OBSERVATIONS SUPPORTING PHOSPHATE REMOVAL BY BIOLOGICAL EXCESS UPTAKE — A REVIEW

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Abstract
The paper briefly reviews the development of the biological excess removal of phosphorus in the activated sludge process, from 1959 when it was first observed to the present. It concludes by proposing, tentatively, a biochemical mechanism whereby excess P uptake and release can be explained.

INTRODUCTION
From the first publication reporting phosphorus removal in excess of metabolic requirements in some activated sludge plants there has been controversy as to the mechanism whereby the excess removal is accomplished, whether the mechanism is a precipitation of inorganic compounds, albeit biologically mediated, or biological through metabolic formation and accumulation of phosphorus compounds in or on the organisms. This paper will discuss evidence that supports the hypothesis that excess phosphorus removal normally is via a biological mechanism. This does not imply that precipitation of inorganic phosphorus salts, due to chemical changes resulting from biological action, e.g. Alkalinity, Acidity and pH, does not take place. Such inorganic precipitation certainly can occur, but it would appear that in the treatment of municipal waste flows by an appropriately designed activated sludge process, within the normal ranges of pH, Alkalinity, Acidity and calcium concentrations in the influent, excess P removal is principally via a biological mechanism.

The types of evidence to be presented are of two kinds, direct and indirect. Direct evidence normally is explicit to the objective; whereas indirect evidence is inferred from behaviour analysed in terms of some hypothesis. However the fact that a particular hypothesis allows correct prediction of an outcome in a particular situation may provide support for the hypothesis, but does not constitute proof of it — other hypotheses may lead to the same prediction. Consequently an hypothesis gains credibility only by the consistency with which it predicts the outcome correctly over a spectrum of situations — much of the evidence favouring biological excess P removal is of the indirect variety.

The presentation is semi-historical; this not only provides a record of the evolution of a concept but also a convenient framework to evaluate the contributions of the various investigators.

CONCEPT EVOLUTION

Discovery
According to Levin and Shapiro (1965), it was Srinath, Sastry and Dilla (1959)
in India and Alarcon (1961) in America, who independently of each other, first drew attention to a phenomenon they observed in the activated sludge process: on occasion, removal of phosphorus (P) took place from the bulk liquid in excess of that indicated by normal metabolic requirements. These investigators concluded that in the plants where they observed excess removal, the magnitude of the removal appeared to be linked to the intensity of aeration. Tests on batches of mixed liquor indicated that if adequate aeration was provided, significant reductions in P, to near zero concentration were observed; however, if aeration was continued then with time a slow release of P back to the bulk of the liquid took place. Figure 1 shows the P uptake behaviour in a batch sample of mixed liquor under aeration, taken from Srinath et al. and is historic in recording the first evidence of biological excess P uptake in the activated sludge process.

Phosphorus Uptake and Release

The scientific father of biological excess P removal undoubtedly is Shapiro. In 1965 he and his pupil Levin reported on an extensive investigation into P uptake and release. As a basis for their investigation they went out from the hypothesis that the uptake is biological, mediated by the metabolic pathways normal to aerobic organisms, namely the sequence of the Embden-Meyerhof pathway and Krebs cycle, particularly the latter because P uptake takes place during aerobic conditions. Furthermore, they noted that in the literature it had been reported that some fungi, algae and bacteria stored phosphates in granular clusters called volutins; if P could be stored in this fashion then the carbon/phosphorus (C/P) ratio normal to microorganisms would not apply and excess uptake would find an acceptable explanation.

In their experimental investigation Levin and Shapiro (1965) obtained mixed liquor from the District of Columbia Sewage Treatment Plant, a "high rate" i.e. short sludge age plant. Samples aerated with, and without wastewater addition both exhibited P uptake, but the magnitude of the P uptake and its rate of uptake were higher for samples with wastewater addition than for ones without, indicating that the carbonaceous energy addition promoted uptake. If aeration was prolonged P release to the bulk liquid commenced, as previously observed by Alarcon (1961), indicating that the stored P was associated with the active fraction of the sludge – endogenous respiration reduces the active mass and the associated stored P will be released. In batch tests on two samples, of which one was aerated and the other not, the aerated one took up P while the unaerated one released P; this they

![Fig.1. P uptake behaviour of a batch of mixed liquor from a plant showing excess P removal. (Taken from Levin and Shapiro of data reported by Srinath et al. 1959).](https://iwaponline.com/wst/article-pdf/15/3-4/15/95470/15.pdf)
hypothesized could be explained by P uptake for ATP formation by oxidative phosphorylation in the (aerobic) Krebs cycle with oxygen as electron acceptor. In contrast the Embden-Meyerhof pathway, which remains operative under "anoxic" (anaerobic)* conditions, with substrate phosphorylation, did not appear to be implicated in the uptake because P was released under anoxic (anaerobic) conditions. However Levin and Shapiro offer no comment on the possible causes for the release. If the Krebs cycle is implicated in uptake then, they reasoned, (1) the oxygen tension in the mixed liquor and (2) poisons that inhibit oxidative phosphorylation (during aeration) should effect P uptake and they accordingly investigated these.

(1) Oxygen tension: Batch experiments at different aeration rates indeed showed that at low rates the P uptake rate was correspondingly low but as the aeration rate increased so uptake rate improved, but at a diminishing tempo to some plateau. From this it can be speculated that inadequate aeration should adversely affect P uptake but aeration beyond a certain rate would not lead to improvement in uptake. However, high aeration intensity should strip CO2 and the consequential rise in pH may cause calcium phosphate to precipitate. To check if high aeration rate did not perhaps promote precipitation, Levin and Shapiro aerated five batches of mixed liquor in which the pH was controlled to remain at 5, 6, 7, 8 and 9, respectively. The results from the batches at the higher pH values are of particular interest: at pH 7 and 8 the uptake rate was rapid whereas at pH 9 the uptake rate was significantly lower. Normally one would expect that if precipitation was due to solid calcium phosphate formation, the rate would be higher at pH 9 than at pH 8; observing the opposite it was concluded that the mechanism was not precipitation.**

(2) Inhibition: With regard to the effects of poisons or inhibiting substances it is well known that 2,4-dinitrophenol inhibits oxidative phosphorylation*** Levin and Shapiro found that on aerating mixed liquor samples with and without dinitrophenol, the sample without the phenol showed hardly any uptake whereas the sample with the phenol took up all the dissolved P. This indicated that when the Krebs cycle is inhibited P uptake does not occur suggesting that the mechanism is biological.

To Levin and Shapiro must be accorded the credit for hypothesizing a biological mechanism for P uptake and providing the first experimental data supporting this concept.

The pioneering work of Levin and Shapiro has been verified and extended by a number of investigators. Sekikawa, Nishikawa, Okazaki and Kato (1966) found that the soluble phosphate in the bulk liquid increased if (i) there is a deficiency of "nutrients" (carbon energy) i.e. when the sample is aerated for a long period of time without COD addition; (ii) aeration is withheld i.e. anaerobic conditions are established and (iii) a poisonous substance such as K2CrO4 is added to the mixed liquor****

* The terms anoxic and anaerobic have acquired a usage in sanitary engineering that differs from that in bacteriology. In sanitary engineering anoxic implies absence of oxygen but presence of nitrate; anaerobic implies absence of both oxygen and nitrate. In bacteriology the term anoxic is not common and the term anaerobic implies absence of oxygen and includes the condition where nitrate is present. In this paper the sanitary usage will apply and when quoting from bacteriological papers the sanitary terms will be added in brackets after the bacteriological ones.

** There is evidence of a species of calcium phosphate precipitant with a low solubility product that forms at pH 7 to 8 but its rate of formation is extremely slow in the presence of organic material.

*** but not reductive phosphorylation.

**** These findings appear to have been obtained independent of Levin and Shapiro although this is not certain.
The inhibitory effect of 2,4-dinitrophenol on P uptake has been repeatedly duplicated by later workers, for example, Yall, Broughton, Knudsen and Sinclair (1970), Fuhs and Chen (1975) and Rensink, Donker and de Vries (1981). In general, in batch tests taken from the reactor showing release if the phenol is added at 1 to 2 mM/l and aerated, either uptake is inhibited or there may be a release of P during aeration; this is dramatically illustrated by the plots in Fig.2 taken from Rensink et al.

The conclusion of Levin and Shapiro, that uptake is not physical-chemical, has been checked by a number of investigators:

(i) Yall et al. (1970) studied the removal of P by monitoring radio active 32P and 45Ca additions to their experiments. Their conclusions, inter alia, were that calcium phosphate precipitation played a minor role in the excess P uptake observed in their experiments.

