The use of simultaneous chemical precipitation in modified activated sludge systems exhibiting biological excess phosphate removal Part 2: Method development for fractionation of phosphate compounds in activated sludge

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Abstract

An experimental investigation was conducted into the use of a simple fractionation (extraction) procedure for distinguishing chemically and biologically stored phosphorus compounds in activated sludge. For this purpose, it was necessary to select appropriate methods for measurement of total phosphate(total P) and orthophosphate (orthoP) and to make approximations in respect of the nucleic acid content of activated sludge. The selected fractionation procedure appeared to be capable of broadly distinguishing between chemical and biological forms of stored phosphorus in activated sludge systems. Satisfactory agreement was obtained with results for biological polyphosphate (polyP) accumulation predicted using a mathematical model applied to such systems. The recovery of chemical phosphate precipitate formed *in vitro* was found to be satisfactory, although the addition of metal precipitant appeared to cause particular artefacts in the fractionation pattern which need to be taken into account when interpreting the data. It was concluded that the chemical fractionation procedure developed would be useful for assessing the relative sizes of biological and chemical phosphorus "pools" in activated sludge simultaneously dosed with chemical precipitants (typically iron or aluminium salts).

Introduction

This series of papers presents an investigation into the effect of simultaneous chemical addition on the biological excess phosphorus removal (BEPR) mechanism in activated sludge systems. The aim was to measure the extent to which the P removal could be ascribed specifically to the biological mechanism, as opposed to a chemical mechanism. Pilot- or laboratory-scale activated sludge systems are most suitable for such research, and were used in this study. Identical systems were operated under identical conditions in the laboratory, with the exception that one unit (the test unit) was dosed with chemical precipitant (e.g. alum or iron salt), while the other served as the control unit (De Haas et al., 2000b,c). Phosphate removal could then be measured in both units from the difference between influent and effluent total P. Phosphate removal in the test unit would be attributable to the combined chemical-biological mechanisms, while that in the control would be mainly due to the biological mechanism. However, cations naturally present in domestic sewage could make a contribution to the P removal of the control (Arvin, 1983, 1985; De Haas, 1989). Therefore, in this investigation it was considered essential to have a phosphorus fractionation procedure which, when applied to activated sludge mixed liquor from both the test and control units, would be capable of estimating the relative magnitudes of phosphate fractions bound chemically vs. those stored biologically. Fractionation procedures used for this purpose have been reviewed in **Part 1** (De Haas et al., 2000a).

The aim of the work described in this paper was to test one phosphorus fractionation procedure, namely that based on cold perchloric acid. This procedure has been applied to activated sludge in various forms (*inter alia* Kerdachi and Roberts, 1985; Mino et al., 1987; De Haas, 1989; Blonda et al., 1994). The intention here was to consolidate and simplify the method where possible, as well as to validate it. Validation of the chemically-removed fractions would be attempted by means of batch tests in which known amounts of chemical precipitant and phosphate are added to samples of activated sludge drawn from laboratory-scale units exhibiting BEPR, followed by application of the fractionation procedure to determine recovery of chemically-bound phosphate. Validation of the biological fractions would be attempted by comparing the fractionation results for the control unit, under defined conditions, with the concentrations of biologically-stored polyphosphate (polyP) predicted by a mathematical model (Wentzel et al., 1992). Moreover, to correctly apply the fractionation procedure, it was necessary to give special attention to the methods for total P and orthoP determination, specifically in samples of activated sludge or the extracts obtained.

Experimental investigation and results

OrthoP method

Vanadate-molybdate spectrophotometric method

This method was most often selected since it has been reported to obey Beer's Law up to 300 mgP/ℓ (Burke et al., 1986), which is a suitable range for mixed liquor samples of activated sludge. It was also suitable for influent and effluent samples from the laboratory-scale plants used in this study since phosphate was added to the influent in most experiments. The performance of the method was only tested up to 100 mgP/ℓ, which was adequate for the experiments performed in this investigation and gave absorbances up to ca. 0.9 and 0.6 respectively for orthoP and total P (see Fig. 1).

The method used was that described by Burke et al. (1986). A colour reagent was prepared as follows:

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Received 10 November 1998; accepted in revised form 20 June 2000.

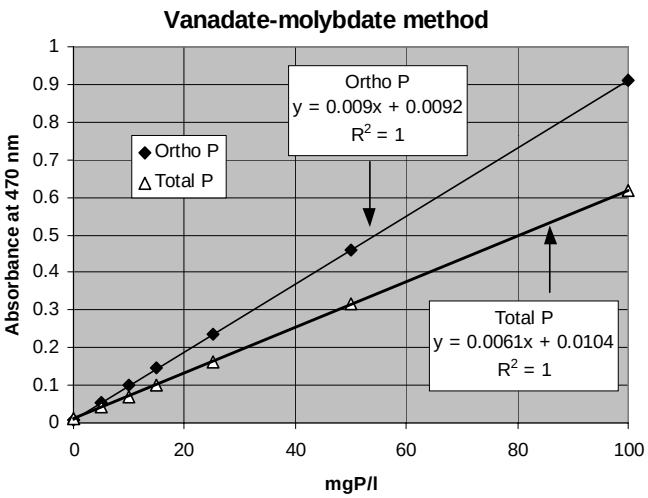

Figure 1
Typical calibration curves for total phosphate and orthoP determination by the vanadate-molybdate method. Path length = 1 cm.

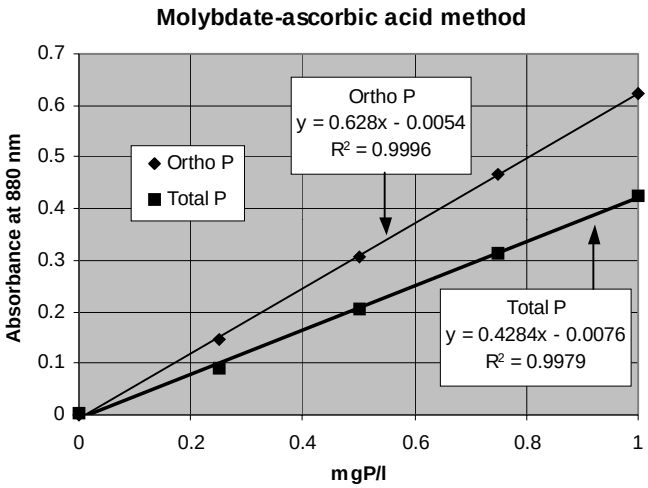

Figure 2
Typical calibration curves for orthoP and total P determination by the molybdate-ascorbic acid method. Path length = 1 cm.

- Solution A: 20 g ammonium molybdate tetrahydrate was dissolved in ± 250 mℓ distilled water;
- Solution B: 1 g ammonium metavanadate was dissolved in ± 200 mℓ distilled water and 40 mℓ conc. nitric acid;
- Solutions A and B were mixed, a further 100 mℓ conc. nitric acid added and diluted to 1 ℓ with distilled water after cooling.

It was found that the mixed vanadate-molybdate reagent has a limited shelf life. It should be kept (in the dark) for a maximum of approximately one week, and should not be used if a precipitate forms in the bottom of the bottle.

Effluent samples containing particulate material were filtered through Whatman no. 41 paper (or equivalent) before the determination. For each orthoP determination, 5 mℓ sample (pre-diluted if necessary into the range 1 to 100 mgP/ℓ) was mixed with 5 mℓ colour reagent and 30 min allowed for colour development at room temperature. The absorbance was read at 470 nm using a spectrophotometer with a flow cell of 1 cm pathlength against a distilled water blank. In cases where samples were coloured or slightly turbid (even after paper filtration), a blank correction was performed in which the absorbance at 470 nm of 5 mℓ sample plus 5 mℓ distilled water (instead of colour reagent) was read against distilled water and subtracted from that for the sample with colour reagent.

