is believed to help prevent future problems and increase the performance of EBPR (Oehmen et al. 2007, Seviour et al. 2003).

The study of EBPR systems has been interesting from an engineering perspective, but it has also been interesting from a microbiology perspective as a lot has also been learned about microbial communities and microbial ecology. The organisms mainly responsible for the EBPR process are called polyphosphate accumulating organisms (PAO), and one of the important PAOs identified has been named *Candidatus* Accumulibacter phosphatis (Hesselmann et al. 1999).

One group of organisms that are thought to cause deterioration of EBPR processes is called glycogen accumulating organisms (GAO). It has been shown that GAOs have a metabolism similar to PAOs, however they do not accumulate polyphosphates. GAOs are considered unwanted organisms because they do not contribute to P removal, and they compete with PAOs for the same carbon source. Therefore a lot of research activity has focused on the interactions between PAOs and GAOs (Oehmen et al. 2007, Seviour et al. 2003).

Questions still remains about the details of the metabolic pathways of the PAO, what is an optimal control strategy of EBPR systems, what other organisms can be classified as PAOs or GAOs and details about the interaction between GAOs and PAOs. This paper will give an overview over what has been learned during the last decade regarding these questions and discuss some further directions.

Overview of EBPR process and PAO

The EBPR process is an activated sludge process, and the basis of the process is to make the bacteria incorporate phosphorus into the biomass, and then the phosphorus is removed together with the wasted sludge in the clarifier. PAOs are able to form energy rich storage compounds called polyphosphates (polyP) in excess of normal cellular requirements under aerobic conditions. PAOs will proliferate if they are exposed to alternating anaerobic and aerobic conditions, since they are able to use their energy reserves that they build up in the aerobic phase in the anaerobic phase. The simplest form of EBPR can be shown in Figure 1. However many different configurations have been developed over the years, and usually the process configurations involve combined nitrogen and phosphorus removal. A review of different configurations can be found in Tchobanoglous et al. (2003).

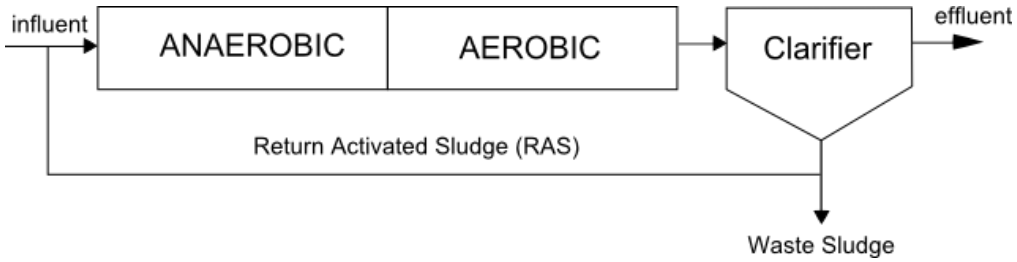

Figure 1 - Basic configuration for EBPR process (Janssen et al. 2002).

Biochemistry of EBPR and PAO

Various biochemical models have been proposed for the EBPR process, which tries to explain the metabolic transformations of PAOs. They agree on the major aspects, however they differ on certain important aspects and will be discussed later. The main biochemical transformations that are widely accepted in the literature are presented here. However, studies have identified *Tetrasphaera* –related PAOs that contribute to EBPR. These microorganisms seems to have a slightly different metabolism (Kong et al. 2005, Nguyen et al. 2011, Kristiansen et al. 2012).

For a simplified overview over the main mechanisms taking place in anaerobic and anoxic/aerobic PAO metabolism, see Figure 2. In the anaerobic zone, PAO utilize volatile fatty acids (VFAs), such as acetate or propionate, to form polyhydroxyalkanoates (PHAs), which is a carbon storage compound. The type of PHA that is formed depend on the type of VFA that is used by the PAO (Seviour et al. 2003). The VFAs are formed by fermentative bacteria in the anaerobic reactor, or already present in the influent. It can be beneficial to provide a prefermentation tank in the process configuration to enhance the levels of VFAs available in the anaerobic zone (Oehmen et al. 2007).

To obtain energy required for the synthesis of PHAs, the energy carrier adenosinetriphosphate (ATP) is needed. ATP is largely generated by the hydrolysis of the stored polyphosphates, but also by degradation of glycogen. As the polyphosphates are hydrolyzed, orthophosphates are formed and released to the liquid phase. For the synthesis of PHA, reducing power in the form of $NADH_2$ is also needed, and is provided mainly by the degradation of stored glycogen (Oehmen et al. 2007, Seviour et al. 2003, Yuan et al. 2012). The oxidation of VFAs by the full or partial tricarboxylic acid (TCA) cycle could also supply part of the reducing power (Zhou et al. 2010). On the left side of Figure 3, the relative changes of the concentrations of the participating compounds in the liquid phase and biomass are illustrated.