(ii) Barnard (1976) in an experiment in which the pH in a Bardenpho process was raised, found no significant increase in excess P removal* as would be expected if a physical-chemical mechanism was operative.

(iii) Hoffman and Marais (1977) set up two identical 2-reactor series configuration laboratory scale activated sludge plants, the one operated anoxic-aerobic, (i.e. nitrate was present in the anoxic zone), the other aerobic-aerobic, and ran batch tests on the mixed liquors from these plants: In the batch test on the aerobic-aerobic mixed liquor, by raising the pH to 7.3 and then lowering it to 6.0 they found that on raising the pH both the concentrations of Ca and P were reduced in the bulk liquid in the approximate molar ratio of [Ca]/[P] = 3 and on

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*In this paper uptake refers to the reduction in P in a batch test or in a reactor whereas removal refers to the reduction achieved by a continuous process, between the influent and the effluent. In a continuous process it is conceivable that in different reactors there can be uptake and release without removal being accomplished by the process.
lowering the pH, Ca and P increased again in the same ratio. They concluded that the phenomena probably were precipitation and dissolution respectively of a calcium phosphate mineral, although the Ca/P ratio did not conform to any of the commonly known calcium phosphate minerals. By making mass balances of P across the continuous processes, taking due account of P requirements for basic metabolic requirements and for precipitation as noted in the batch tests, it was possible to account for the P removal in the aerobic-aerobic system but not in the anoxic-aerobic system. The latter system always gave the higher P removal irrespective of whether the plants were run at pH 7,3 or 6,0 and the \([\text{Ca}]/[\text{P}]\) was always very much less than 3. Furthermore it was noted at times that when the pH of a system was lowered suddenly from pH 7,3 to 6 the Ca concentration in the effluent temporarily could exceed that of the influent and concomitantly the P removal temporarily was reduced; this behaviour pattern indicated that calcium phosphate that had precipitated at the higher pH, dissolved at the lower pH. In general however, for this influent (low Alkalinity and \(\text{Ca} = 40 \text{ mg/l}\)) calcium phosphate precipitation at pH 7 could account for only about 1 mg P/l removed; the major removal was judged to be due to the biological excess phenomena.*

(iv) Rensink et al. (1981) clearly demonstrated the complete lack of association between Ca and P in plants giving excess P removal attributable to the biological mechanism. They operated a 10 in-series reactor activated sludge plant with the first 5 reactors not aerated and the underflow recycle discharging to the first reactor. The plant was operated at a low sludge age of 5,3 days, at 14°C, and no nitrification was observed so that the first 5 reactors were anaerobic. Plots of the concentrations of phosphorus, calcium and magnesium in the bulk liquid are shown in Fig.3. Evidently the increase and decrease of soluble P was completely independent of the concentration of Ca so that the behaviour cannot be ascribed to dissolution and precipitation of phosphate minerals.

In 1967 Shapiro, and Shapiro, Levin and Zea (1967) focussed attention on the P release aspect under anaerobic conditions. They (1) verified the earlier findings of Levin and Shapiro (1965) that although there was P uptake during aeration, P was again released during "anoxic" (anaerobic) conditions, i.e. uptake and release were reversible; (2) found that the release apparently was triggered by a lack of oxygen and/or a low redox potential, but, of these two they concluded that release appeared to be controlled more by the redox potential than the oxygen tension - Shapiro observed that rapid release appeared to be triggered off once the redox potential fell to \(-150 \text{ mV}\); (3) found that in batch tests the P appeared to be released from the acid-soluble fraction of the sludge cells and to a minimal degree from the RNA and DNA protein fractions, in general the total protein was hardly affected; (4) found that during anaerobiosis, apart from the observed P release, the BOD of the liquid medium hardly changed, soluble TKN also showed no change for the first 40 minutes but thereafter increased rapidly, mostly in the NH3 form. The behaviour of these three parameters during a batch test is plotted in Fig. 4.

There are several aspects of the above observations that merit discussion -

* It is of interest here to note that irrespective of whether the conditions in the "anoxic" reactor are anaerobic or anoxic some excess P removal always will be obtained. Generally if the system is aerobic-aerobic the P concentration in the active mass is about 3 percent, if anoxic-aerobic about 6 percent and if anaerobic-aerobic (provided a number of other conditions are satisfied, see later) can range from 6 to 35 percent. The concentration of P in endogenous residue and in inert volatile material in the influent, is about 1,5 percent.
Fig. 3. P and Ca behaviour in a 10 series reactor system with the first 5 reactors anaerobic to illustrate lack of association of P and Ca. (Taken from Resink et al. 1981).

Fig. 4. Changes in soluble BOD, TKN and P in a batch under unaerated conditions. (Taken from Shapiro, 1967).
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Fig. 5. Phosphorus uptake and release in a batch test under alternating aerated and unaerated periods. Sludge sample taken from a high rate plant (after Wells, 1969).

(i) **Reversibility of uptake and release**: Wells (1969) using batches of mixed liquor from the San Antonio plant (which exhibited P removal) verified that if a batch is sequentially aerated during the day and left unaerated during the night, phosphorus was taken up during the day and released during the night, but, that the uptake decreased progressively (Fig. 5). If one plots the P uptake each day versus the relative active mass for that day, calculated by means of the activated sludge kinetic theory of Dold, Ekama and Marais (1981), one has the interesting result that the uptake appears to be linearly related to the active mass—a behaviour pattern more in keeping with biological mechanism than a physical-chemical one, and indicating that the biological uptake is limited by the active mass even though the factors controlling the magnitude of the uptake are not identified (see Fig. 6).

(ii) **Redox potential and oxygen**: Redox potential as parameter controlling P release has a deceptive persuasiveness about it. In samples from long sludge age plants, P is not immediately released when dissolved oxygen becomes zero, release may only commence after a considerable time has elapsed. In contrast, in samples spiked with influent, release is virtually immediate when the dissolved oxygen becomes zero. Consequently, the reasoning is that it is not the dissolved oxygen *per se* but the redox potential that needs to be depressed to a sufficiently low value before P is released even though the mechanism of release is not described. Barnard (1975a) and Siebritz, Ekama and Marais (1980) followed Shapiro *et al.* in accepting this explanation for P release. In order to test this hypothesis, one problem is that reliable measurement of redox potential in biological systems is difficult; as a consequence the hypothesis has not been conclusively proved or disproved. To bypass the difficulties with direct measurement, Siebritz *et al.* (1980) substituted the redox potential by a parameter called the anaerobic capacity; this parameter is defined as the mass of nitrate that can be removed in an anaerobic reactor if sufficient nitrate is available. From this definition the anaerobic potential is an extensive parameter whereas redox potential is an intensive one; replacing the redox potential by the anaerobic potential is analogous to replacing pH by the total alkalinity. Such a replacement is allowable for pH under
restricted conditions only and this will be true also for the redox potential, but the conditions which allow such a substitution are not clear. However Siebritz, Ekama and Marais (1980) found that the anaerobic capacity was not a consistent parameter and was only of restricted use in predicting P release. (see later).

Randal, Marshall and King in 1969, specifically investigated the connection between redox potential and P release and concluded that there is no evidence supporting the hypothesis that the redox potential controls the release. From spiked batch tests they found that in all cases but one, release always took place immediately the dissolved oxygen became zero, but a reduction in the redox potential lagged by about 40 to 60 minutes; they concluded that the redox potential is only weakly linked to P release. The late changes in redox potential, observed by Randal et al. very likely are due to the commencement of "fermentation", in which CO₂ or perhaps H⁺ in the bulk liquid commences to serve as electron acceptor and thereby giving rise to a change in the redox potential evident in the bulk liquid - it would appear that the causes for P release must be sought inside the organism, as will be discussed later.

(iii) Insensitivity of soluble BOD: The insensitivity of the soluble BOD under anaerobic conditions as reported by Shapiro is not unexpected. Dold, Ekama and Marais (1980) in their death-regeneration model for bacterial metabolism concluded that during death of organisms the lysed products are particulate and become enmeshed in the sludge mass or adsorbed onto the active mass fraction; consequently these products will not be reflected in a COD or BOD test conducted on a filtered sample. The fact that Shapiro reported that in the filtered supernatant the NH₃ increased after about 40 minutes (Fig.4) indicates that the particulate TKN (or protein) set free by the death and lyses of the organisms commenced to be broken down to NH₃ by anaerobic action, and/or direct anaerobic destruction of some organism started to occur due to a qualitative shift in the population from facultative to anaerobic -

![Fig.6. P uptake versus relative active mass as a percentage of the initial active mass as from the batch test results shown in Fig.5.](image1)

![Fig.7. Typical orthophosphate and dissolved oxygen concentration profiles along the length of a full scale plug flow activated sludge process (after Scalf et al., 1969).](image2)
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Subsequent research by Shapiro and Levin was oriented to the development of their Phostrip method and does not contribute materially to the development of the biological excess P removal concept.