A stock standard (100 mgP/ℓ) was prepared from 562.8 mg dipotassium hydrogen phosphate (K_2HPO_4) dissolved in ca. 500 mℓ distilled-deionised water to which conc. nitric acid (2 mℓ) was added as preservative, and the solution made up to a final volume of 1 ℓ with distilled-deionised water. Working standards of 5, 10, 15, 25 and 50 mgP/ℓ were prepared from the stock standard by dilution with distilled-deionised water also containing 2% v/v nitric acid. Standards were included in each run for orthoP or total P. A typical standard curve for this method is given in Fig. 1.

Molybdate-ascorbic acid spectrophotometric method

For samples containing <1 mgP/ℓ, this method was selected. Also, for certain samples, it was not possible to use the vanadate-molybdate orthoP method because interference was caused by the sample matrix. For example, 1 M sodium hydroxide extracts of activated sludge produced a dark blue complex with the vanadate-molybdate colour reagent. Neutralisation of the undiluted extract prior to the analysis was not possible since this produced a precipitate in the sample (probably protein-nucleic acid complexes and potentially including phosphate species). The simplest alternative was to dilute the sample approximately 20-fold and then to neutralise with an appropriate aliquot of HCl. This was followed by further dilution to a total of 25-fold, and determination of orthoP using the molybdate-ascorbic acid method. This method has a linear range of approximately 0.01 to 1 mgP/ℓ (*Standard Methods*, 1985). Details of the exact method used here have been described by De Haas et al. (1990). In the case of coloured samples (e.g. NaOH extracts of activated sludge were coloured brown), a blank correction was performed by substituting distilled-deionised water for colour reagent, and subtracting the absorbance from that for the sample with colour reagent.

A typical standard curve for this method is shown in Fig. 2.

Total phosphate method

Total phosphate (total P) is determined by some form of sample digestion to convert all phosphate forms to orthophosphate (orthoP), followed by determination of the orthoP, most commonly by either the vanadate-molybdate or molybdate-ascorbic acid methods described above. This section describes the digestion method adopted and its effect on the subsequent colorimetric determinations of orthoP, particularly in respect of its application to samples of activated sludge in the fractionation procedures to be developed. Attention was given to the concentrations of acid and persulphate used in the digestion procedure, since both can influence the recovery of total P and/or produce interference in the subsequent colorimetric reactions (De Haas et al., 1990).

Digestion method

The method for total P digestion used for a number of years in the Water Quality Engineering laboratory at the University of Cape Town (UCT) Dept. of Civil Engineering has been described by Burke et al. (1986). It involves digestion of the sample (20 mℓ) with sulphuric acid (5 mℓ of 0.11 M H_2SO_4) and potassium persulphate (5 mℓ of 3% m/v $K_2S_2O_8$) in a pressure cooker at 100 kPa for 30 min. This method gives final concentrations (after admixture with sample) of 0.018 M H_2SO_4 and 0.5% $K_2S_2O_8$. Whilst these concentrations may be adequate for wastewater influent or effluent samples with a low solids

content, incomplete digestion may occur when the method is applied to activated sludge mixed liquor samples. For such samples, De Haas et al. (1990) found that the final concentrations of sulphuric acid (0.1 M) and persulphate (0.8% as ammonium persulphate) recommended in *Standard Methods* (1985) were the minimum that gave acceptable recovery of total P, compared to a magnesium nitrate fusion method. De Haas et al. (1990) recommended a higher final sulphuric acid concentration (1 M) for surety of complete total P recovery from mixed liquor. However, this necessitated larger dilutions (at least 20-fold) of the digest in order to avoid interference in the subsequent colorimetric orthoP determination. This aspect was further investigated for the purpose of this study.

As a means of comparing different digestion regimes, an experiment was set up in which the final acid concentrations (prior to digestion) were 0.02 M (Burke et al., 1986), 0.1 M (*Standard Methods*, 1985) and 1 M (De Haas et al., 1990). The final persulphate concentration was fixed at 1% potassium persulphate (0.037 M) since this corresponded closely with 0.8% ammonium persulphate (0.035 M) in *Standard Methods* (1985). Standard curves were prepared from standard solutions of dipotassium hydrogen orthoP (K_2HPO_4) carried through the respective digestion regimes. The same sample of activated sludge mixed liquor (from a laboratory unit exhibiting BEPR) was subjected to all three digestion regimes at dilutions of 6.66, 4 and 2-fold. Following digestion, the vanadate-molybdate determination for orthoP (after total P digestion) was adopted since it offered the most suitable range for activated sludge phosphate concentrations (Burke et al., 1986 – see above).

In order to establish a control procedure against which the persulphate digestion regimes could be tested, a carbonate fusion digestion procedure was used. This method had been the applied in the past for total P digestion in the Water Quality Engineering Laboratory at UCT, as described in detail by Rabinowitz and Marais (1980). In summary, it involves the following steps:

- Mixed liquor (5 mℓ) plus distilled water (20 mℓ) were placed in a platinum crucible and evaporated to dryness by gentle heating over a boiling water bath.
- Sodium carbonate (approx. 2.5 mℓ dry powder) was added and the crucible was heated strongly over a Bunsen burner until the carbonate melted and turned clear.
- The crucible was cooled and the residue dissolved in 50% (v/v) nitric acid (ca. 10 mℓ), using a watch glass to catch splashing due to CO_2 evolution. Small increments of the nitric acid solution were added until CO_2 evolution stopped and the residue was completely dissolved.
- The contents of the crucible were transferred quantitatively to a 25 mℓ volumetric flask, diluted to the mark with distilled water, and the orthoP concentration determined by the vanadate-molybdate method described above. It was found that a blank consisting of 50% v/v nitric acid gave an absorbance to within 0.002 units of that for very dilute (0.2% v/v) nitric acid only. OrthoP standards made up in 0.2% v/v nitric acid were therefore considered appropriate for the carbonate fusion method.
- The above steps were carried out for six replicates.

TABLE 1
Results of total P determination of one mixed liquor sample (from BEPR plant) using digestion by sulphuric acid- persulphate and carbonate fusion methods

Method	Linear regression for standard curve of absorbance (470 nm)		Result for mixed liquor total P (mgP/ℓ)	Dilution used	% Recovery
Carbonate fusion	1/Slope: Y-Intercept: R^2:	80.49 0.011 0.9998	146.9	5	100% (assume)
0.02 M H_2SO_4 **1% m/v $K_2S_2O_8$**	1/Slope: Y-Intercept: R^2:	118.83 0.066 0.9999	107.0 112.2 110.1	6.66 4 2	73% 76% 75%
0.1 M H_2SO_4 **1% m/v $K_2S_2O_8$**	1/Slope: Y-Intercept: R^2:	118.85 0.068 0.9997	140.2 140.2 156.2 (Ave. 145.5)	6.66 4 2	95% 95% 106% (99%)

The results of the above-mentioned experiment are given in Table 1. The digestion method using 1 M final sulphuric acid concentration failed because all tubes (including blanks and standards) produced a uniform orange colour upon addition of the colour reagent for orthoP determination. Presumably this was due to the high acid strength and is analogous to the inhibition of colour development at high acid concentrations in the molybdate-ascorbic acid method (De Haas et al., 1990).

Table 1 shows that, relative to the carbonate fusion method, sulphuric acid-persulphate digestion at 0.1M H_2SO_4 gave acceptable recovery of total P, while that at 0.02 M H_2SO_4 did not. This indicates a deficiency in the method of Burke et al. (1986), making it inadequate for determination of phosphorus in mixed liquor samples. For such samples, the recommendations in *Standard Methods* (1985) should be followed.

