The effect of media on evaluating the phosphate uptake capacity of activated sludge bacterial isolates

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Abstract

Since the identification of *Acinetobacter calcoaceticus* as the primary agent responsible for biological phosphorus removal, much research has been conducted to either confirm or refute this claim. The majority of these studies was conducted under defined laboratory conditions using various artificial media; as a result, many conflicting reports exist regarding the role of *A. calcoaceticus* and other activated sludge isolates in their possible roles as true polyphosphate-accumulating bacteria. This study was therefore conducted to determine the effect of media type on biological phosphate removal when performed in the laboratory under defined conditions. Five isolates were obtained from a full-scale biological nutrient removal activated sludge system and a previously identified *A. calcoaceticus* isolate was inoculated into three different media types. The inoculated media were subjected to anaerobic and aerobic conditions to stimulate the phosphate removal mechanism. The test micro-organisms released phosphate, under anaerobic conditions, in the three test media. Synthetic wastewater was found to promote the release of higher concentrations of phosphate by the micro-organisms than in the other two media. During the aerobic phase, phosphate uptake was highest in synthetic wastewater and settled sewage. Due to inconsistencies obtained in the readily biodegradable chemical oxygen demand, total chemical oxygen demand and soluble oxygen demand tests, it was concluded that the medium formulation may skew qualitative or quantitative data regarding biological phosphate removal. It can be concluded that the media used for P uptake studies are of importance as it was observed that the organisms' removal/uptake capabilities were enhanced or reduced depending on the media used.

Introduction

Enhanced biological phosphorus removal (EBPR), in an activated sludge system, is a process used to remove phosphorus from wastewater before being discharged into natural water bodies. Removal of phosphate (P) is of significance in preventing eutrophication (excessive algal growth and related negative consequences) of inland water systems. EBPR is achieved by mixing activated sludge, containing phosphate-accumulating organisms (PAOs) with the wastewater, and subjecting the activated sludge to alternating anaerobic and aerobic conditions (Fuhs and Chen, 1975).

Preconditioning of bacteria for P uptake in excess of what is deemed as normal metabolic requirements under aerobic conditions, occurs as activated sludge passes through the anaerobic zone situated at the head of the biological wastewater treatment systems. (Muyima et al., 1997). During anaerobiosis, PAOs take up short-chain fatty acids and store them intracellularly as polyhydroxy alkonates (PHA). Hydrolysis of polyphosphate (polyP) to orthophosphate, which is subsequently released to the external environment, and simultaneous degradation of intracellular glycogen are thought to provide the required energy for this process (Smolders et al., 1994; Nielsen et al., 1999).

The aerobic zone provides an environment in which P previously released in the anaerobic zone and P entering the plant in the feed sewage can be removed biologically from the biomass solution (Pitman, 1984). PAOs utilise stored PHA to take up soluble P to restore polyP and glycogen pools, during the aerobic phase (Nielsen et al., 1999). In most bacteria, polyP serves as a phosphorus pool during periods of P starvation. In some bacterial strains, polyP also acts as an energy source similar to organic storage products (PHB and glycogen). The role of polyP as intracellular energy storage material is decisive for the mechanism of EBPR, as well as for the metabolic selection of bacterial species in activated sludge systems within the anaerobic zone (Muyima et al., 1997).

Members of the *Acinetobacter* spp. were first proposed as the bacteria responsible for EBPR (Fuhs and Chen, 1975). Subsequently, many researchers reported the predominance of these organisms in EBPR processes based on culture-dependent identification methods (Lötter, 1985; Wentzel et al., 1986).