Phosphorus accumulating organisms

In 1975 Fuhs and Chen presented the results of an intensive investigation into excess P removal undertaken from 1973. It would appear that they went out from the hypothesis that (1) if excess removal is biological then some organism(s) in the sludge mass must be capable of accumulating the phosphorus, and (2) an anaerobic-aerobic sequence is important in some, as yet, undefined manner.

In preliminary work they utilized an aerobic activated sludge laboratory unit which had been run for a number of years on an artificially constituted influent substrate. They converted the operation to run on an anaerobic-aerobic cycle for two weeks but could obtain no evidence of excess P removal. On examining the organisms in the sludge microscopically after suitable straining they concluded that this behaviour was not unexpected as only few of the organisms in the mass were capable of storing polyphosphates. Thereupon they studied samples of activated sludge from the Black River Treatment Plant of Baltimore and the Seneca Falls Plant as both these plants exhibited excess P removal. The samples of sludge were stored unaerated the night after collection and the following day unsettled influent from the respective plants was added to the respective batch samples of sludges, and aerated for 4 hours. (The reported mass of sewage added indicated a very high COD load per unit mass of sludge). A dramatic decrease in phosphorus, from about 32 to near zero mg P/ℓ, was observed over the 4 hours. Subsequent storage of the batch unaerated for 20 hours caused release to about 40 mg/ℓ. Parallel tests, in which the one test sample was spiked with 2 mM/ℓ 2,4-dinitrophenol, indicated virtually complete inhibition of the uptake mechanism during aeration. Microscopic examination of the sludge mass during aeration indicated the presence of intercellular accumulations of inorganic polyphosphates in some bacteria; during the subsequent 20 hour anaerobic period the inclusions disappeared and the P in the bulk liquid increased. Repeated cycles of aeration (4 h) and anaerobiosis (20 h) indicated that uptake and release were associated; when P release was high so was the subsequent uptake, when P release was indifferent or zero (even after 20 h of anaerobiosis) so also was the subsequent uptake. Generally P release and uptake decreased as the number of cycles increased.

Microscopic study of the P accumulating organisms indicated that the accumulations were specific to one morphological bacterium type. From a series of identification tests Fuhs and Chen concluded that the organism associated with the phosphorus accumulations belonged to the Actinetobacter genus. Pure batch cultures of this organism fed on acetate when subjected to the anaerobic-aerobic cycle gave rise to a behaviour pattern very similar to that with the mixed liquor sludge samples. By the third cycle no release was obtained during the anaerobic period and under the subsequent aerobic period a slow release was observed. However when acetate was added during the final aerobic period, rapid uptake of P again took place.

The eventual slow release even under aerobic conditions is not unexpected; elsewhere it has been noted, in Fig. 5, that the uptake and release decrease as the number of cycles increases. If continued long enough then, consistently, little or no release and uptake should be observed within the cycle sequence; the release under aerobic conditions then could be accounted for by a greater loss of P due to endogenous conditions than due to excess uptake. With regard to the P uptake on addition of acetate, if one accepts that release and uptake are linked, as surmised by Fuhs and Chen, then the uptake finds explanation in that the preceding anaerobic period "conditioned" the sludge mass for uptake when a carbon energy source became available. (It
should be noted that the P uptake on acetate addition is possibly magnified due to the pure culture organism mass; in mixed cultures the effect would be much smaller due to competition for the acetate by other organisms).

Assessing Fuhs and Chen's work the following important aspects must be credited to them: (1) Although not explicitly stated they were the first to surmise a possible link between P release and subsequent P uptake; (2) they implicated an organism group, the Acinetobacter genus, as responsible for the P uptake and release; (3) they suggested that the growth of the Acinetobacter was as a result of the anaerobic-aerobic sequence; in their own words "Anaerobic conditions preceding aerobiosis in sewage treatment .... could well be related to the appearance of Acinetobacter", and (4) they speculated on a biochemical model that explained the growth of the Acinetobacter in a more explicit fashion than Shapiro. Again in their own words: "The principal function of the anaerobic treatment .... is to establish a facultatively anaerobic microflora. .... During anaerobiosis this flora would tend to produce compounds such as ethanol, acetate and succinate which serve as carbon source for Acinetobacter .... without anaerobiosis, an obligately aerobic assemblage is likely to develop; and the intermediate products would not be formed. Acinetobacter, which cannot attack sugars* or polysaccharides and are likely to be subject to heavy competition for the utilization of amino acids, would not then develop."

Their proposed mechanism (4 above) however is open to reservations: Their argument, that the function of the anaerobic period is to produce intermediates which can be utilized by the Acinetobacter in the aerobic phase, does not explain why the Acinetobacter appear to grow preferentially in anaerobic-aerobic system - these organisms on entering the aerobic phase would still be subject to the same competition with other organisms as if these intermediates were added from external sources to an aerobic system. Their argument also does not explain why an anaerobic-aerobic sequence appears to be essential for P uptake. Under steady state aerobic conditions Harold (1966), for example, found that Aerobacter Aerogenes (an organism that also can accumulate phosphorus) accumulated P only minimally, so that it would appear that some stress situation in the anaerobic-aerobic cycle triggered the P release and uptake. However despite these unresolved questions Fuhs and Chen did clearly demonstrate that the cycles of anaerobic-aerobic phases gave rise to a sludge with specific organisms for P uptake and release, even though the reasons for this remained unknown.

It is of interest to note that in 1967 Wells already came to the conclusion that the sludge in plants giving excess P removal differed from those in plants that did not. He states: "The rapid uptake of phosphates by the Rilling Road plant sludge and the almost complete absence of uptake by the East and West plant sludges when treated under identical conditions, indicate a fundamental difference in the nature of the sludge itself over and above plant differences which produced the sludge".

Osborn and Nicholls (1977) pointed out that besides Acinetobacter a large number of organisms capable of accumulating P have been reported in the literature. Furthermore in P removal plants, as designed in South Africa, it has been repeatedly observed that considerable P is taken up in the anoxic reactor receiving the outflow from the anaerobic reactor; Acinetobacter being an obligate aerobe should find the anoxic reactor quite unfavourable for growth thereby suggesting that the facultative denitrifying bacteria might also include P accumulating organisms and play a role in P removal.

Despite the difficulties in explaining the presence of the Acinetobacter

*For example, Acinetobacter cannot utilize glucose, a most readily biodegradable substrate.
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there is little doubt that this organism is an important one in P excess uptake. Osborn and Nicholls (1977) studying activated sludge plants in the Johannesburg area noted the presence of Acinetobacter type organisms in all the plants having an anaerobic zone. Rensink et al. (1981) found the P accumulating organisms increased under anaerobic-aerobic plant operation with consequential increases in P removal.

Buchan (1981) investigated the morphology of the phosphate-accumulating organisms and the chemical composition of phosphorus accumulates. From mixed liquor samples taken from the Brits, Goudkoppie, Umhlatuzana and laboratory scale units all showing excess P removal, he positively identified that these processes all contained organisms of the Acinetobacter group. To investigate whether this bacterium group is in fact able to accumulate large amounts of phosphorus, he grew pure cultures of species of this group under batch test conditions. His results indicated that the Acinetobacter can accumulate P in excess of 30% of the organism mass. Ohsumi, Shoda and Uduka (1980), Shoda, Ohsumi and Uduka (1980) and Lawson and Tomhazy (1980) all investigated pure cultures of the Acinetobacter group and all found that the group can accumulate phosphorus in excess of 20 percent by mass.

Lawson and Tomhazy (1980) and Buchan (1981), utilizing a scanning electron microscope confirmed the findings of Harold (1966) that metachromatic cell clusters (volutin) were rich in phosphorus. Buchan noted a consistent association of phosphorus and calcium in the phosphate-rich inclusions indicating that calcium may be an essential requirement for biological phosphate accumulation so that calcium is important not only for inorganic calcium phosphate precipitation but also for biological excess P uptake.