In view of the above, the standard method adopted for total P digestion in this study was as follows:

- Sample (20 mℓ) pre-diluted with distilled-deionised water to contain 1 to 100 mgP/ℓ was transferred to a 50 mℓ rimless test tube [*Note: Samples of extracts containing 1 M NaOH were pre-neutralised by adding 10 mℓ 1 M HCl to 10 mℓ sample, giving an initial dilution of twofold]*.
- 0.6 M H_2SO_4 (5 mℓ) and freshly dissolved 6% m/v $K_2S_2O_8$ (5 mℓ) were added.
- The tube was covered with aluminium foil and autoclaved in a pressure cooker at 100 kPa for 1 h.
- The tubes were cooled to room temperature and filtered (where necessary) through ashless filter paper (Whatman No. 41 or equivalent).
- An aliquot (5 mℓ) of each was taken for orthoP determination using the vanadate-molybdate method (see below).

Orthophosphate (orthoP) standards in the range 5 to 100 mgP/ℓ were included with every total P run and passed through the above-mentioned digestion procedure. A typical standard curve for this method is given in Fig. 1.

Molybdate-ascorbic colorimetric reaction

In cases where the effluent total P was <1 mgP/ℓ, the vanadate-molybdate colorimetric reaction became unreliable. For such samples, the molybdate-ascorbic acid method (*Standard Methods, 1985,* as described by De Haas et al., 1990) was applied with one exception: orthoP standards (range 0.2 to 1.5 mgP/ℓ) were carried through the acid-persulphate digestion process described above for total P. It was found that with 1% potassium persulphate used in the digestion, colour development at room temperature was delayed. Accordingly, a period of 2 h for colour development at room temperature was allowed, after which a standard curve comparable to that for orthoP was obtained, after taking dilution from the digestion reagents into account (Fig. 2).

Fractionation procedures

As outlined in the **Introduction**, a suitable fractionation procedure was required to distinguish biologically-stored forms of phosphate (mainly polyP in enhanced P removal systems) from chemically precipitated forms of phosphate (mainly orthoP) in activated sludge. A procedure using cold perchloric acid was selected on the basis that it appeared to meet this requirement, was relatively simple to perform, and did not require specialised analytical equipment.

Cold perchloric acid (PCA) procedure

The use of cold perchloric acid (PCA) for extraction of phosphate compounds from activated sludge has been previously reviewed (De Haas et al., 2000a). PCA has been found to be effective in dissolving chemical precipitates of phosphate (e.g. ferric and calcium precipitates), thereby releasing orthoP into solution. Evidence has been put forward that the orthoP content of cold PCA extracts of activated sludge is a measure of the chemically bound fraction (De Haas, 1989). Since PCA is a strong acid, one could expect hydrolysis of polyP, even at cold temperatures. De Haas (1989, 1991) showed that fragmentation of polyP to shorter chain lengths apparently does occur in extracts from activated sludge. However, the rate of hydrolysis of polyP to orthoP in cold PCA has been found to be slow (De Haas, 1989; Kerdachi and Roberts, 1985). Thus, provided orthoP analysis follows the extraction step directly, the contribution of polyP hydrolysis to the orthoP result will be negligible (De Haas, 1989). This implies that in the cold PCA extract, orthoP originates largely from chemically bound P, while non-orthoP (Total P – orthoP) is largely of biological origin.

De Haas (1989) found that the extraction times with cold PCA for activated sludge samples could be reduced to around 5 min, in three or four replicate steps, without significantly compromising the extraction of chemical precipitates or polyP. Since shorter extraction times would reduce potential polyP hydrolysis and speed up the fractionation procedure, three 5 min extractions at 0 to 3°C were used in the basic procedure adopted. Several preliminary experiments showed that the fractionation procedures described by De Haas (1989) could be further simplified as follows:

- The nucleic acid fraction is the third major group of phosphorus-containing compounds in activated sludge biomass (after polyP and orthoP of chemical origin) (De Haas, 1989). Nucleic acids or nucleotides may be extracted into cold PCA; the remainder will be largely extracted in a subsequent alkaline step (if used), or resort to the residue (De Haas, 1989). In the extracts, polyP could be loosely grouped with the nucleic acids and termed "complex P" to distinguish it from orthoP. Hence, in the PCA extract (or subsequent NaOH extract):

  polyP + nucleic acid P + (phospholipids + minor organic P)
  = complex P
  = total P - orthoP

- For the purposes of comparison with the mathematical model predictions of activated sludge polyP content, the estimated (or "measured") polyP content of the extracts is required. Since the nucleic acid content of a range of sludges was found to be relatively constant and the minor organic P fractions relatively insignificant (De Haas, 1989), the "measured" polyP content of the extracts was obtained by subtracting the expected nucleic acid P from the sum of the complex P fractions (PCA+ residue, or PCA + NaOH + residue).

The above simplifications led to the basic PCA procedure described in Table 2.

Testing the cold PCA fractionation procedure

The cold PCA procedure was tested on various samples of activated sludge:

- Samples of activated sludge were taken from the aerobic zone of a modified UCT (MUCT) laboratory system operated at a 20 d sludge age with cyclic (12 h) feed and exhibiting BEPR (Wentzel et al., 1993). These were compared with a sample taken from a completely aerobic 2.5 d sludge age unit, also with cyclic (12 h) feed, used for readily biodegradable COD (SBSI) determination (WRC, 1984).
- Samples were taken from a unit set up for the development of an "enhanced culture" of BEPR organisms by feeding incremental concentrations of sodium acetate (50; 100; 250 mg/ℓ as COD) and decremental amounts of sewage such that the target influent total COD was a constant 500 mg/ℓ.
- Samples of mixed liquor taken from the above-mentioned "enhanced culture" unit were spiked with dissolved orthoP (50 mgP/ℓ or 1.62 mmol P/ℓ) and $FeCl_3$ (524 mg/ℓ or 3.23 mmol Fe/ℓ) to give a 2:1 molar ratio of Fe:P, based on the spiked orthoP. A control sample of mixed liquor was treated with the same dose of $FeCl_3$, but without phosphate spike. In both cases, the pH was corrected to 7.0 with a known volume (8 to 12 mℓ) of 10% (m/v) sodium bicarbonate solution. A mixing period of 15 min was allowed for precipitation to proceed.
- Some of the enhanced culture samples were subjected to an anaerobic P-release batch test prior to the fractionation procedure. The batch test involved the following steps:
  - Aliquots (50 mℓ) of mixed liquor were each placed in centrifuge tubes used for performing the extractions and spiked with a known amount of sodium acetate.
  - The amount of acetate added was equivalent to 1 000 mg/ℓ as COD when dissolved in the mixed liquor. This was a stoichiometric excess, allowing up to 20 mgN/ℓ as nitrate and up to 300 mgP/ℓ polyP in the starting mixed liquor (WRC, 1984; Wentzel et al., 1990).
  - The acetate was dissolved by inverting the tubes several times over the first 5 min.
  - The tubes were allowed to stand for 4 h, inverting approximately once every hour.
  - The tubes were then centrifuged to start of the fractionation procedure (Table 2, Step 2).