Isolation of PAOs has been reported to be problematic due to their slow growth rates. In environments with diverse microbial populations and limited organic resources, it is possible that PAOs are often out-competed by organisms capable of utilising available nutrients more rapidly. Another limiting factor in the isolation of PAO species from activated sludge is the lack of suitable media that would support the growth of all viable nutritional types (Muyima et al., 1997). It has been reported that isolated PAOs do not show the same characteristics in their P uptake capabilities under laboratory conditions, compared with full-scale *in situ* studies (Van Loosdrecht et al., 1997). A possible explanation could be that in their natural environment the organisms are continuously exposed to changes in critical factors such as the nature and concentration of substrates. However, laboratory conditions imposed on the organism may differ vastly from conditions in a full-scale EBPR plant. Therefore, micro-organisms may behave differently in the laboratory environment when compared to those in a full-scale EBPR activated sludge plant (Muyima and Cloete, 1995).

Many full-scale EBPR process plants have demonstrated the ability to reduce P concentrations to very low levels. However, studies done under defined and controlled laboratory conditions were unsuccessful (Nielsen et al., 1999; Cech and Hartman, 1990). In most laboratory experiments, batch tests are normally done to

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study a continuous system (as found in an EBPR plant). This could be one of the fundamental flaws of theses experiments. Failure of laboratory experiments could also perhaps be due to the type of media used in the batch tests, although no experimental evidence exists. Independent studies have shown what is presumably the identical wild type isolated from activated sludge to have vastly different P uptake capabilities under laboratory conditions. The one striking feature which differs in these bioassays is the use of different media to conduct the experiment Although mixed liquor is derived from the natural environment of PAOs, synthetic media of different formulations are popular for experimental study of PAOs, due to their controllable consistencies. Mixed liquor and synthetic media have been used in microbial P uptake research (Ohtake et al., 1985; Appeldoorn et al., 1992; Muyima and Cloete, 1995), but no conclusive studies were conducted to compare the effect of these various media types on bacterial response, or if a particular medium causes results to be inflated or exaggerated. The use of synthetic media and mixed liquor in different studies resulted in conflicting reports on the P uptake capabilities of PAOs isolated from activated sludge. Lack of information about the influence of different media types on P removal experimentation provided the motivation for this study. Therefore, this experimental study was conducted to determine the effect of different media types on P removal.

Experimental

Sampling and isolation

Activated sludge samples were obtained from the aerobic zone of Darvill Wastewater Works. It was assumed that PAOs in the aerobic zone would possess the required intercellular polyP needed to survive the aerobic starvation procedure, as the PAOs would have already accumulated P, thus the rationale for sampling from this zone (the presence of polyP granules was confirmed by Neisser stain**).** One litre mixed liquor was decanted into a conical flask (2 l) and agitated on an orbital shaker for 10 d at 25°C without supplementation of organic substrate. It was assumed that this would result in a decrease in non-PAO bacterial species simultaneously allowing the relative proportion of PAOs to increase due to intracellular energy stores (polyP) as found by Ubukata and Takii (1998). They stated that to simplify isolation of PAOs, activated sludge must be subjected to aerobic starvation for about one week, because under these conditions PAOs survive and other bacteria die, and the percentage of PAOs in mixed cultures increases. Soluble reactive phosphate (SRP) concentrations were measured using Merck phosphate test kits (Cat. No. 1.14842) and the Merck spectroqaunt Nova 60.

Following the period of aerobic starvation, 1 ml of sample was transferred, using the Gilson pipetteman, into a 2 ml poly - propylene micro-test tube containing 0.1 mm diameter glass beads. The sample was bead beaten (10 min at 5 000 r·min^{-1}) to disintegrate and disperse floc structures. Serial dilutions (10^{-1} to 10^{-8}) of the dispersed samples were prepared in 10 ml test tubes. Samples were aseptically inoculated on casitone glycerol yeast-autolysate agar (CGYA) using the spread plate technique. To obtain bacterial cultures, isolates were continuously subcultured on the basis of their predominance on the plates, colony morphology and pigmentation. Plates were incubated for 5 to 7 d at 20°C. Five isolates were randomly selected based on their ability to outgrow other organisms on the initial growth medium.