Phosphorus Release Prerequisite

In the late 1960's and early 1970's considerable data were collected on full scale plants exhibiting excess P removal, for example, by Vacker, Connel and Wells (1967), Scalf, Pfeiffer, Lively, Witherow and Priesing (1969), Witherow (1970) and Millbury, McCauley and Hawthorne (1971). All these plants were of the conventional type i.e. with long plug flow reactors, graduated aeration from the inlet to outlet, and all were operated under high loading rates or equivalently very short sludge ages, from 1½ to 6 days. Underflow recycle ratios (where these are given) ranged from 0,25:1 to 0,5:1. Dissolved P profiles along the reactor axis characteristically showed P values well in excess of those in the influent near the influent end (indicating release) up to the point where the oxygen concentration becomes positive, whereafter there was a rapid drop in P (indicating uptake) up to the effluent end, see Fig.7. It was generally accepted that the release was due to a lack of oxygen arising from the high oxygen demand near the influent end, but no special significance was attached to this beyond the possible reduction in P removal by the process if release should commence in the secondary clarifier. The reports principally focussed attention on the uptake of P, and in particular the effect of aeration intensity at the effluent end on the rate of uptake; Milbury et al. for example noted that reverse aeration, i.e. higher intensity aeration at the effluent than at the influent end produced improved P removal. However, the plug flow régime per se was recognized as conducive to uptake and Milbury et al. accordingly recommended length: width ratios of 25:1 in design of the reactor. Step feeding to the reactor positively was to be avoided; when operated with step feeding Milbury et al. observed a rapid loss of the P removal propensity. As step feeding provides a mixing régime approaching that of complete mixing, completely mixed régimes accordingly also were not recommended. A further observation of note was that the filtrate of digested sludge from these plants needed to be treated to remove P prior to returning it to the head of the works for further treatment; if this was not done the plants lost their P removal propensity even though release and uptake continued as
before. This observation is important in that it indicates that the sludge has a limited P accumulation propensity, an observation that lends support to the biological as against the physical-chemical excess P uptake hypothesis.

The observations on the behaviour of these full scale plants were of significant importance in the evolution of the excess P removal concept. Descriptions of the plant behaviour generally displayed sharp insight; although they did not immediately lead to basic solutions, they influenced all subsequent investigations and served, and serve still, as measures against which various hypotheses on excess P removal can be evaluated.

The first surmise that P release may be a prerequisite to excess P uptake was, as we have seen in the previous section, due to Fuh and Chen. However it is to Barnard (1975, 1976) that an explicit statement to this effect is due. He hypothesized that, for excess P uptake, the mixed liquor needs to be subject to an anaerobic state at some point in the process of such intensity that P release is obtained, then, if the mixed liquor is adequately aerated, P uptake in the aerated reactor, and P removal by the process, will be obtained. Barnard came to this hypothesis while testing a 4 reactor Bardenpho (nitrification-denitrification) pilot plant with nominal reactor retention times of 2 h primary anoxic, 6 h aerobic, 3 h secondary anoxic and 1 h reaeration respectively, operated at an unspecified long sludge age with a 4:1 mixed recycle ratio and 1:1 underflow recycle ratio. With an influent total P concentration of 9 to 12 mg/L he noted that the filtered P in the 4 reactors typically were 2,5; 2,5; 30 and 0,3 mg/L indicating a massive release in the second anoxic reactor and a massive uptake in the reaeration reactor.* He also established that the magnitude of the P removal was very adversely affected by nitrate; as nitrate in the effluent increased so the P removal declined. The confounding effect of nitrate first noted by Barnard henceforth was to be a constant source of difficulty in all investigations into nitrification-denitrification-P removal processes.

From the background of the experiment above, and after reviewing the literature Barnard (1976) stated "The foregoing discussion leads to the inevitable conclusion that the activated sludge returned from the clarifier or the mixed liquor must pass through an anaerobic phase where the oxygen demand exceeds the supply of both oxygen or nitrates at some stage except the final stage before clarification at which point it should be aerated. In this anaerobic zone or stage, a certain degree of anaerobiosis or a certain minimum level of the oxidation-reduction potential must be reached. At this level of the oxidation-reduction potential phosphates will be released to the liquid in the form of dissolved ortho-phosphates and whereas it would be difficult to measure the oxidation reduction potential, it would be a simple matter of control to measure the release of phosphates in the anaerobic zone as a means of ensuring that the necessary conditions for the removal of phosphates would prevail." Referring to previous reported investigations on high rate P removal plants in America he was "... led to the conclusion that the only common feature of all the plants that could be responsible for removal of the phosphates was the intentional or unintentional creation of an anaerobic zone in the plant as opposed to an anoxic zone. The sludge or mixed liquor passing through this anaerobic zone would then release phosphate to solution in the form of orthophosphates." To achieve the prerequisite low redox potential for P release most expeditiously he proposed the Phoredox process, Fig.8. He modified the Bardenpho process by introducing an anaerobic reactor ahead of the first anoxic reactor to receive the underflow recycle and the influent waste flow. It was hypothesized that the waste organic load entering the anaerobic reactor would create the necessarily low redox potential for P release, furthermore, the Bardenpho section of the plant would reduce the nitrate sufficiently low that any nitrate in the underflow would have negligible effect on attaining the required low redox potential level in the

*Curiously enough, in all subsequent reports by several investigators no P release has ever been reported in a secondary anoxic reactor even if no nitrate were present.
Barnard's proposed process and his P release hypothesis was experimentally investigated by a number of research workers, McLaren and Wood (1976), Nicholls (1977), Simpkins and McLaren (1978) and Davies and Wiechers (1978). These investigations verified that P release led to P uptake and P removal in the Phoredox process. Also it was observed that the magnitude of the removal appeared to increase with increase in the nominal retention time of the anaerobic reactor, that the denitrification in the secondary anoxic reactor per unit volume was relatively inefficient compared to the primary anoxic reactor and that nitrate in the underflow recycle had a pronounced adverse effect on excess P removal by the process. The inefficiency of the secondary anoxic reactor prompted Simpkins and McLaren to suggest that this reactor could be left out under certain circumstances and the primary anoxic reactor appropriately enlarged, to give the Modified Phoredox process.

Stern and Marais (1974), Martin and Marais (1975), Marsden and Marais (1976), Ekama, van Haandel and Marais (1979), Siebritz, Ekama and Marais (1980), Van Haandel, Ekama and Marais (1981), and Van Haandel, Dold and Marais (1982) focussed attention particularly on the denitrification aspects of the Bardenpho and Phoredox processes. They found that it was not possible to increase the anaerobic zones in these processes ad lib in order to ensure low nitrate in the effluent and underflow recycles; if, for a selected sludge age and temperature the unaerated mass fraction of the sludge was increased beyond a certain magnitude, the process stopped nitrifying. They showed that the maximum anoxic mass fraction allowable was determined by the maximum specific growth rate of the nitrifiers, \( \mu_{\text{nm}} \), at the lowest temperature the process would be required to operate, and the sludge age. Limiting the anoxic mass fraction (to ensure nitrification) necessarily limits the magnitude of the denitrification achievable. They developed equations whereby the denitrification can be estimated and concluded that the Phoredox process can be designed to achieve nitrification and complete denitrification only if the TKN/COD (mg (TKN-N)/mgCOD) ratio of the influent is below about 0,08. For TKN >0,08 the nitrate concentration in the effluent and hence in the recycle to the anaerobic reactor increases causing a disproportionate decrease in the P removal. Usually the TKN/COD ratio of raw sewage is in the range 0,06 to 0,08 and for settled sewage 0,09 to 0,11; the 0,08 upper limit for the Phoredox process severely restricts the application of this process.

Siebritz, Ekama and Marais (1980) and Rabinowitz and Marais (1980) thereupon investigated modifications to the Phoredox process that would make the anaerobic reactor independent of the effluent nitrate concentration; this lack of independence, they perceived, was the main obstacle to developing a viable process suitable to treat high TKN/COD ratios. Their efforts led to the development of the University of
Cape Town (UCT) process, see Fig. 9. The underflow return discharges to the primary anoxic reactor (instead of the anaerobic reactor) and a further recycle (r-recycle) is introduced from the primary anoxic reactor to the anaerobic reactor; the secondary anoxic zone is left out so that the system contains 3 reactors only, anaerobic, anoxic and aerobic in series. By appropriate operation of the r-recycle the nitrate in the anoxic reactor can be maintained at approximately zero, and consequently, the anaerobic reactor receives a nitrate free recycle irrespective of the nitrate concentration in the aerobic reactor and effluent. This situation theoretically can be maintained for TKN/COD ratios up to about 0.14. Extensive experimentation (Siebritz, Ekama and Marais 1980, 1982) has confirmed that the UCT process can always ensure zero nitrate flow to the anaerobic reactor for TKN/COD < 0.14. Surety of a nitrate free recycle to the anaerobic reactor allowed a rational attack on the excess P removal phenomenon without having to contend with the confounding effect of the nitrate on the anaerobic reactor.