In the aerobic or anoxic zone, the PHAs that were formed in the anaerobic phase are oxidized to CO_2 by using oxygen or nitrate as an electron acceptor. The released energy is used for the formation of polyphosphate, glycogen and for growth of new cells (Oehmen et al. 2007). See the right side of Figure 3 for an illustration of the change in participating compounds in the aerobic zone. Because the amount of phosphorus that is accumulated by the PAOs in the aerobic zone is greater than the amount released in the anaerobic zone, a net phosphorus removal is achieved (Yuan et al. 2012).

It is generally accepted that at least a fraction of the PAOs are able to use nitrate as an electron acceptor, and therefore they are able to denitrify as they accumulate phosphorus (Oehmen et al. 2007). See section on denitrifying PAOs for more information.

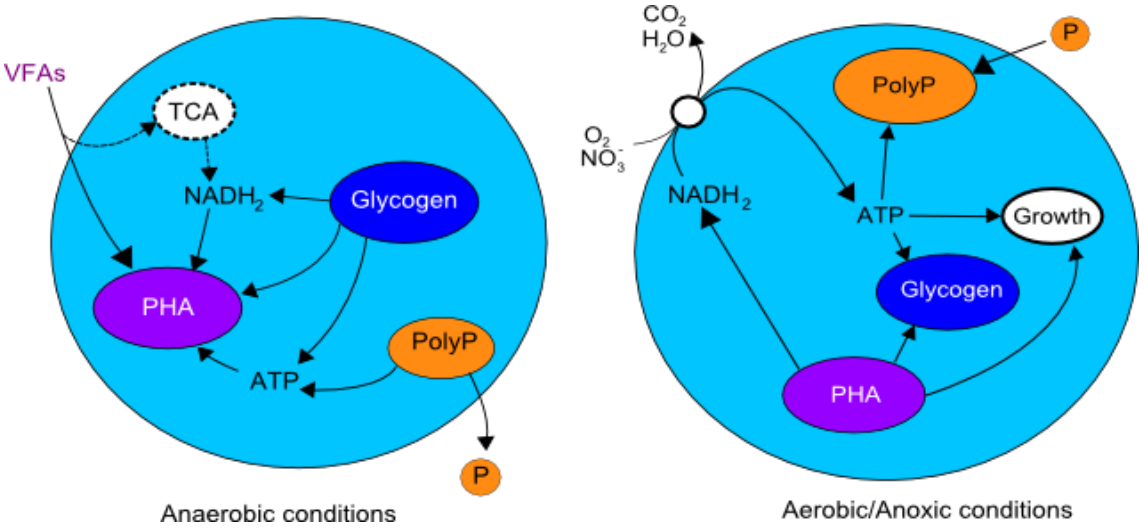

Figure 2 - Schematic diagram of anaerobic and aerobic PAO metabolism (Yuan et al. 2012).

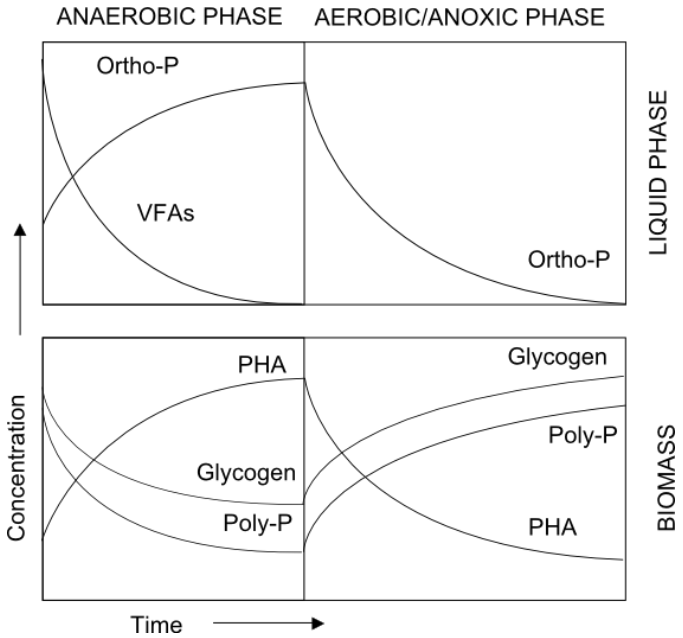

Figure 3 - Illustrative changes in the concentrations of the participating compounds in the anaerobic and aerobic reactor (Janssen et al. 2002).