TABLE 1
Composition of synthetic wastewater as formulated by Appeldoorn et al. (1992)

Ingredients	g/l
NH_4Cl	0.32
$MgSO_4.7H_2O$	0.60
$CaCl_2.2H_2O$	0.007
K_2HPO_4	0.09
KH_2PO_4	0.05
Glucose	0.09
Trace solution	
$FeCl_3.6H_2O$	1.5
H_3BO_3	0.15
$CuSO_4.5H_2O$	0.03
KI	0.03
$MnCl_2.4H_2O$	0.12
$Na_2MoO_4.2H_2O$	0.06
$ZnSO_4.7H_2O$	0.12
$CoCl_2.2H_2O$	0.15

Phosphate uptake media

A mixed liquor sample (25 l) was obtained from the anaerobic zone at Darvill Wastewater Works. The mixed liquor (6 l) was allowed to settle for 2 h and centrifuged using a Beckman J6 - MC centrifuge for 20 min (3480 ×g). The pellet was discarded and the supernatant was filtered through Whatman No.1 filter paper. Supplements ($MgSO_4.7H_2O$; 0.5 g·l^{-1} and KNO_3; 0.18 g·l^{-1}) were added to the supernatant, to increase nutrient content. Settled sewage was obtained from the overflow of the primary clarifiers at Darvill Wastewater Works. The synthetic wastewater medium was formulated according to Appeldoorn et al. (1992) (Table1). Initial SRP concentrations were measured using Merck phosphate test kits (Cat. No. 1.14842) and the Merck spectroqaunt Nova 60. The pH of the different media was adjusted to ~ pH 7.0 using 2 M HCl or 2 M NaOH before autoclaving at 121°C for 15 min. Before inoculation mixed liquor and settled sewage were analysed for SRP and readily biodegradable chemical oxygen demand (RBCOD). Both were of sufficient quantities in the media to carry out the experiment, so no acetate or P sources needed to be added.

Phosphate uptake batch tests

Growth rates of the organisms under investigation were measured to determine the time required to reach a standardised phase of growth. This was done so that the test organisms would be in approximately the same phase of growth for P uptake studies. Each isolate, including the *Acinetobacter calcoaceticus,* was inoculated into 200 ml of Biolab nutrient broth and incubated for 24 h, at room temperature (~22 to 25°C) on an orbital shaker. After 24 h, 20 ml inoculum of each organism was transferred into fresh nutrient broth. Samples were taken every 30 min from each of the nutrient broth cultures, from the time of inoculation. The optical densities were recorded on the Pharmacia Biotech Ultrospec200 UV/VIS spectrophotometer at 600 nm. Results were used to plot a growth curve using Sigma plot (Fig. 1). From the growth curve it was

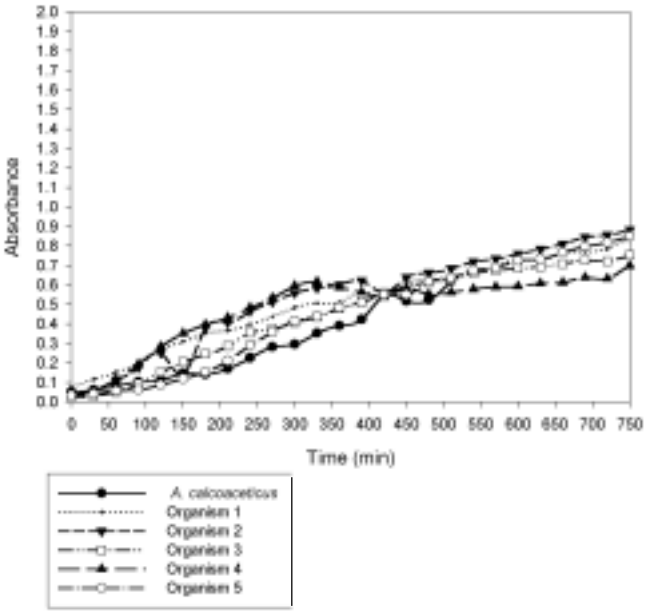

Figure 1
Growth curves of Acinetobacter calcoaceticus *and test isolates*

determined that it took 430 to 450 min (~ $7^1/_2$ h) for organisms to reach a standardised phase of growth.