The importance of Barnard’s contribution lies in his (1) recognition of a link between phosphate release under anaerobic conditions and subsequent uptake under aerobic conditions, (2) hypothesizing a process configuration which has the propensity to remove both nitrogen and phosphorus. This latter contribution is all the more significant for it required moving outside the ambit of the then existing activated sludge practice from, low sludge ages to long sludge ages, series plug flow reactors to completely mixed series reactors that include anaerobic, anoxic and aerobic reactors that allow nitrification and denitrification. His contribution can be said to have given a new direction to waste water treatment.

Readily Biodegradable Substrate Hypothesis
Barnard’s hypothesis, that biological excess P uptake requires an anaerobic condition such that P release is obtained, is not helpful in design for it does not provide guidance as to how the required "intensity" of the anaerobic state can be predicted. The difficulties in this regard led him to suggest an empirical guideline - the anaerobic reactor should be provided with nominal retention time of about one hour; presumably this would reduce the redox potential to the level necessary to give P release. A further difficulty in design is that no guidance is given to predict the mass concentration of P that the system will remove in a given design situation. In fact, it was not possible to give any firm assurance, at the design stage, that the plant would in fact fulfill its design expectations. These difficulties are present to various degrees in sanitary engineering design and are to be expected in development of a new and rather novel process - they are symptomatic of a lack of a model that links the performance of the plant to some measurable input parameters. Research efforts towards resolving the process designer's problems now will be reviewed.

In developing a general model for the activated sludge process the University of Cape Town group established that normal municipal influents contain two biodegradable COD fractions, a readily biodegradable soluble and a slowly biodegradable particulate fraction (Dold, Ekama and Marais, 1980). They found that the denitrification behaviour of the plant was crucially affected by the magnitudes of these two fractions. When the influent readily biodegradable COD, $S_{bsi}$, entered the primary anoxic zone it was rapidly metabolized, in a few minutes, with the nitrate proportional to the mass of $S_{bsi}$. In contrast the influent slowly biodegradable COD, $S_{bpi}$, gave rise to a long term low rate reduction of nitrate. In the second anoxic reactor only the particulate COD, derived from the influent but principally from death and lysis of the organism mass, was operative. These effects gave rise to a two phase denitrification behaviour in the primary anoxic reactor and a single phase in the secondary anoxic, see Fig. 9. It was also observed that where an anaerobic reactor preceded the primary aerobic reactor the readily biodegradable COD in its passage through an anaerobic reactor (if no nitrate was introduced) apparently was
not affected and gave rise to the same reduction in nitrate in the primary *anoxic* reactor as if the anaerobic reactor were not present. It was further established that for every mg of nitrate reduced by the readily biodegradable COD, 8.6 mg of readily biodegradable COD was utilized, for synthesis and energy production. In the anaerobic reactor, therefore, entry of say 10 mg \((\text{NO}_3^- - \text{N})\) removed 86 mg readily biodegradable COD.

Van Haandel, Ekama and Marais (1981) extended the general aerobic model to incorporate the kinetic behaviour of the process under anoxic and anaerobic conditions and derived steady state equations by means of which the denitrification behaviour of a plant can be readily predicted. This in turn opened up a way to quantify the anaerobic state in the anaerobic reactor: Siebritz, Ekama and Marais (1980) proposed a parameter, the anaerobic capacity, which is defined as the difference between the denitrification potential of an anaerobic reactor and the nitrate discharged to the reactor where the denitrification potential is the maximum mass concentration of nitrate that the reactor can remove if sufficient nitrate is discharged to it. It was hoped that this parameter, because it could be estimated from the process kinetics and the input data, would substitute for the redox potential, which could not be predicted. Accordingly it was hypothesized that if the anaerobic capacity reached a sufficiently great magnitude, then P release would be induced in the anaerobic reactor.

Application of the anaerobic capacity to the UCT process indeed appeared to indicate that if the potential exceeded about 10 mg \((\text{NO}_3^- - \text{N})/l\) in the anaerobic reactor then P release was observed with associated P removal. However when applied to a two reactor nitrification-denitrification system, Fig. 10, with an un aerated mass fraction of up to 70 percent, operated such that the anaerobic capacity was 34 mg/l, no release in the first reactor was obtained and only minimal P removal by the process was achieved. In contrast a UCT process operated in parallel, with only 7.5 percent anaerobic mass fraction and an anaerobic capacity of 12 mg \((\text{NO}_3^- - \text{N})/l\) gave rise to P release and excess P removal. The only evident difference between the two systems was that in the UCT process an appreciable concentration of readily biodegradable COD was present in the anaerobic reactor whereas in the two reactor process the nitrate recycled to the first or anaerobic reactor had completely utilized the readily biodegradable COD, so that the anaerobic capacity arose totally from the utilization of slowly biodegradable COD. These observations led to the hypothesis that the presence of readily biodegradable COD in the anaerobic reactor induced P release and excess removal.

Extensive research into the utility of this hypothesis over a year by Siebritz, Ekama and Marais (1982) established that release is induced if the readily biodegradable COD in the anaerobic reactor, \(S_{\text{BSA}}\), exceeds about 25 mg/l, the release and excess removal increasing as \(S_{\text{BSA}} - 25\) increases. This opened the way for enquiry into other factors affecting the release and excess removal, and quantification of the excess removal. It appeared that excess P removal depended on (1) \(S_{\text{BSA}} - 25\), (2) the fractional mass of sludge in the system passing through the anaerobic reactor, and (3) the actual time a unit of sludge is retained in the anaerobic reactor; if any one of these is zero, no excess removal is obtained. Empirically these three factors were combined in a *phosphorus removal propensity factor* and it was found that the fractional mass of phosphorus relative to the active mass, \(\gamma\), could be functionally related to the P removal propensity factor.* Further investigation showed that in the Phoredox and UCT processes, due to the recycle systems and their interactive effects on the anaerobic retention time, the fractional mass of the sludge in the process passing through the anaerobic reactor per day and the concentration of \(S_{\text{BS}}\) in the anaerobic reactor, the three parameters can be reduced to two, (1) \(S_{\text{BS}} - 20\) and (2) the *anaerobic mass fraction*, defined by (mass of

* Once \(\gamma\) is available the removal can be calculated from the sludge wasted each day, for, under steady state operation the removal can only take place via the waste sludge.
sludge in the anaerobic reactor)/(total mass of sludge in the system).

Extensive testing of the concepts embodied in the P removal propensity factor has, in every instance, verified the utility of this approach. At laboratory scale, employing the UCT process the concepts were tested at different sludge ages, temperatures, anaerobic mass fractions, influent COD concentrations in which the readily biodegradable fraction of the influent (unsettled municipal sewage) was augmented by addition of glucose or acetate. All these tests have given results remarkably consistent with the predictions of the theory. At full scale, in a joint research project with the Johannesburg City Council, on the Goudkoppies and Northern Works, analysis of the process in terms of these concepts has explained the poor and erratic P removal obtained in these plants and provided a basis for suggesting procedures to improve the removal where this was possible (Nicholls, Osborn and Marais, 1982). It would seem that the readily biodegradable hypothesis and its consequences allow for the first time, a rational quantitative approach to optimal design of proposed P and N removal plants, and a basis for evaluating the performance of existing plants (Ekama, Siebritz and Marais, 1982).

Biochemical Mechanism for P Release and Uptake

The models for excess P removal presented by Barnard and by Marais and his group are virtually totally heuristic because the behaviour is not explained in terms of, or directly linked, to any basic biological or biochemical phenomena, except perhaps that in Marais' approach the removal is linked to the mass of active material generated per day (Siebritz et al. 1982). Until a satisfactory biochemically based model of P release and uptake is evolved it is unlikely that the full potential of processes, utilizing these phenomena for excess P removal, will be attained. Such a model to be successful should, in our opinion, provide answers to the behavioural pattern observed in P removal plants listed below:

1. P release to the bulk liquid takes place under anaerobic conditions if readily biodegradable COD is present in the bulk liquid.*

2. P uptake takes place if P release had been obtained and nitrate or oxygen is present.

3. Release, uptake and excess removal are sensitively related to the concentration of readily biodegradable COD in the influent - addition of acetate or glucose to the influent is reflected in improved P removal performance.