MUCT vs. SBSI unit

Results for the fractionation are set out in Table 3. It can be seen from Table 3 that the orthoP content of the PCA extract (on a VSS basis)

TABLE 2
Basic PCA fractionation procedure as applied to activated sludge

Step	Procedure	Sample preparation and analysis	Interpretation
1	Transfer one aliquot (50 mℓ) of mixed liquor to each of two centrifuge tubes with screw cap lids for sealing.	Analyse original mixed liquor for: • total P • MLSS, VSS *Notes 1 & 2*	
2	Centrifuge at 3 000 r/min (max. 2000 *g*) for 5 min. Collect and pool supernatant and retain pellet.	Label supernatant *SUP* and retain.	Original supernatant corresponding to "effluent" at point of sampling
3	Add 0.9% (m/v) NaCl (10 mℓ) to pellet in each tube. Cap and shake well to mix/ re-suspend.	-	-
4	Centrifuge at 3 000 r/min (max. 2000 *g*) for 5 min. Collect and pool with supernatant with that marked *SUP (Step 2)*. Retain pellet.	Filter about half *SUP* sample through ashless filter paper (Whatman No. 41 or equivalent). *SUP* and *filtered SUP* (*fSUP)* analysed for • orthoP immediately • total P Correct by a factor of 1.2 i.e. 60 mℓ extract per 50 mℓ original mixed liquor.	"Interstitial" loosely bound phosphate pooled with that present in the original supernatant.
5	Extract pellet three times with ice-cold 0.5 M PCA (20 mℓ) in a refrigerated water bath (0 to 3°C) for 5 min each time. Centrifuge between extractions (as before, preferably with centrifuge refrigerated at 5°C) and keep the extracts ice cold. Retain residue.	Pool extracts and label *PCA*. Filter about half *PCA* sample (see step 4). *PCA* and *filtered PCA* (*fPCA)* analysed for: orthoP immediately**;** total P Correct by a factor of 1.2 i.e. 60 mℓ extract per 50 mℓ original mixed liquor.	Acid-soluble complex P (polyP and nucleic acids) extracted as well as chemical precipitates (orthoP). *Note 3*
6	Residue resuspended by serial washing in a total of 50 mℓ distilled water per centrifuge tube.	Analyse residue (*RES)* for: total P *Note 1*	Non acid-extractable P compounds grouped. Allows check for overall P mass recovery.

Note 1: Take precaution to mix sample well and perform determinations in duplicate at least.
Note 2: The mixed liquor sample should contain ca. 2500 mg VSS/ℓ. Refer to Table 9 for optional pre-concentration step.
Note 3: "Fatty" fragments may float in the PCA extract but these have a small total P content, as judged from the difference: *PCA- fPCA.*

from the MUCT unit (4.41 and 5.52 mgP/gVSS) was very similar to that of the SBSI unit (4.32 mgP/gVSS), despite the two units exhibiting very different BEPR behaviour. The low value for this fraction suggests that chemical precipitation mechanisms were not strongly present in either unit. This can be explained by the low hardness of water in the Cape Town area from which the influent sewage to these units was derived, and the fact that no chemical precipitant was dosed. Furthermore, the fractionation procedure appeared to be capable of correctly distinguishing biologically stored phosphate (polyP) in mixed liquor. The PCA extract from the MUCT unit contained significant amounts of complex P (25.18 and 30.26 mgP/gVSS), which may be reasonably explained by the storage of

TABLE 3
Fractionation results for activated sludge samples from MUCT and SBSI unit

Sample/ Extract	OrthoP		Total P		Complex P (Total P - OrthoP)	
	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS
MUCT unit 13/8/93 VSS = 2 772 mg/ℓ						
SUP.	2.41	0.87	2.11	0.76	(-0.3)	0
PCA	**12.21**	**4.41**	96.10	34.67	**83.89**	**30.26**
RES.	-	-	47.71	17.22	(47.71)	(17.22)
Mixed liquor	-	-	**140.04**	**50.52**		
Sum of extracts	-	-	145.92	52.65		
Recovery	-	-	104%	104%		
MUCT unit 27/8/93 **VSS = 2 908 mg/ℓ**						
SUP.	15.84	5.45	15.67	5.39	(-0.17)	0
PCA	**16.04**	**5.52**	**89.26**	**30.69**	**73.22**	**25.18**
RES.	-	-	67.52	23.22	(67.52)	(23.22)
Mixed liquor	-	-	**166.62**	**57.30**		
Sum of extracts	-	-	172.45	59.30		
Recovery	-	-	103%	103%		
SBSI unit 27/8/93 **VSS = 1 106 mg/ℓ**						
SUP.	7.22	6.53	5.86	5.30	(-1.36)	
PCA	**4.78**	**4.32**	**6.77**	**6.12**	**1.99**	**1.80**
RES.	-	-	16.25	14.69	(16.25)	(14.69)
Mixed liquor	-	-	**28.12**	**25.43**		
Sum of extracts	-	-	29.05	26.11		
Recovery	-	-	103%	103%		

polyP in the mixed liquor of this unit. In contrast, the PCA extract from the SBSI unit contained very little complex P (1.80 mgP/gVSS); this was expected since this unit did not exhibit BEPR. Thus the method would seem to be capable of correctly distinguishing polyP in broad terms, although polyP is not distinguished from other forms of complex P (e.g. nucleic acid).

Assuming that the residue contains a negligible amount of orthoP (see results below in Table 8 with inclusion of the NaOH fractionation step), with little error the total P content of the residue may be taken to be equal to complex P. On this basis, the SBSI unit contained a total complex P (PCA extract + residue) of about 18 mgP/gVSS, which is in good agreement with the nucleic acid content of sludges from full-scale activated sludge plants measured by more direct methods (De Haas, 1989). Since the polyP content of the sludge from the SBSI unit is negligible, what removal did occur can be explained in terms of biomass growth: the observed total P content of SBSI unit sludge was 25.4 mgP/gVSS, which agrees reasonably well with the P content of heterotrophic non-polyP organism active and endogenous masses (30 mg P/gVSS) accepted by Wentzel et al. (1990) for modelling purposes. From the data presented here, approximately 5 mgP/gVSS of this may actually be due to natural P precipitation reactions for Cape Town (low hardness) sewage.

Overall, the data in Table 3 suggests that the cold PCA fractionation method gave reasonable results which were in agreement with general observations of the BEPR phenomenon.

Enhanced BEPR organism culture development

Table 4 shows that as the enhanced culture developed in the laboratory-scale system due to increasing acetate addition to the influent, the complex P fraction in the PCA extract increased by nearly three-fold, from 38.2 to 109.5 mgP/gVSS. This can be ascribed to the increase in polyP storage in response to the increased acetate supplement in the influent. In contrast, the orthoP content of the PCA fraction remained virtually constant, indicating that the chemical precipitate fraction remained largely unchanged while the biological P removal mechanism developed. This provides further evidence that the fractionation procedure can correctly distinguish chemically and biologically bound P. It also indicates that the degree of polyP hydrolysis in the fractionation procedure is negligible in so far as it does not significantly affect the orthoP result of the PCA extract (refer to De Haas et al., 2000a concerning the findings of Müssig-Zufika et al.,1994).

Ferric chloride addition in batch tests

Table 5 shows the effect of $FeCl_3$ addition on fractionation results for two of the original sludge samples given in Table 4. P mass balances for the respective fractions after P and/or Fe addition are shown in Table 6, relative to the fractions from the original sludge.

Table 6 shows that good recovery of added phosphate was obtained in the sludge fractions and the mixed liquor analyses. Iron addition at a 2:1 molar ratio relative to the added orthoP was apparently in excess of the stoichiometric amount since orthoP removal from the original sludge supernatant also occurred. The observed Fe:P removal molar ratio was close to 1.6. Hence, although the increment in the PCA orthoP fraction was numerically similar to the added orthoP, more complex minor transformations apparently took place in the sludge. Comparing the results in Tables 4 and 5 (before/ after iron addition), an apparent decrease in the PCA complex P fraction but an increase in the residue (RES) total P fraction was observed. In the second experiment, more orthoP was precipitated from the supernatant since the concentration of dissolved orthoP in the starting mixed liquor was higher (Table 4). This may have left less iron to react with other fractions. Taking into account that the recovery of total P in these experiments was not exactly 100%, the possibility cannot be ruled out that these observations were due to experimental limitations. Alternatively, two explanations may be put forward: either iron addition caused rapid hydrolysis of a portion of the acid-soluble polyP pool, with the resultant orthoP binding with iron in a non-acid-soluble form; or iron became complexed with a part of the

polyP pool and converted it into a form not soluble in cold PCA.