Before P uptake batch tests the test organisms were initially grown as done for the growth curves. However, after the organisms were transferred into the fresh nutrient broth (after 24 h), they were allowed to grow for ~ $7^1/_2$ h.Upon reaching the desired point of growth (according to the constructed growth curves), the organisms were centrifuged. The supernatant was discarded and the biomass pellets retained. Seven Erlenmeyer flasks (500 ml) were used as batch reactors for each media type. The biomass pellets were then resuspended into the respective experimental medium (supplemented mixed liquor, synthetic wastewater and settled sewage), individually. The 7th flask for each medium was set up as a control, containing the medium only. Both the control and inoculated media were subjected to an anaerobic phase (4 h) prior to an aerobic phase (4 h). The flasks were agitated on an orbital shaker. Aerobic conditions were achieved through constant aeration with an air stone attached to an aquarium pump, while nitrogen gas was purged into the media (15 min) to achieve anaerobic conditions. SRP was quantified initially and at the end of the anaerobic and aerobic phases. The pH, during the experimentation, was monitored and maintained at 7.2 by addition of 2 M NaOH or 2 M HCl. Temperature during the experiment was about 22 to 25°C. Experimentation was repeated three times for each of the media with each organism. An average of the three results was used to plot the P release and uptake graphs. The P values had a margin error of about 2 to 3%.

Results and discussion

The results illustrated in Figs. 2 to 7 indicate varying degrees of P release and aerobic uptake by *A. calcoaceticus* and the isolates in the different media types. The initial bar in the figures represents the SRP present in the medium before experimentation. P release was calculated as the difference between the initial and the measured value at the end of the anaerobic phase. P uptake was calculated as the difference between the total P (initial + P released) and the measured value at the end of the aerobic phase. Initial P in mixed

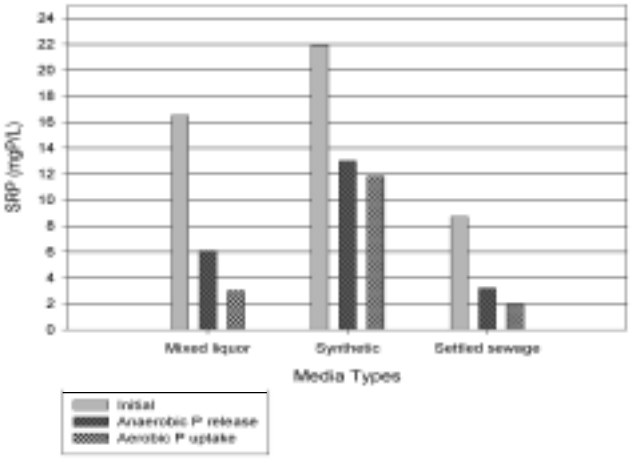

Figure 2
Phosphate release and uptake by Acinetobacter calcoaceticus *in three media types*

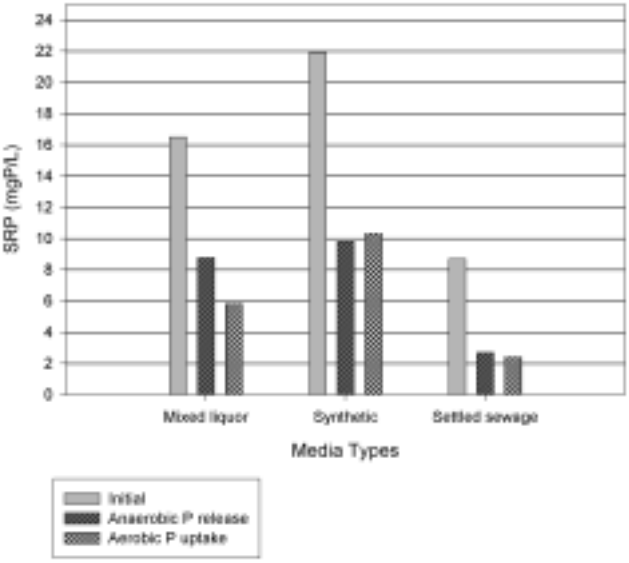

Figure 3
Phosphate release and uptake by Organism 1 in three media types

liquor was 16.5 mg P/l, in synthetic wastewater; 21.9 mg P/l and settled sewage; 8.7 mg P/l.