4. Anaerobic-aerobic sequencing appears to be essential in order to obtain P removal - in an anaerobic-aerobic plant that is removing P, if it is changed to operate as an aerobic-aerobic one, or, if sufficient nitrate is added to the influent of the anaerobic-aerobic plant so that nitrate is present in the anaerobic reactor, P removal immediately ceases. This indicates that, even though the appropriate organism may be present, specific conditions also must be present to obtain P removal.

5. Anaerobic-aerobic conditions appear to promote the growth of P accumulating organisms. This aspect is particularly intriguing for many of the organisms implicated are obligate aerobes, for example Acinetobacter, yet these organisms apparently find considerable advantage for growth by passing through the anaerobic phase with substrate present (as indicated by their abundance in P removal plants, Buchan 1981). This behaviour is the more remarkable when it is noted that acetate promotes the growth of these organisms in the system even though this substrate is completely unavailable as an energy source to aerobes in the anaerobic zone, being one of the end products of the Embden-Meyerhof pathway.

* Readily biodegradable COD implies, in a biochemical sense, a substrate that can readily pass through the cytoplasmic membrane of the cell by diffusion or osmotic pressure.
From denitrification rate and oxygen utilization rate studies in the anoxic or aerobic reactors of a P removal process there is heavy competition for substrate between the polyP and other microorganisms whereas in the anaerobic reactor there can be competition only for substrates that can be utilized through anaerobic glycolysis e.g. the Embden-Meyerhof pathway.

Phosphate accumulation by organisms in P removal plants is but one instance of this phenomenon. Harold (1966) in an authoritative survey of the state of the art reports that polyphosphate accumulation is widespread among microorganisms and has been observed amongst others in bacteria, yeasts, fungi and photosynthetic algae. Phosphorus plays such a crucial role in biology that it must have been associated with life from the beginning. There is the suggestion that polyphosphates (polyP) and/or pyrophosphates predate adenosine triphosphates (ATP) as an energy carrier, that polyphosphate is a metabolic fossil which in time has lost its original function to assume new ones "which still elude us" (Harold 1966). From an evolution point of view if one considers the rapid adaptability and the frequency of mutation in microorganisms, any such new functions would have developed only if they accrued some advantage to the organism in its competitive struggle for survival otherwise their presence would have diminished with time. It seems therefore that if one could identify the function(s) of polyP accumulations it would go a long way to elucidating their biochemical behaviour.

With regard to function, Harold states that there is no clarity as to whether the polyP accumulations serve the function of phosphate storage or energy storage. He tended towards the phosphate storage hypothesis on the basis that the accumulation of polyP will suffice for several doublings of the cell mass should subsequent phosphate starvation occur and starvation of P is not unlikely, in nature generally P availability is low; also "polyP could constitute an accessible reserve for synthesis of messenger RNA, ribosomes and metabolic intermediates to facilitate the initiation of growth"). Furthermore, he was of the opinion that storage of polyP "would minimize disturbance of the osmotic equilibrium and of the concentrations of the critical intermediates, Pi*, and adenine nucleotides."

Considering synthesis and degradation of polyP, Harold states that: "There appears to be only a single pathway for the biosynthesis of long-chain polyP. This is the reaction catalyzed by polyphosphate kinase, in which the terminal phosphoryl group of ATP is transferred to polyP according to the reaction (a) : ATP + (Pi)ₙ ⇄ ADP + (Pi)ₙ₊₁. With regard to the breakdown of polyP both polyphosphate kinase and Polyphosphatase are implicated. Polyphosphate kinase can act reversibly for both synthesis and degradation - there is evidence that some types of polyphosphate kinase may be involved in the transfer of phosphate from polyP to adenosine monophosphate (AMP) or for phosphorylation of glucose. Polyphosphatase, however, acts only in the degradation of polyP to phosphate.

The regulating mechanisms that control these reactions are but poorly understood. As an example of this lack of understanding, consider the mechanism that controls the accumulation of polyP in the so-called "polyphosphate overplus" phenomenon. This phenomenon is observed in organisms that can accumulate P when the organism is inoculated into a medium that contains all the substances necessary for growth i.e. salts and carbon energy, with the exception of a deficiency in phosphate. This deficiency means that nucleic growth cannot occur but observations show that polyphosphate kinase and polyphosphatase increase in concentration. On addition of phosphate to the medium a rapid accumulation of polyP is catalyzed by the kinase, whereupon the kinase concentration commences to decrease but the phosphatase concentration remains high probably to break down the polyP to phosphate for nucleic acid synthesis. Regulation of these reactions poses the question: Is the increase in kinase during P deficiency regulated by the low concentration of P in the medium

*Pi represents the phosphate radical; (Pi)ₙ a polyP chain of n Pi units.
or by the ATP-ADP-P equilibrium within the organism? This same kind of question crops up where any other essential metabolite is absent, such as nitrogen or sulphur.

The behaviour patterns described above generally point to the hypothesis that P accumulation is a response to a stress condition in which the propensity for P accumulation confers an advantage on the organism in a way not evident.

The first endeavour to construct a biochemical model of P uptake and release in the activated sludge process is due to Osborn and Nicholls (1977) of Johannesburg City Council. Cognizant of the past work (as set out by Harold) they approached their subject from the standpoint that the polyP phenomenon is a response by the organism to the imposition of an anaerobic stress, that the polyP pools assist the organism in surviving the stressed state. This approach in itself constituted a bold extension on the work reported by the bacteriologists - they considered only aerobic systems, and the stresses they applied were by imposing deficiencies in selected metabolites.

In 1978 the Johannesburg group extended their work by proposing that "poly-β-hydroxybutyrate also plays an important role in maintaining the life cycle of aerobic and denitrifying organisms during their passage through the anaerobic zone", Hall, Nicholls and Osborn (1978). They state: "Under normal aerobic conditions sewage (as typified by glucose in the following equation) is oxidised in a series of steps to pyruvate, which results in the production of 4 hydrogen atoms and the liberation of 4 electrons

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CO COOH} + 4\text{H}^+ + 4\text{e}^-
\]

Glucose pyruvic acid

These hydrogen ions and electrons are then passed through the Krebs (citric acid) cycle and finally meet up with oxygen from either the air or from nitrate, and are eliminated from the system as water.

"Under anaerobic conditions aerobic bacteria can metabolise substrate up to the formation of acetyl coenzyme A, and will die at this point if no alternative route is available to relieve the system of the accumulated hydrogen ions and associated electrons. Some bacteria have the ability to absorb these hydrogen ions and convert them into water insoluble poly-β-hydroxybutyrate where they can be, as it were, temporarily stored until aerobic conditions are restored. The poly-β-hydroxybutyrate reverts back to acetyl coenzyme A which then passes the liberated hydrogen out of the system as water via the aerobic Krebs cycle.

"Energy requirements for obligate aerobes finding themselves in an anaerobic environment can be supplied as indicated in the following equation:

\[
\text{Acetyl CoA} + \text{ADP} + \text{Pi} + \text{ATP} + \text{acetic acid} + \text{CoA} \text{H}
\]

where the ATP formed is used to support life-sustaining reactions. The phosphorus requirements are probably preferentially supplied from the polyphosphate pool.

"Many aerobic bacteria have the ability to form poly-β-hydroxybutyrate and the concept of this compound acting as a balancing facility for hydrogen ions, or as an electron sink, as it is now more commonly called, is probably the basis of survival of many bacteria when exposed to temporary conditions of anaerobiosis."

From the abstract above the Johannesburg group appears to advance two mechanisms for survival in the anaerobic zone: (i) energy for ATP formation derived from the break up of polyP chains to phosphate and (ii) creation of a balancing facility for hydrogen ions and electrons by the formation of PBR. Generally the orientation of the Johannesburg model is towards survival. Judged against the six observations on P

* The authors have quoted the writers at length for the passage above neatly summarises their approach to this problem. Details of the biological pathways are to be found in their papers.
Phosphate removal by biological excess uptake

removal process behaviour the main aspect in which the model is not persuasive is that it does not explain satisfactorily the evident *advantage* the polyP organisms appear to have in processes having anaerobic zones as indicated by their proliferation in these processes. However, this imputed deficiency aside, the model showed its worth in practical terms immediately*: Noting the rôle of the intermediates in poly-ß-hydroxybutyrate formation and further, that the retention time in the anaerobic reactor is too short to process the particulate COD input via the Embden-Meyerhof pathway to produce intermediates, the group proposed that the intermediates be augmented as follows: Subject the underflow from the primary settling tank to acid digestion and feed the supernatant to the P removal plant. They experimented by introducing the acid digester supernatant to the anaerobic, anoxic or aerobic zone and found that the most promising results were associated with addition to the anaerobic zone. This outflow from their model has provided a means whereby the P removal propensity can be extended to plants treating influents deficient in readily biodegradable influent COD and merits serious attention in future research. The behavioural pattern, with acid addition, also provides direct support for the readily biodegradable COD hypothesis of the Cape Town group.