In summary, the results in Tables 4 and 5 are summarised in Table 6 and confirm that the cold PCA fractionation procedure is able to quantitatively recover ferric (hydroxy) phosphate precipitate formed from added Fe and orthoP. These results also suggest that the addition of Fe causes an apparent shift in P from complex PCA fraction to the residue. It raises the question of whether an alkaline soluble complex between Fe and polyP forms in the mixed liquor biomass, or whether such complexation is an artefact of the fractionation procedure itself. This aspect will be further examined below (see **Effect of iron addition during fractionation procedure**).

TABLE 4
Fractionation results for samples of "enhanced BEPR culture" activated sludge for different stages of incremental acetate COD (mg/ℓ) supply in the feed

Sample/ Extract	OrthoP		Total P		Complex P (Total P - OrthoP)	
	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS
50 mg/ℓ acetate 24/9/93 VSS = 2 395 mg/ℓ						
SUP.	13.90	5.80	14.02	5.85	0.12	0.05
PCA	**13.01**	**5.43**	**104.39**	**43.59**	**91.38**	**38.15**
RES.	-	-	58.64	24.48	-	-
Mixed liquor	-	-	**181.15**	**75.64**	-	-
Sum of extracts	-	-	177.05	73.93	-	-
Recovery	-	-	98%	98%	-	-
100 mg/ℓ acetate 5/10/93 VSS = 2 350 mg/ℓ						
SUP.	11.49	4.89	11.79	5.02	0.30	0.13
PCA	**15.67**	**6.67**	**186.24**	**79.25**	**170.58**	**72.58**
RES.	-	-	68.93	29.33	-	-
Mixed liquor	-	-	**246.48**	**104.89**	-	-
Sum of extracts	-	-	266.96	113.60	-	-
Recovery	-	-	108%	108%	-	-
250 mg/ℓ acetate 31/10/93 VSS = 2 088 mg/ℓ						
SUP.	27.63	13.23	28.34	13.57	0.71	0.34
PCA	**12.57**	**6.02**	**241.20**	**115.52**	**228.63**	**109.50**
RES.			39.29	18.82	-	-
Mixed liquor			**316.11**	**151.39**	-	-
Sum of extracts			308.83	147.91	-	-
Recovery			98%	98%	-	-

Anaerobic P release batch tests

Table 7 gives the results of a fractionation experiment conducted on mixed liquor taken from the aerobic zone of a "semi-enhanced culture" system fed 100 mg/ℓ acetate COD. The mixed liquor sample was subjected to a 4 h anaerobic batch test in the presence of excess acetate.

From the results in Table 7 it can be seen that almost 80% of the PCA extract complex P was degraded in the anaerobic period. This phosphate could be accounted for entirely as orthoP released to the supernatant, taking total P recovery into account. The PCA orthoP and residue fractions remained virtually unchanged before and after the anaerobic period. Thus, P release in the anaerobic test is derived solely from the PCA extract complex P (i.e. polyP). On release, this P moved to the supernatant as orthoP; very little was precipitated.

The data in Table 7 further corroborate the view that the cold PCA fractionation procedure is able to reliably distinguish between activated sludge orthoP (chemical precipitate) and complex P (mainly polyP) fractions. The observed P release (corresponding to 60% of the mixed liquor total P) was in approximate agreement with the finding by Wentzel et al. (1989) for fully enhanced cultures that about 70% of the mixed liquor total P could be released in the presence of excess acetate. *[The average extent of P release found in approximately twenty such anaerobic batch tests for mixed liquor samples from the control pilot plant in this study (data not shown here) was 62% of the mixed liquor total P].*

Inclusion of NaOH step in PCA fractionation procedure

The cold PCA fractionation procedure described in Table 2 was applied to two identical enhanced BEPR organism culture systems strongly exhibiting the BEPR mechanism. The two systems were operated in parallel: one system (R1, the test unit) was dosed with chemical precipitant, while the other (R2, the control unit) was not (De Haas et al., 2000b; c). During periods with chemical precipitant addition (alum or iron salts), for the test unit it was observed that the complex P content of the PCA extract decreased but the total P content of the residue (RES) fraction increased, compared to the control. This suggested that the metal ions encouraged the formation of a P fraction which was not extractable into cold PCA. A similar observation was noted in batch tests with $FeCl_3$ addition (Table 6). Röske and Schönborn (1994 a; b) and Psenner et al. (1984) noted that complexes/precipitates of Fe and orthoP or Fe and polyP are considered extractable into alkaline bicarbonate-dithionite solutions. *[Dithionite is a strong reducing agent]*. The Psenner extraction procedure (Röske and Schönborn, 1994 a; b) did not include a preceding acid step. For the purposes of this study, it was considered important to determine whether an increase in the RES fraction denoted an increase in the orthoP content of the sludge (as reported for iron addition by Röske and Schönborn, 1994 a; b), or whether this fraction represented an alkaline-extractable form of complex (poly) P. For this reason, an alkaline extraction step was included in the basic fractionation procedure. It was found that extraction into 1M NaOH over a fairly short period (30 min + 15 min + 15 min in three steps) extracted virtually all the residue phosphate.

Table 8 shows the effect of including the NaOH step in the fractionation procedure. It should be noted that problems were

TABLE 5
Fractionation results for samples of "enhanced culture" activated sludge (developed with influent acetate supplement) after addition of $FeCl_3$ with or without added dissolved orthoP in two batch tests. Refer to Table 4 for comparison with control sludges (before dosing)

Sample/ Extract	OrthoP		Total P		Complex P (Total P - OrthoP)	
	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS
100 mg/ℓ acetate 5/10/93 VSS = 2 350 mg/ℓ 3.23 mmol/ℓ Fe added as $FeCl_3$	**No P added**					
SUP.	0.29	0.12	0.73	0.31	0.44	0.19
PCA	**24.56**	**10.45**	**175.94**	**74.87**	**151.38**	**64.42**
RES.	-	-	86.86	36.96	-	-
Mixed liquor	-	-	**248.15**	**105.60**	-	-
Sum of extracts	-	-	263.53	112.14	-	-
Recovery	-	-	106%	106%	-	-
100 mg/ℓ acetate 5/10/93 VSS = 2 350 mg/ℓ 3.23 mmol/ℓ Fe added as $FeCl_3$	**50 mgP/ℓ added as K_2HPO_4**					
SUP.	5.57	2.37	5.73	2.44	0.16	0.07
PCA	**68.66**	**29.22**	**213.09**	**90.68**	**144.43**	**61.46**
RES.	-	-	93.72	39.88	-	-
Mixed liquor	-	-	**295.22**	**125.63**	-	-
Sum of extracts	-	-	312.54	133.00	-	-
Recovery	-	-	106%	106%	-	-
250 mg/ℓ acetate 31/10/93 VSS = 2 088 mg/ℓ 3.23 mmol/ℓ Fe added as $FeCl_3$	**No P added**					
SUP.	0.41	0.20	0.24	0.12	(-0.17)	0
PCA	**36.61**	**15.62**	**247.44**	**118.51**	**214.83**	**102.89**
RES.	-	-	58.19	27.87	-	-
Mixed liquor	-	-	**317.20**	**151.92**	-	-
Sum of extracts	-	-	305.87	146.49	-	-
Recovery	-	-	96%	96%	-	-
250 mg/ℓ acetate 31/10/93 VSS = 2 088 mg/ℓ 3.23 mmol/ℓ Fe added as $FeCl_3$	**50 mgP/ℓ added as K_2HPO_4**					
SUP.	12.93	6.19	13.45	6.44	0.52	0.25
PCA	**65.11**	**31.18**	**281.25**	**134.70**	**216.14**	**103.52**
RES.	-	-	62.41	29.89	-	-
Mixed liquor	-	-	**368.12**	**176.30**	-	-
Sum of extracts	-	-	357.11	171.03	-	-
Recovery	-	-	97%	97%	-	-

experienced in applying the vanadate-molybdate colorimetric method for orthoP to the NaOH extracts, even after pH neutralisation. These problems were overcome by using the molybdate-ascorbic acid method after neutralisation with suitable dilution of the NaOH extracts.