P release and uptake by *A. calcoaceticus* (positively identified from a previous study) in the different media types is shown in Fig. 2. The optimal P release occurred in synthetic wastewater (13 mg P/l) followed by mixed liquor (6.07 mg P/l) and settled sewage (3.2 mg P/l). P uptake was observed to have been best in synthetic wastewater (11.8 mg P/l). In mixed liquor 3 mg P/l was taken up while in settled sewage 1.9 mg P/l was removed.

Figure 3 shows the performance for Organism 1. P release was 9.8 mg P/l in synthetic wastewater, 8.8 mg P/l in mixed liquor and 2.7 mg P/l in settled sewage. In synthetic wastewater it was observed that complete removal of the P released as well as slight removal of the initial P occurred, i.e. 10.3 mg P/l. Relatively good removal was observed in mixed liquor and settled sewage, 5.8 mg P/l and 2.4 mg P/l respectively.

In Fig. 4, 12.3 mg P/l of P was released in synthetic wastewater by Organism 2. In mixed liquor and settled sewage, 6.4 mg P/l and

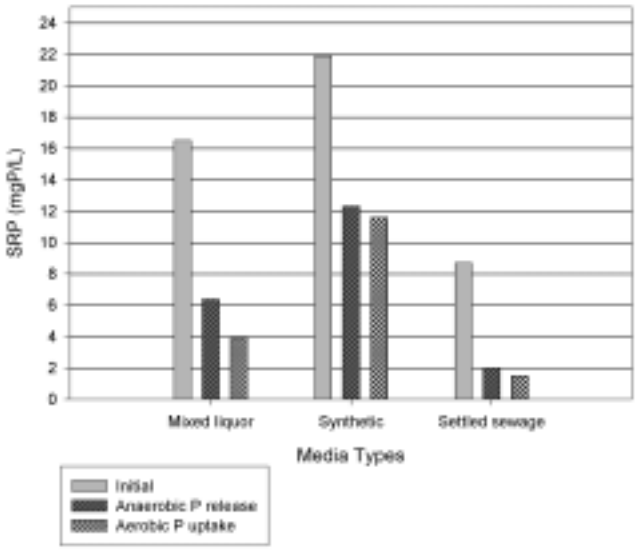

Figure 4
Phosphate release and uptake by Organism 2 in three media types

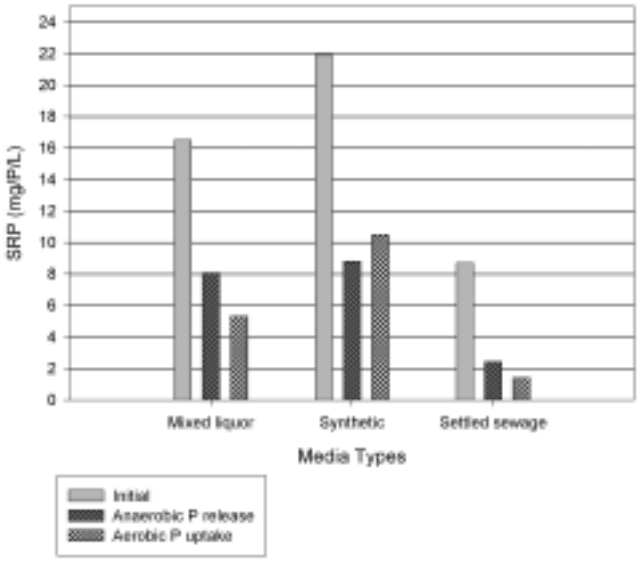

Figure 6
Phosphate release and uptake by Organism 4 in three media types

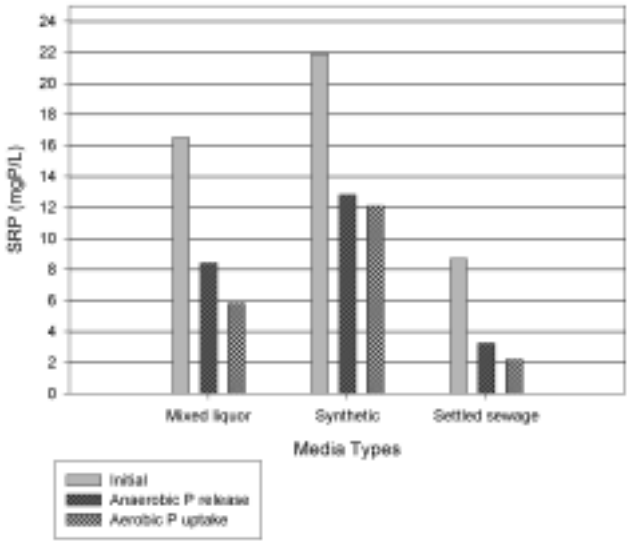

Figure 5
Phosphate release and uptake by Organism 3 in three media types

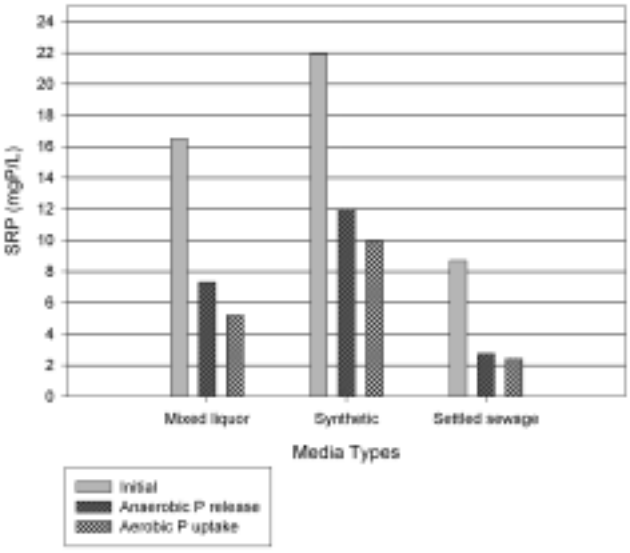

Figure 7