Rensink (1981) reported on the work on excess P removal at the Technical University of Wageningen, Holland. Following on the work of Osborn and Nicholls they concurred that the presence of polyphosphates served as a survival mechanism for polyP organisms during the anaerobic phase, but went further by hypothesizing that during the anaerobic phase the lower fatty acids present in the liquid phase are stored as PHB, the energy for formation of PHB being obtained by conversion of polyP to PO₄ via ATP and ADP. In the aerobic zone the stored PHB is utilized for the dissimilation and assimilation functions of the organism, i.e., the PHB accumulated during the anaerobic phase provides a source of energy for growth of the polyP organisms in the aerobic zone over and above that available in competition with other non-polyP organisms in that zone. The storage mechanism indeed is of crucial importance; Rensink notes that *Acinetobacter*, for example, is a relatively slow growing organism so that in the aerobic zone, in the absence of the storage mechanism, this organism will show relatively minor growth in competition with other faster growing facultative organisms. The storage mechanism therefore explains the growth of polyP organisms in anaerobic-aerobic systems. Also, in terms of this hypothesis the mass of polyP organisms should be strongly linked to the mass of lower fatty acids available to this organism in the anaerobic zone.

Rensink demonstrated the conversion of the lower fatty acids to a higher energy form in the anaerobic reactor as follows: To an anaerobic sample of mixed liquor, from a P removing plant, acetate was added and monitored with time. In both filtered and unfiltered samples taken from the batch, the acetate concentration diminished with time and concomitantly the phosphate concentration in the filtered liquid increased. Disappearance of acetate from the filtered sample indicates absorption of the acetate by the organisms; disappearance from the unfiltered sample indicates transformation of the acetate to some other organic material, see Fig.10. To show that the acetate removal and P release and uptake are associated with the polyP organisms, a 10 in-series reactor system was operated, initially all aerobic, and it was found that P removal was that expected for normal metabolic requirements. On changing to an anaerobic/aerobic system (5 anaerobic, 5 aerobic), initially no P was released and uptake was normal, but gradually over a six-week period, release and excess uptake developed in an increasing degree. Over the same period the plant was periodically given a dose of acetate in the influent; at the start of the six-week period little or no acetate disappeared in the anaerobic reactor series but by the end of six weeks the acetate disappeared, to zero, by the third anaerobic reactor, see Fig.11. Over the six-week period the polyP organisms also were found to increase indicating a correlation between the polyP organism growth and acetate disappearance. These results are very revealing and constitute a significant contribution towards elucidating the phenomenon of P uptake and release.

* and justifies the effort in constructing such models.
Rensink ascribes the presence of the lower fatty acids to the redox state in the anaerobic reactor - the lower this state the more the growth of facultative anaerobes will be promoted in the system, the greater the production of lower fatty acids and logically the higher the P removal. In this regard Rensink follows the same line of thought as Fuhs and Chen and Shapiro, that the fatty acids are produced in the anaerobic state at low redox potentials. This approach would appear to be in direct contrast to that proposed by the University of Cape Town group which proposes that the substrate that promotes excess release and uptake is present in the influent (the so-called rapidly biodegradable COD), and that the redox potential is not a significant parameter. A brief discussion on these two points of view will assist in clarifying the situation:

Generally all substrates, except the lower fatty acids, must pass through the Embden-Meyerhof or some other equivalent pathway to convert the substrate to a form (for example, acetate or formate) suitable for entry into either the aerobic Krebs cycle or anaerobic fermentation pathway. It is important to note that this step is essential in both aerobic and anaerobic processes. The conversion rate is dependent on the type of substrate; Dold et al. (1980) has shown that in aerobic processes the utilization rate of particulate organic material is slow, whereas for some soluble substrates the rate is extremely rapid, approximately 7 to 10 times faster than for the particulate substrate. With the particulate substrate the rate limiting step appears to be the solubilization of the particulate matter whereupon it can pass into the
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organism for energy production and fatty acid formation via the Embden-Meyerhof pathway. Similarly Eastman and Ferguson (1981) have shown that in anaerobic processes the rate limiting step for particulate substrate also lies in the solubilization of particulate organic material prior to lower fatty acids production. In both aerobic and anaerobic processes soluble substrates like glucose pass readily through the cytoplasmic membrane and are rapidly utilized for energy production, and for production of lower fatty acids via the Embden-Meyerhof pathway. As this pathway is operative in both aerobic processes, with high redox potential, and anaerobic processes, with low redox potential, it must be concluded that the Embden-Meyerhof reactions at best are only weakly affected by the redox potential.

From the discussion above there is good reason to believe that the production of lower fatty acids in the anaerobic reactor of P removal plants, under the usual modes of operation, will be minimal for particulate COD and probably is derived almost exclusively from the rapidly biodegradable COD.* It would be instructive therefore to enquire more closely into the fate of the rapidly biodegradable substrates and their interaction with the polyP organisms.

Interaction of the polyP organisms with the substrate cannot be done effectively unless a model of polyP behaviour is hypothesized. Then if it can be shown in biochemical terms that the model is consistent, this would provide a basis for experimental investigation to test the model. A model for the polyP organism behaviour can be constructed if the following hypothesis is accepted:

PolyP accumulation serves as an energy reservoir, to sustain the organism during the anaerobic stressed state, but principally to gain a positive advantage over non-P accumulating organisms by partitioning of readily biodegradable COD (in the lower fatty acid form) in the anaerobic state for its exclusive use subsequently in the aerobic state.

The biochemical behaviour is envisaged as follows:

In the formation of a polyP chain the linking of two phosphate radicals requires the input of about 1 ATP (7 kcal/mole P). When this bond is broken the same quantity of energy is released for work, and a phosphate radical is released. Under anaerobic conditions with readily biodegradable COD surrounding the organisms, when polyP is degraded it provides P and energy to form ATP. The ATP is used to bring substrate into the cell and to convert the substrate to a lipid form available for storage as acetoacetate and/or poly-β-hydroxybutyrate. Two types of substrates are to be considered, first, a relatively 'low energy' short chain fatty acid which cannot yield biological energy through anaerobic glycolyses, e.g. acetate, and second, a relatively 'high energy' substrate which can be used by facultative and aerobic organisms to yield energy through anaerobic glycolysis, e.g. glucose.

Acetate: Acetate entering the cell will be complexed by the enzyme CoA to form acetyl CoA, a reaction requiring an input of energy of 2 ATP. This is achieved as follows: An ATP is formed from ADP + Pi + 7 kcal/mole P where Pi and the energy is produced by breaking of a phosphate radical (Pi) from the polyP chain (Pi)n which yields 7 kcal/mole P., i.e.

* Under high anaerobic mass fractions and low recycles giving rise to long actual anaerobic retention times, acid formation from the slowly biodegradable COD fraction may become of significance and probably is contributory to excess P removal for example in the process of Kerdachi and Roberts (1982); however, their mode of operation results in a process behaviour that is (i) oxygen limited rather than the normally COD limited state, and (ii) the anaerobic mass fraction is so large, 80 to 90 percent, that the metabolic processes appear to deviate significantly from that normally observed, for example, the sludge produced per day in their plant appears to be 75 to 100 percent more than that normally expected.
2ADP + (Pi)\textsubscript{2} = 2ATP

acetate + 2ATP + Co-A + acetyl CoA + 2ADP + 2Pi

This step would in fact reduce the acetate concentration within the organism allowing an osmotic pressure to be created for further entry of the substrate. The organism has only a limited supply of Co-A so that acetyl Co-A will not be a convenient means of storing the ingested substrate. Storage is more likely as a lipid of the form acetoacetate (or perhaps hydroxybutyrate, see below), i.e.

2 acetyl Co-A + 2ADP + 2Pi → acetoacetate + 2ATP + 2Co-A

The overall energy required in the storage of acetate as acetoacetate is given by 2 times Eq (2) plus Eq (3) i.e.

2 acetate + 2ATP → acetoacetate + 2ADP + 2Pi

Thus, to store a molecule of acetoacetate (originally from 2 molecules of acetate) requires that 2Pi are released, these originating from the polyphosphate store, thereby increasing P continuously in the cell, creating an osmotic pressure of P which diffuses through the cytoplasmic membrane and increases the P concentration in the bulk liquid.