From Table 8 it can be seen that the unit with iron dosing showed significantly more total P in the RES fraction compared with the Control. However, with the NaOH step included, only about 1% of the mixed liquor total P remained in the residue. The NaOH extract contained small amounts of orthoP, but mainly complex P. The complex P forms extracted with NaOH were not determined but could be polyP associated in some manner with proteins or polysaccharides. High molecular weight extracellular polysaccharides are produced in large amounts in activated sludge and are known to have the propensity to bind metal ions (Brown and Lester, 1979).

The full fractionation procedure

With the inclusion of the NaOH step in the basic PCA fractionation procedure, the full procedure is given in Table 9, along with the interpretation thereof.

Testing the full fractionation procedure

In order to test whether the results obtained for polyP using the fractionation procedure were reasonable, the results for the complex P fractions of the mixed liquor from the Control "enhanced culture" system (R2) were compared with the polyP content predicted using the UCTPHO model for biological P removal (Wentzel et al., 1992). In order to estimate the measured polyP content of sludge extracts, the complex P fractions of the PCA and NaOH (where applied) extracts were lumped, and an amount of 10 mgP/gVSS (De Haas, 1989) was subtracted to make allowance for the expected nucleic acid content (Fig. 3).

The results in Fig. 3 suggest that in cases where mass balances were satisfactory, good correlation between the modelled and estimated polyP content by fractionation was obtained. Thus, the fractionation procedure would seem to provide a useful analytical tool for calibration and validation of the mathematical models.

Effect of iron addition during fractionation procedure

As noted previously from the results of batch tests (Table 6), iron dosing had a tendency to increase the fraction of "complex" P not extracted with cold PCA, but extracted in a

TABLE 6
P mass balances for the fractions obtained in $FeCl_3$ batch experiments (refer to Tables 4 and 5)

FRACTION	ΔOrthoP mgP/gVSS	ΔComplex P mgP/gVSS	ΔOrthoP mgP/gVSS	ΔComplex P mgP/gVSS
Date: / Batch test:	5/10/93 Fe only	5/10/93 Fe only	5/10/93 P + Fe	5/10/93 P + Fe
SUP	- 4.77	0.06	- 2.52	- 0.06
PCA	+ 3.78	- 8.16	+ 22.55	- 11.52
RES	ND	+ 7.63	ND	+ 10.55
Σ	-0.99	- 0.47	+ 20.03	- 0.57
Σ overall	- 1.46		+ 19.46	
(Recovery %)	(N/A)		(92%)	
Δ Mixed liquor total P	+ 0.53		+ 20.74	
(Recovery %)	(N/A)		(97%)	
P added	0		21.28	
Date: / Batch test:	31/10/93 Fe only	31/10/93 Fe only	31/10/93 P + Fe	31/10/93 P + Fe
SUP	- 13.04	- 0.34	- 7.04	- 0.09
PCA	+ 9.60	- 6.61	+ 25.16	- 5.98
RES	ND	+ 9.05	ND	+ 11.07
Σ	- 3.44	+ 2.10	+ 18.12	+ 5.00
Σ overall	- 1.34		+ 23.12	
(Recovery %)	(N/A)		(97 %)	
Δ Mixed liquor total P	- 0.53		+ 24.91	
(Recovery %)	(N/A)		(104%)	
P added	0		23.95	

(N/A) : Not applicable
ND: Not determined

TABLE 7
Fractionation results before and after batch anaerobic P release test with excess acetate addition. Mixed liquor sample taken from semi-enhanced culture system fed 100 mg/ℓ acetate COD.

Sample/ Extract	OrthoP		Total P		Complex P (Total P - OrthoP)	
	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS	mgP/ℓ	mgP/gVSS
BEFORE 22/10/93 VSS = 2 522 mg/ℓ						
SUP.	16.28	6.46	16.68	6.61	0.40	0.16
PCA	**18.30**	**7.26**	**191.26**	**75.84**	**172.96**	**68.58**
RES.	-	-	40.85	16.20	-	-
Mixed liquor	-	-	**275.34**	**109.18**	-	-
Sum of extracts	-	-	248.33	98.65	-	-
Recovery	-	-	90%	90%	-	-
AFTER 22/10/93 VSS = 2 522 mg/ℓ						
SUP.	166.43	65.99	163.14	64.69	(-3.29)	0
PCA	**19.99**	**7.93**	**57.83**	**22.93**	**37.84**	**15.00**
RES.	-	-	42.16	16.72	-	-
Mixed liquor	-	-	**275.34**	**109.18**	-	-
Sum of extracts	-	-	**263.13**	104.33	-	-
Recovery	-	-	96%	96%	-	-
PolyP estimate from anaerobic P release :					*150.15*	***59.54***

TABLE 8
Effect of inclusion 1M NaOH step in fractionation procedure. Mixed liquor samples from laboratory-scale units fed settled sewage containing 150 mg/ℓ acetate COD. $FeCl_3$ dose to R1 was 10 mg/ℓ as Fe. Precipitant not dosed to Control (R2).

Sample/ Extract	OrthoP		Total P		Complex P (Total P - OrthoP)	
	% Total P	mgP/ℓ	% Total P	mgP/ℓ	% Total P	mgP/ℓ
4/7/95, R1						
$FeCl_3$ dosed						
VSS = 2 396 mg/ℓ						
SUP. (filtered)	3%	16.40	-	17.72	-	-
SUP. (unfiltered)			4%	18.21	<1%	1.81
PCA (filtered)	26%	134.90	(73%)	374.01	47%	239.11
PCA (unfiltered)			79%	399.60	-	-
RES.	-	-	**17%**	**85.63**	-	-
Mixed liquor	-	-	-	**509.34**	-	-
Sum of extracts	-	-	-	503.44	-	-
Recovery	-	-	99%	-	-	-
4/7/95, R2						
CONTROL						
VSS = 2 499 mg/ℓ						
SUP. (filtered)	5%	20.43	-	21.82	-	-
SUP. (unfiltered)		-	5%	21.82	<1%	1.39
PCA (filtered)	9%	39.07	(81%)	352.36	72%	313.29
PCA (unfiltered)			84%	364.17		
RES.	-	-	**13%**	**57.09**	-	-
Mixed liquor	-	-	-	**433.89**	-	-
Sum of extracts	-	-	-	443.08	-	-
Recovery	-	-	102%	-	-	-
18/7/95, R1						
$FeCl_3$ dosed						
VSS = 2 184 mg/ℓ						
SUP. (filtered)	2%	11.11	-	11.08	-	-
SUP. (unfiltered)		-	2%	12.26	<1%	1.15
PCA (filtered)	27%	139.37	(77%)	397.50	50%	258.13
PCA (unfiltered)			79%	403.44		
NaOH	2%	9.5	**18%**	**93.35**	16%	83.85
RES.	-		**1%**	**6.92**	-	-
Mixed liquor	-		-	**514.19**	-	-
Sum of extracts	-		-	515.97	-	-
Recovery	-		100%	-	-	-
18/7/95, R2						
CONTROL						
VSS = 2675 mg/ℓ						
SUP. (filtered)	5%	26.08	-	23.93	-	-
SUP. (unfiltered)			5%	25.51	0%	0
PCA (filtered)	9%	45.12	(82%)	431.12	73%	386.00
PCA (unfiltered)			85%	450.90		
NaOH	1%	4.00	**6%**	**30.32**	5%	26.32
RES.			**1%**	**3.96**	-	-
Mixed liquor			-	**529.02**	-	-
Sum of extracts			-	510.69	-	-
Recovery			97%	-	-	-

Note: Small errors in total P recovery (5%) unavoidably occur where filtered/ unfiltered total P results differ significantly.

subsequent alkaline step. This tendency was noted, to a variable degree also for dosing with alum, $FeCl_3$ and a $FeCl_2$-$FeCl_3$ blend, both at pilot scale and full scale.