Phosphate release and uptake by Organism 5 in three media types

2 mg P/l of P were released, respectively. Extremely good uptake occurred in synthetic wastewater (11.6 mg P/l) and settled sewage (1.5 mg P/l), as practically most of the P released was removed. However, in mixed liquor 3.9 mg P/l of P was removed.

Figure 5 shows that Organism 3 had released 12.8 mg P/l of P into synthetic wastewater, 8.4 mg P/l into mixed liquor and 3.2 mg P/l into settled sewage. Excellent removal of P was observed in synthetic wastewater, where 12.1 mg P/l was taken up. Good P removal of 5.8 mg P/l and 2.2 mg P/l was observed in mixed liquor and settled sewage, respectively.

Although Organism 4 (Fig. 6) released a higher concentration of P in synthetic wastewater (12 mg P/l), the greatest P removal occurred in settled sewage. In settled sewage 2.4 mg P/l of P was taken up from 2.7 mg P/l of P that was released, while in synthetic wastewater 9.9 mg P/l was removed. Moderate P removal occurred in mixed liquor as 5.2 mg P/l of 7.3 mg P/l was removed.

Organism 5 displayed modest P removal in mixed liquor and settled sewage. Only 5.3 mg P/l was removed from 8.1 mg P/l released in mixed liquor while 1.4 mg P/l was removed from 2.4 mg P/l released in settled sewage. As with Organism 1, Organism 5 also was observed to have removed both released and influent P in synthetic wastewater; 8.8 mg P/l was released but 10.5 mg P/l was removed (Fig. 7).