TCA Cycle vs. Glycolysis as a source of reducing power

The main difference between the popular biochemical models for EBPR is the source of reducing power for PHA synthesis in the anaerobic metabolism of PAO (Zhou et al. 2010). It was first suggested that the TCA cycle was responsible for providing reducing equivalents (Comeau et al. 1986, Wentzel et al. 1986). Acetate was used as the model volatile fatty acid, and it was proposed that acetyl-CoA, which was formed from acetate, would be converted through the TCA cycle to provide $NADH_2$. However, Mino et al. (1987) suggested that degradation of glycogen by glycolysis would provide sufficient reducing equivalents for PHA synthesis. For the last decades, glycolysis has been accepted as the main source of reducing equivalents for the synthesis of PHA from VFAs (Zhou et al. 2010).

However, various lab studies have shown that PAOs are able to use both TCA cycle and glycolysis to provide reducing power, but still used glycolysis as the main source of reducing equivalents (Pijuan et al. 2008, Zhou et al. 2009). Few full scale studies of the activity of TCA cycle have been conducted, however a recent study (Lanham et al. 2013) showed that the TCA cycle plays an important part in full scale EBPR systems. It was found that limited glycogen availability promoted the activity of the TCA cycle, as was found by Zhou et al. (2009) as well. It was also found that EBPR systems with a higher activity of glycolysis showed a more efficient P removal, indicating that an important operational strategy could be to promote glycolysis.

Searching for PAO organisms

A lot of effort has been put in to the search for organisms that are responsible for EBPR. *Acinetobacter* was first proposed to be a PAO by Fuhs et al. (1975) as it was isolated from EBPR systems, and was long accepted as the main organism responsible for EBPR process. However, as new molecular techniques was used, such as fluorescent *in* situ hybridization (FISH), it was found that *Acinetobacter* compromised only a small part of the activated sludge in EBPR systems. After several studies it was concluded that *Acinetobacter* is not capable of performing the simultaneous VFA uptake and phosphorus release under anaerobic conditions, which is essential to the PAOs (Kang et al. 2013, Tandoi et al. 1998).

Many advances has been made in understanding the microbiology of EBPR and the PAOs, but one of the main challenges is that no one has managed to culture a microorganism that can perform EBPR. The importance of obtaining pure cultures that are able to perform EBPR has been emphasized by various authors (Kang et al. 2013, Seviour et al. 2003). Obtaining a pure culture of an EBPR organism is crucial as it would most likely reveal many of the unanswered questions about the metabolic pathways and regulation mechanisms of an EBPR organism.

Because of the unsuccessful attempts to obtain a pure EBPR culture, molecular culture independent techniques have been used to advance the knowledge of PAO. Among the important culture independent techniques that are used in the investigation of PAOs, it is worth to mention FISH, quinone profiles, microautoradiography (MAR) (Mara et al. 2003). It is beyond the scope of this article to describe the different techniques in details. However it is important to be aware of the purpose of the different techniques.

In studies of PAO and EPBR studies, FISH technique is used to quantify the different type of microorganisms that is present in the activated sludge. Quinone profiles are used to characterize microbial communities and monitor shift in populations. To identify active

microorganisms and their functions in microbial communities a combination of MAR and FISH has also been developed (MAR-FISH) (Nielsen et al. 1999).

By using FISH, two studies (Hesselmann et al. 1999, Crocetti et al. 2000) reported that *Rhodocyclus*-related organisms represented the majority of the cells in the activated sludge and the organism believed to be mainly responsible for EBPR was called *Candidatus* Accumulibacter phosphatis (from now referenced as *Accumulibacter*). It has later been demonstrated through a MAR-FISH study, that *Accumulibacter* are able to perform the metabolism that is typical for PAOs (Kong et al. 2004).

By using MAR-FISH technique, Kong et al. (2005) and Nguyen et al. (2011) were able to show that another group of microorganisms called *Tetrasphaera*, are present in EBPR in high numbers and are able to accumulate polyphosphate. A recent study proposed a metabolic model for *Tetrasphaera* organisms, and suggested that they have a different metabolism than *Accumulibacter* (Kristiansen et al. 2012). This model suggests that *Tetrasphaera* are able to perform fermentation of glucose, and use glycogen as a storage polymer. They also suggest that they are able to denitrify.

Denitrifying PAOs

Various studies have showed that some types of PAOs are able to use nitrate as an electron acceptor instead of oxygen (Kerrn-Jespersen et al. 1993, Kuba et al. 1993, Oehmen et al. 2010), and therefore simultaneous denitrification and phosphorus removal can be achieved. These have been called denitrifying polyphosphate accumulating organisms (DPAO). Denitrifying phosphorus removal is very attractive economically as it can reduce aeration costs, when nitrate can be used as an electron acceptor instead of oxygen. When using nitrate as an electron acceptor, the energy generation efficiency of DPAOs is generally lower so the cell yield is estimated to be 20-30% lower compared to when oxygen is used as an electron acceptor. This will lead to a lower sludge production (Kuba et al. 1994). It has also been found that the COD requirement can be lowered when using a configuration where anoxic phosphorus uptake is utilized (Kuba et al. 1996). All these benefits make a good argument for investigating the opportunity for simultaneous denitrification and phosphorus removal.