It was hypothesised that the observed effect of metal ions on complex P extraction patterns in acid vs. alkali may be an artefact of the fractionation procedure. That is, the metal ions may result in the formation of complexes between biopolymers (e.g. polysaccharides; proteins; and polyP) which tend to be insoluble in cold PCA but are soluble in alkali. These complexes may be formed during the fractionation procedure, rather than in the activated sludge systems *per se*. In this hypothesis, metal ions would be solubilised in cold PCA from metal-orthoP or metal hydroxide precipitates formed in the activated sludge systems. The metal ions thus extracted would rapidly complex with polyP and other biopolymers. The biopolymers (e.g. proteins, polysaccharides and polyP) would be exposed to metal complexation to a greater degree as the cellular structure is disrupted due to denaturation in the PCA solution. Because of their relatively large physical size, such metal-complex P/biopolymer complexes would probably be precipitated during centrifugation after each PCA extraction step, and hence reach the NaOH extraction step. Solubilisation in NaOH at room temperature would follow.

In order to test the above hypothesis, an experiment was conducted in which $FeCl_3$ was added directly to the 0.5M PCA solution used in the fractionation procedure and compared to a parallel control fractionation conducted as normal with 0.5M PCA (refer to Table 9). For both the test (ferric added to PCA *in vitro*) and control (no ferric added to PCA), the mixed liquor source was the last aerobic reactor of the R2 pilot plant system which had never received simultaneous chemical addition, but which received feed containing supplemental sodium acetate (100 mg/ℓ COD), and which exhibited BEPR.

The amount of $FeCl_3$ added to the PCA extract in the test fractionation was based on an estimated daily dose of 6.2 mmol Fe/d to a pilot plant system with a process volume of 32ℓ, operating at a 10 d sludge age. Assuming that all the metal dosed would be bound in the mixed liquor (Rabinowitz and Marais, 1980) the expected metal concentration in the PCA extract could be calculated, taking into account the volume of PCA used and the associated dilution. This gave an estimate of 225 mg Fe/ℓ in the PCA extract. In the test fractionation, $FeCl_3$ was added to a concentration very close to 220 mg Fe/ℓ in the 0.5M PCA solution. The $FeCl_3$ was added to the PCA solution prior to the fractionation procedure.

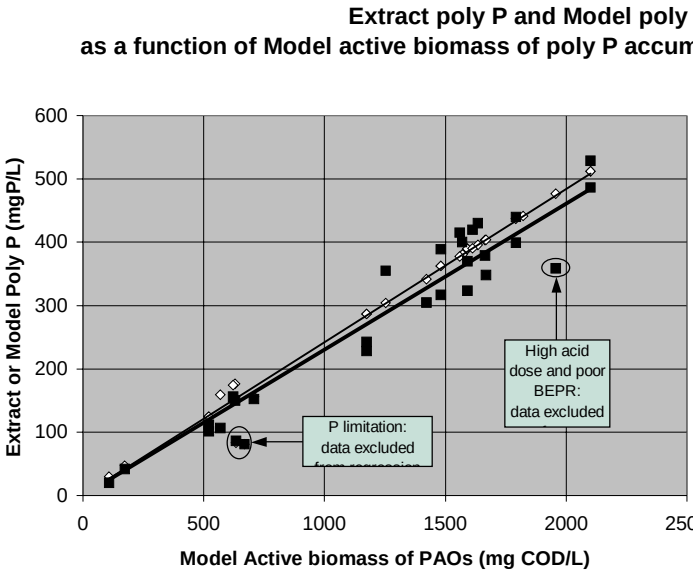

Figure 3

Relationship between estimated extract polyP (by fractionation) and polyP content predicted by UCTPHO model, plotted as a function of the modelled content of active polyP organism biomass. Encircled data excluded from the regression calculations, for reasons highlighted in the text box insert.

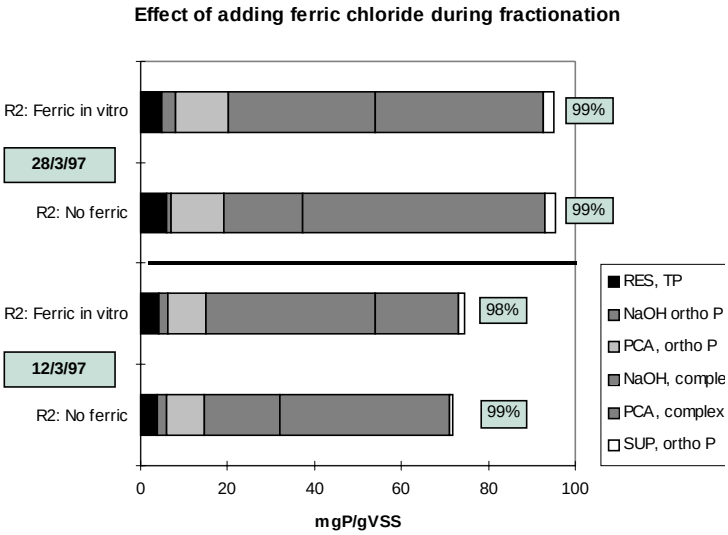

Figure 4

Fractionation pattern in the presence and absence of ferric chloride added to the 0.5M perchloric acid used in the PCA step. The mixed liquor used in this experiment was taken from a control reactor (R2) exhibiting BEPR but not dosed with metal precipitant. Percentage figures quoted are for % TP recovery in the fractionation procedure compared to the original mixed liquor.

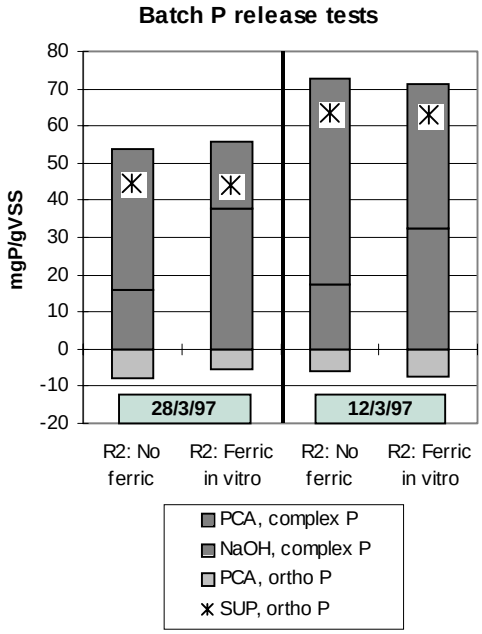

Figure 5

Results of P release batch tests for the fractionation experiments shown in Fig. 4. Results represent P release from respective fractions during anaerobic batch test (after-before) in the presence of excess acetate. Negative release represents apparent uptake (precipitation) in minor fractions.