It was assumed that micro-organisms would have shown best P uptake capabilities in either mixed liquor or settled sewage. This assumption was based on the fact that mixed liquor and settled sewage were sampled from a full - scale BNR plant and thus would be the media type ideally suited to micro-organisms isolated from

activated sludge. However, evidence from this study suggests that composition of synthetic wastewater catered for best P release and removal. This implies that the type of media used affects P removal capabilities of micro-organisms. Although release of P was better in mixed liquor than in settled sewage, better P uptake was evident in settled sewage. Therefore, it can be assumed that P uptake does not depend on the quantity of P released in the anaerobic zone. These findings contradict the report by Momba and Cloete (1996a). They reported, in studies done using mixed liquor, that excess P uptake was related to the amount of P released. When comparing P release using synthetic wastewater and mixed liquor, both showed a high degree of P release. However, when using synthetic wastewater as a medium, P removal capabilities were comparatively better

When using mixed liquor the findings could be considered to be more representative of the actual P removal capacities in the environment. Because this medium is obtained from wastewater of a full-scale BNR plant, it is considered to correspond closely to the isolates in the natural environment. Although mixed liquor should theoretically give the best indication of P activity, by its nature it is highly variable in composition (inhibiting factors may be present or absent at different times). This may be responsible for the modest P removal/uptake activity in mixed liquor and settled sewage. Although the composition of settled sewage allowed for low P release, almost all the organisms were able to remove most of the P that was released. In the batch assay the failure of mixed liquor to show optimum P removal can be explained as follows:

In a full-scale EBPR plant the activated sludge mixed liquor is continuously recycled until complete or near complete P removal has been achieved. This allows the activated sludge enough time to take up P in the wastewater. However, during this batch test the mixed liquor and micro-organisms were only run through one cycle. This may have prevented the micro-organisms from functioning in the mixed liquor batch test in the same manner as they would have done in a full-scale process.

When compared to a full-scale BNR plant another possible cause for lack of EBPR could be the reduced aeration rate during the aerobic phase. Pitman (1984) stated that excessive P should sometimes be avoided as it might prove difficult to take up all the released P in the aeration time available during the aerobic phase. This implies that mixed liquor requires longer aeration times, for better P uptake, than the other two media.

Synthetic wastewater was formulated to contain most of the nutrients generally found in domestic wastewater. In the mixed liquor some of the required nutrients may have been limited, due to the varying sources (domestic or industrial wastes) which make up this medium. Settled sewage may have also been nutrient-deficient because it was obtained from the clarifiers, which are found at the end of the EBPR process plant. Therefore most of the nutrients in treated wastewater would possibly have been utilised by the organisms in the EBPR process before reaching the clarifiers. The growth rate is of importance to P activity as a high concentration of P can be released or removed depending on the cells' growth phase. Momba et al. (1996b) observed that *Pseudomonas fluorescens* released P immediately on entering the mixed liquor, while in the logarithmic growth phase. They reported that the release of P was enhanced in the active phase of growth. Ohtake et al. (1985) reported that maximum P was removed when cells reached the stationary growth phase. This could explain modest removal in the natural media compared to the synthetic medium. This could imply that the nutrient composition of the media used for P uptake experimentation is of importance due to the fact that nutrient availability affects growth rates of organisms.

Based on investigations, it can be concluded that synthetic wastewater is not the ideal medium for comparing P removal capabilities of micro-organisms. The results could possibly be inflated due to the fact that synthetic wastewater is an artificial laboratory medium, containing nutrients needed to enhance growth which enhances P activity, and is a medium not found in a full-scale EBPR system. While cultures in synthetic medium might give a good indication of comparative P removal/uptake performance of different organisms, it should be noted that the organisms may not necessarily perform in the same manner under the conditions present in an actual wastewater treatment works. Therefore, future researchers using a synthetic medium to show P uptake capabilities of micro-organisms isolated from activated sludge, should be cautious.

It can be further concluded from these findings that the P uptake capabilities of isolates from activated sludge are affected by the type of media used.

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