Even though denitrifying phosphorus removal can have various benefits, the anoxic phosphorus uptake has been observed to be generally lower, due to the lower energy generation efficiency when using nitrate as an electron acceptor. It is also argued that the lower phosphorus uptake is because only a fraction of PAOs are able to use nitrate (Oehmen et al. 2007, Oehmen et al. 2010). On this basis, it has been argued that EBPR systems with denitrification doesn't represent a significant advantage (Hu et al. 2002).

One of the main questions is whether PAOs and DPAOs are different microorganisms. An early study by Kerrn-Jespersen et al. (1993) suggested that PAOs and DPAOs are two different groups of microorganisms. Recent studies (Flowers et al. 2009, Oehmen et al. 2010) strongly suggested that only some clades of *Accumulibacter* are able to use nitrate as an electron acceptor, and therefore support the hypothesis of two different groups of PAOs.

Environmental factors affecting the EBPR process and competition between GAO and PAO

As mentioned in the introduction, competition between PAO and GAO is believed to be a possible cause of deterioration or sub optimal performance of an EBPR system. For

optimization of the EBPR system, it is crucial to understand how different environmental factors affect the metabolism of PAOs and the competition between GAO and PAO. The factors such as pH, temperature and carbon source has been shown to play a complex and interdependent role in the competition between PAOs and GAOs (Lopez-Vazquez et al. 2009, Oehmen et al. 2007). The most important results regarding the different environmental factors are presented here.

Temperature

In the literature, various authors have reported that a decrease in temperature can increase the performance of an EBPR system (Oehmen et al. 2007). This is different from conventional biological systems were an increase of the temperature results in an increase of the performance of the system because of increase in the biological kinetics. In various lab-studies, it has been shown that PAO is the dominating microorganism at lower temperatures, and this was observed together with an improved performance of the EBPR system (Erdal et al. 2003, Lopez-Vazquez et al. 2009, Panswad et al. 2003). It is important to note that Lopez-Vazquez et al. (2009) found that PAOs were the dominating microorganism at 10 ^{o}C regardless of carbon source or pH, which gives an indication that temperature is one of the most important factor for the competition between PAO and GAO. It has also been observed that PAOs are the dominating microorganisms between PAO and GAO at low temperature in full scale plants in Netherlands (López-Vázquez et al. 2008).

Carbon source and wastewater composition

The composition of the VFAs is essential to the competition between PAO and GAO. It has been shown that PAOs are able to efficiently use a mixture of acetate and propionate, while the known GAOs prefer a sole carbon source, such as only acetate or propionate (Lopez-Vazquez et al. 2009, Oehmen et al. 2007). Especially it has been demonstrated by lab studies that by alternating the carbon source between acetate and propionate it is possible to obtain a highly enriched culture of *Candidatus* Accumulibacter Phosphatis, were GAOs were out-competed (Lu et al. 2006). While this is useful for obtaining highly enriched cultures of PAO in lab studies, it most likely would be difficult to implement at full-scale plants. If a prefermenter is used to produce VFAs, it would be beneficial to control the operating conditions to vary the production of acetate and propionate (Oehmen et al. 2007).

pH

Through various studies it has been observed that a high pH (7.5 – 8) in the EBPR system is beneficial for the removal of phosphorus in EBPR system (Filipe et al. 2001, Oehmen et al. 2005). It has been observed that at a higher pH, PAO will have a competitive advantage against GAOs, and an increase in pH shifted the population to where PAO was the dominant microorganism (Oehmen et al. 2007).

Conclusion and future directions

Due to increased research efforts and the development of new molecular techniques, our knowledge of PAOs and their role in EBPR systems have increased immensely the last decades. The impact of environmental factors affecting the EBPR process has been well documented and the understanding of PAO-GAO competition has increased significantly.

Since microorganisms identified as *Tetrasphaera* seems to play an important role in EBPR, but have a slightly different metabolism than the current PAO model, further investigation is needed and possibly revision of current established PAO models.

Currently, no PAO has been obtained in pure culture, and this is preventing us from investigate many of the details regarding the genetics, physiology and biochemistry of a PAO organism. For instance, little is known about the regulatory mechanisms of *Accumulibacter* that makes this organism able to rapidly shift their metabolism in anaerobic and aerobic environments.

However much have been learned about the microbiology, now the challenge is to use this knowledge to increase the effectiveness and stability of EBPR systems.

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