Glucose: Where glucose is the readily available substrate two situations may arise, (a) the polyP organisms may not be able to utilize glucose (or any other simple sugar) as the carbon source - according to Fuhs and Chen (1975) this is the case with regard to Acinetobacter; or (b), polyP forming organisms can utilize glucose as a substrate.

(a) PolyP organisms cannot utilize glucose:
For this situation in a mixed culture the facultative organisms only will utilize glucose as an anaerobic energy source via the Embden-Meyerhof pathway, the two principal steps being

(i) Glucose to pyruvate:

1 Glucose + 2ADP + 2NAD\textsubscript{OX} → 2 Pyruvate + 2ATP + 1NAD\textsubscript{RED} (5a)*

(ii) Pyruvate to short chain fatty acids:
The reaction is depicted as follows:

2 Pyruvate + 2NAD\textsubscript{RED} + 3 Acetate + 2NAD\textsubscript{OX} (5b)

In this step pyruvate is reduced (i.e. accepts the electrons from NAD\textsubscript{RED}) to form the by-product of fermentation - acetate, and NAD\textsubscript{OX} is regenerated. The overall reaction, for (i) and (ii) above, for the Embden-Meyerhof breakdown of glucose, is given by the sum of reactions (5a and 5b) above, i.e.

1 Glucose + 2ADP + P + 3 Acetate + 2ATP (6)

(Note that P in Eq (6) does not originate from polyP but from P present in the cytoplasm).

At the completion of reaction (6), the facultative organism is in the same electron state as before the reaction, except that it is at a higher energy level, by 2ATP/molecule of glucose; the bulk concentration of acetate has also increased by three molecules. The acetate cannot be utilized further by the facultative organisms while in an anaerobic state. If polyP organisms are present in the mixed culture, the acetate is available for storage as acetoacetate, as set out under acetate utilization above. Consequently, by being

* NAD (nicotinamide adenine dinucleotide) is an electron carrier in the cell, the reduced and oxidized states being depicted as NAD\textsubscript{RED} and NAD\textsubscript{OX} respectively. The ratios of NAD\textsubscript{OX}/NAD\textsubscript{RED} and ADP/ATP both serve as regulating parameters in feed-back mechanisms controlling many of the transformation reactions in which they are involved.
in a mixed culture in the anaerobic state the polyP organism receives substantial benefit by being able to utilize the products of the glycolysis reaction (by the facultative organisms) of an input substrate it cannot use directly. If glucose was fed to a pure culture of polyP organisms (of the polyP strain under discussion) the mechanisms set out above would not be possible.

(b) PolyP organisms can accept glucose as a carbon source:

In this situation both the facultative and polyP organisms will compete for the glucose molecules surrounding them. For both species the glucose will be broken down in the Embden-Meyerhof pathway according to reaction (5a) above. Thereafter the action of the two groups of organisms may differ. The facultative organism continues via reactions (5b and 6) to discharge acetate. In some strains of polyP organisms there may exist a pathway whereby utilizing the electrons (as NAD\textsubscript{RED}) and pyruvate generated in reaction (5a), the pyruvate is transformed to poly-\(\beta\)-hydroxybutyrate (PHB) which can be stored in these organisms. The storage mechanism can be depicted as follows:

(i) The pyruvate is oxidized to acetyl Co-A with NAD\textsubscript{OX} acting as the electron acceptor:

\[
2 \text{Pyruvate} + 2\text{NAD}_{\text{OX}} + 2 \text{Co-A} + 2 \text{Acetyl Co-A} + 2 \text{CO}_2 + 2\text{NAD}_{\text{RED}} \quad (7)
\]

(ii) Acetyl Co-A is converted to acetoacetate:

\[
2 \text{Acetyl Co-A} \rightarrow \text{acetoacetate} + 2 \text{Co-A} \quad (8)
\]

(iii) Acetoacetate acts as electron acceptor to form \(\beta\)-hydroxybutyrate:

\[
\text{Acetoacetate} + \text{NAD}_{\text{RED}} \rightarrow 1 \beta\text{-hydroxybutyrate} + \text{NAD}_{\text{OX}} \quad (9)
\]

The overall reaction is formulated by adding reactions (5a, 7, 8 and 9), i.e.

\[
\text{Glucose} + 3\text{NAD}_{\text{OX}} + 2\text{ADP} + 2\text{P} + 1\beta\text{-hydroxybutyrate} + 2 \text{CO}_2 + 2\text{ATP} + 3\text{NAD}_{\text{RED}} \quad (10)
\]

The overall reaction indicates that 2ATP and 3NAD\textsubscript{RED} are produced from 1 Glucose. Unless the organism can find a sink for the 3NAD\textsubscript{RED} the organism would not be viable. From this it would appear that the pathway above is unlikely in a pure culture of this organism. However, in a mixed culture the facultative organisms (in competition for the glucose) would discharge acetate but the polyP organisms utilizing its polyP store to generate ATP (according to reaction (1)) can use the excess NAD\textsubscript{RED} in reaction (10) to transform the acetate to acetoacetate and then to PHB in accordance with reactions (2 and 3), and reaction (9) respectively. The proportions of the relative mass fractions of acetoacetate and PHB stored probably will be controlled by the relative fractions of facultative and polyP organisms and the mass and type of substrate available. Here again, it is apparent that a mixed culture while in the anaerobic state confers an advantage on the polyP organisms in that the mixed nature of the culture promotes the sequestration of the readily available substrate for the subsequent exclusive use by the polyP organisms.

PolyP formation and Pi storage: When the polyP organism with its stored substrate enters an aerobic (or anoxic) region, the stored substrate will be oxidized immediately giving rise to a rapid increase in the ATP/ADP ratio which in turn will act as a feed-back mechanism activating (or producing) polyP kinase to catalyze polyP production in order to refill the polyP pool. (The same type of control probably also acts under anaerobic conditions: the ATP/ADP ratio will decrease acting as a feed-back mechanism activating (or producing) polyphosphatase to catalyze the breakdown of stored polyP to Pi for ATP production).

Energy considerations: To illustrate the advantage accruing to the polyP organism by its sequestrering action, acetate will be used as an example. From reaction (4) it is shown that 2ATP (from the polyP pool) is required for two molecules of acetate to be converted to the storage form of acetoacetate. Under aerobic conditions, through the Kreb cycle and subsequent oxidative phosphorylation, the overall
reaction is

$$1 \text{ Acetoacetate} + 4 \text{ O}_2 + 8\text{NAD}_{\text{RED}} + 22\text{ADP} + 22P + 4\text{CO}_2 + 8\text{H}_2\text{O} + 8\text{NAD}_{\text{OX}} + 22\text{ATP} \quad (11)$$

Now two acetates are needed to form one acetoacetate, consequently, from reaction (11) for each acetate stored in the cell 11ATP will become available under aerobic conditions. To store this acetate requires 1ATP. Hence, from an investment of 1ATP from its polyP pool, 11ATP is returned. This would not have been possible if the organism did not have the polyP storage propensity.

The analysis above was restricted to two substrates, acetate and glucose, to illustrate the different pathways required by the polyP organism to utilize these. In the real world, the substrate will consist probably of many types but the mechanisms described above very likely will still apply.

The bioenergetics model described above would appear to explain the six behavioural patterns observed on large plants listed at the beginning of this section. Also, it supports the proposal from the Johannesburg group that addition of acid digestion supernatant would have a significant effect on P release, uptake and excess removal, even though the last two have not been dealt with in depth in the model presented. Having an hypothesized model should facilitate development of experimental designs to test its validity, and more important, to optimize P removal in full scale plants.

CONCLUSION

This paper has traced the development of the concept of biological excess phosphorus removal in activated sludge from its first reported manifestation in 1959 to the present. The cumulative evidence towards its existence is persuasive and there should not remain much doubt that the phenomenon is biological. Its theoretical description, in a biochemical or bioenergetic sense however, is still in a rudimentary stage. The parametric black box approach in its development and application to design and optimization of P removal plants, which up to now has dominated enquiry, probably has past the peak of its usefulness as a research tool. The direction of future research is likely to be influenced increasingly by the biochemical aspects of the phenomena; any significant advances in the use of this phenomenon in waste water treatment will, in the opinion of the authors, be contingent on greater understanding of the biochemical mechanisms controlling the phenomenon.

ACKNOWLEDGEMENT

This research was carried out under contract with the Water Research Commission of South Africa. The authors wish to thank the Commission for permission to publish this paper.

REFERENCES


Phosphate removal by biological excess uptake


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