The fractionation results are depicted in Fig. 4. From these results, it can be seen that addition of ferric ions *in vitro* via the PCA step of the fractionation procedure caused an "artificial" shift in the solubility of a portion of the complex P from the PCA to the NaOH fraction. It is most likely that the apparent shift in solubility from the acid to alkaline step is caused by metal ions present in the PCA step, whether solubilised from chemical precipitates in the starting mixed liquor solids, or added to the PCA solution (as in this experiment) .

Figure 5 shows the results of P release batch tests using the same mixed liquor samples represented in Fig. 4. Figure 5 shows that P release to the supernatant is in fact an observation of net release from the respective fractions. A minor fraction (the orthoP fraction of the PCA extract) showed negative release (uptake), presumably due to chemical precipitation. The net P release from the respective fractions

TABLE 9
Full PCA-NaOH fractionation procedure as applied to activated sludge.
Pre-concentration step (Step 0) is optional.

Step	Procedure	Sample preparation and analysis	Interpretation
0	Pre-thickening of mixed liquor: For starting VSS = ca. 1 000 mg/ℓ, take 2 ℓ mixed liquor and allow to settle in a measuring cylinder. When sludge has settled, withdraw 1 200 mℓ supernatant and discard. Re-suspend solids into remaining 800 mℓ using magnetic stirrer.	Mixed liquor should contain ca. 2 500 mg VSS/ℓ. *Note 1*	Allows suitable dilution of extracts to ensure minimal interference in orthoP and total P determination from ionic strength, colour and turbidity.
1	Transfer one aliquot (50 mℓ) of well stirred mixed liquor to each of two centrifuge tubes with screw cap lids for sealing.	Analyse original mixed liquor for: • total P • MLSS, VSS	Recovery of summed extract total P will be related to mixed liquor total P. Allows extract P content to be expressed on VSS basis.
2	Centrifuge at 3 000 r/min (max. 2 000 *g*) for 5 min. Collect and pool supernatant and retain pellet.	Label supernatant *SUP*. Retain.	Supernatant corresponds to point of sampling.
3	Add 0.9% (m/v) NaCl (10 mℓ) to pellet in each tube. Cap and shake well to mix/ re-suspend.		"Interstitial" loosely bound phosphate washed out with osmotically neutral saline.
4	Centrifuge at 3 000 r/min (max. 2 000 *g*) for 5 min. Collect and pool with supernatant with that marked *SUP (step 2).* Retain pellet.	Filter about half *SUP* sample through ashless filter paper (Whatman No. 41 or MN 615). *SUP* and *filtered SUP* (*fSUP)* analysed for • orthoP immediately • total P Correct by a factor of 1.2 for 60 mℓ extract per 50 mℓ original mixed liquor.	Loosely bound phosphate extracted with saline is pooled with that present in the original supernatant.
5	Extract three times with ice-cold 0.5 M PCA (20 mℓ) in a refrigerated water bath (0 to 3°C) for 5 min each time. Centrifuge between extractions (as before but preferably with centrifuge refrigerated at 5°C) and keep the extracts ice cold. Retain residue.	Pool extracts and label *PCA*. Filter about half *PCA* sample (see step 4). *PCA* and *filtered PCA* (*fPCA)* analysed for: • orthoP **immediately** • total P Correct by a factor of 1.2 for 60 mℓ extract per 50 mℓ original mixed liquor.	Acid-soluble complex P (polyP and nucleic acids) extracted as well as chemical precipitates (orthoP). *Note 2*
6	Extract three times with 1 M NaOH (20 mℓ) at room temperature (e.g. 30 min; 15 min, 15 min). Centrifuge as before between extractions with centrifuge at room temp.	Pool extracts (brown coloured) and label *NaOH*. Analyse for: • total P (*Note 3)* • orthoP (*Note 4)* Correct by a factor of 1.2 for 60 mℓ extract per 50 mℓ original mixed liquor.	Formation of phosphate compounds extractable into NaOH appeared to be favoured by dosing of iron salts. Minor fraction of orthoP occurs in this extract; mainly complex P is extracted. PolyP or metal hydroxide complexes with proteins or other biopolymers suspected.
7	Residue resuspended by serial washing of centrifuge tube in a total of 50 mℓ distilled water per centrifuge tube.	Analyse residue (*RES)* for total P. *Note 1*	Minor fraction of P compounds not extractable into cold PCA or NaOH. Allows check for overall P mass recovery.
8	Expose original mixed liquor to excess acetate under anaerobic conditions for 4 h.	Add acetate in excess of requirements for denitrification and P release. *Note 5*	Anaerobic batch P release test indicates which complex P fraction(s) are principally labile in the BEPR mechanism.
9	Repeat Steps 1 through 7 on mixed liquor after P release.	-	-

Note 1: Take precaution to mix sample well and perform determinations in duplicate at least.
Note 2: "Fatty" fragments may float in the PCA extract but these have a small total P content, as judged from the difference: *PCA- fPCA.*
Note 3: Neutralise extract with 1M HCl.
Note 4: Use molybdate-ascorbic acid method on neutralised, diluted sample (e.g. 25-fold dilution).
Note 5: For denitrification allow 8.6 mg COD/mg NO_3-N. For P release allow 2 mg COD/mg P released.

compares well with the release of orthoP to the supernatant. However, in the Test procedure, with ferric ions added to the PCA solution, P release from the NaOH complex P fraction appeared to be more significant, compared to the Control procedure using the normal PCA solution.

The data in Figs. 5 and 6 support the hypothesis that the relative distribution of complex P (or polyP) between acid or alkaline soluble fractions is of little significance in the biological P removal mechanism, but must be seen (at least in part) as an artefact of the fractionation procedure. The primary value of crude chemical fractionation techniques such as that used here (Table 9) is to broadly distinguish chemically precipitated orthoP from complex P fractions (including polyP) of biological origin. The data presented here do not rule out the possibility that complexes between metal ions, phosphates and biopolymers can exist in nature, including the mixed liquor of activated sludge systems.

Conclusions

In this paper, methods were investigated for quantifying the biological vs. chemical P fractions in activated sludge. These methods require total P and orthoP measurement and these were evaluated. The importance of aspects such as the range of the method for measurement of orthoP, and the concentration of acid or persulphate during the digestion step for total P were highlighted when dealing with mixed liquor as opposed to wastewater samples. A P fractionation procedure based on extraction with perchloric acid (with an optional subsequent extraction step using alkali) was tested using activated sludge samples subjected to various conditions which influenced biological or chemical P removal in batch tests or continuous-flow laboratory-scale systems. The P fractionation procedure appeared to be capable of broadly distinguishing between chemical and biological forms of stored phosphorus in activated sludge. The P fractionation results for estimated biological polyP content showed satisfactory agreement with the results predicted with a mathematical kinetic model applied to such systems. It may be concluded that the chemical fractionation procedure developed would be useful in measuring the relative sizes of chemically-bound versus biologically-stored forms of phosphorus in activated sludge systems with or without simultaneous addition of metal precipitants. However, caution in interpretation of the fractionation data is advised since artefacts imposed by the fractionation procedure itself may be difficult to avoid. In particular, it appears that the distribution of so-called complex P (i.e. mainly polyP) between acid and alkaline fractions is heavily influenced by the presence of metal ions (e.g. Fe^{3+}).

In subsequent papers in this series (De Haas et al., 2000b,c), the fractionation procedure developed here will be applied to pilot plants operated to test the effect of addition of alum or $FeCl_3$ on the biological P removal mechanism.

Acknowledgements

This work was conducted while the principal author was employed by Umgeni Water (South Africa). The financial assistance and support of Umgeni Water is gratefully acknowledged. The technical assistance of the following persons is also gratefully acknowledged: Paul Gaydon, Gordon Borain, ZT Jikazi, Emma Parkhouse, Rose Smith and Taliep Lakay, the last mentioned at the University of Cape Town.

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