Significance of fractionation methods in assessing the chemical form of phosphate accumulated by activated sludge and an *Acinetobacter* pure culture

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Abstract

Two chemical fractionation procedures aimed at the determination of phosphorus compounds were used to analyse the same activated sludge sample from a five-stage modified Bardenpho plant. In both procedures, taking due account of polyphosphate interference in the orthophosphate (orthoP) assay and polyphosphate (polyP) hydrolysis during acid extraction, at least 14% of the total phosphorus (totalP) was measured to be orthoP. Since intracellular orthoP levels were estimated to be negligible, it was concluded that the origin of the measured orthoP was chemical precipitates. Thus chemical precipitates of orthoP can form naturally in modified activated sludge systems not dosed with chemical precipitants, and, for the sludge tested, appeared to make a minor contribution to the overall P-removal. Comparing the two fractionation procedures, different chain lengths of polyphosphate (polyP) were measured in trichloroacetic and in perchloric acid extracts of the same sample. Furthermore, the chain lengths of polyP measured in subsequent (alkaline) steps were not greater than those found in the acid step. From these observations it was concluded that classification of acid-soluble polyP as "low molecular" and acid-insoluble polyP as "high molecular" is not justified. Also, quantification of acid-soluble and acid-insoluble polyP extracted from activated sludge was challenged since it was found that the distribution of polyP between the two fractions was influenced by the nature of the extractant and by hydrolysis of polyP to orthoP during extraction. Attention was also given to the occurrence of chemically precipitated orthoP in a stationary phase culture of *Acinetobacter* at pH ca. 9. Such precipitation occurred to a much lesser degree in an early log phase culture which had remained at pH ca. 7. Accordingly, it was recommended that metabolic studies of polyP formation by this organism should be conducted in a medium with pH control near neutrality.

Introduction

Modified Bardenpho and UCT activated sludge systems were designed for enhanced biological phosphorus removal as well as nitrogen and carbon removal from waste water (Barnard, 1976; Siebritz *et al.*, 1980). Characterisation of the forms of accumulated phosphorus in the activated sludge of such systems has been the subject of numerous studies (Fuhs and Chen, 1975; Buchan, 1981, 1982; Kerdachi and Roberts, 1985; Lötter, 1985; Mino and Matsuo, 1985; Mino *et al.*, 1985; Murphy and Lötter, 1986; Kerdachi and Healey, 1987; De Haas, 1989a). In most of these studies, polyphosphate (polyP) has been found to constitute the major storage form of phosphorus in the biomass with nucleic acids being quantitatively the next most important biochemical form of phosphorus. Mino *et al.* (1985), from chemical fractionation and radiotracer studies of activated sludge, concluded that two principal forms of polyP exist in the sludge microorganisms; "low molecular polyP (L-PP)" and "high molecular polyP (H-PP)". They derived the designation of these two "pools" from two fractions in their fractionation procedure: the L-PP was extracted with cold 0,5M perchloric acid (PCA) and determined as non PO_4-P, while H-PP was extracted with hot PCA and, after removal of nucleic acids by carbon adsorption, determined as labile P by degradation with 1N sulphuric acid at 100°C.

Experiments performed by Mino *et al.* (1985) on ^{32}P-radioisotopes produced evidence of a turnover of phosphorus in H-PP under aerobic conditions but not (or to a lesser extent) in L-PP. Under anaerobic conditions, turnover in neither polyP form occurred. Mino *et al.* (1985) concluded that H-PP and L-PP fulfil different physiological functions. Because of the observed aerobic turnover observed in H-PP, it was considered to function as the phosphorus pool for microbial growth. (They noted, however, that since uptake and release of P to, or from, H-PP was found in some batch experiments, it was unclear whether H-PP served solely as a phosphorus pool). On the other hand, the L-PP was considered to function as the stored energy pool of the microbial cells. The concept of these two distinct polyP forms has not been incorporated in the biochemical models for enhanced P-removal (Comeau *et al.*, 1986; Wentzel *et al.*, 1986). The first aim of this paper is to investigate the validity of the conclusions drawn by Mino *et al.*, (1985) with the objective of assessing whether these two distinct forms of polyP should be included in future biochemical models.

The second aim of this paper is to investigate whether chemical fixation of orthoP in the activated sludge biomass plays a part in enhanced biological phosphorus removal from waste water. Numerous researchers (Fuhs and Chen, 1975; Arvin and Kristensen, 1985; Kerdachi and Roberts, 1985; Lötter, 1985; Mino and Matsuo, 1985; Mino *et al.*, 1985; Murphy and Lötter, 1986; Kerdachi and Healey, 1987; De Haas, 1989a) have found that variable amounts of orthoP are extractable from activated sludge with cold 0,5 M PCA, cold 0,061 M trichloroacetic acid (TCA) or 0,05 M EDTA. The origin of this orthoP has been ascribed to chemical precipitation (Arvin and Kristensen, 1985; Mino and Matsuo, 1985; Mino *et al.*, 1985; Kerdachi and Roberts, 1985; Kerdachi and Healey, 1987) and to "intracellular" orthoP (Lötter, 1985; Murphy and Lötter, 1986). Clearly, a re-examination of the magnitude and likely origin of orthoP in extracts obtained from activated sludge would be expedient. Any such re-examination should take due account of criticisms of previous experimental techniques: The storage of activated sludge samples by freezing (Arvin and Kristensen, 1985) has been criticised by De Haas and Dubery (1989); the possible interference of polyP in the orthoP assay (De Haas *et al.*, 1980a) and the possible hydrolysis of polyP to orthoP during acid extraction (Kerdachi and Roberts, 1985) have generally not been taken into account in previous studies.

In this paper the magnitude and likely origin of orthoP in extractions of activated sludge and pure culture samples are to be investigated, taking cognisance of criticisms of previous experimen-

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tal techniques, with the objective of assessing the role of chemical fixation of orthoP in enhanced P removal.

Materials and methods

All glassware was washed in dilute hydrochloric acid (ca. 0,01 M) and thoroughly rinsed with distilled water.

Activated sludge samples

A sample (2,0 ℓ) was withdrawn from the primary aerobic zone of the five-stage modified Bardenpho system at Goudkoppies Works (Johannesburg) on 25 April 1988. In the period 4 March to 29 April 1988, this plant had removed orthoP from a mean influent concentration of 6,6 mgP/ℓ to a mean effluent concentration of 0,2 mgP/ℓ. Chemical oxygen demand (COD) was removed from a mean influent concentration of 462 mg O_2/ℓ to a mean effluent concentration of 73 mg O_2/ℓ. Aspects of this plant's performance have been discussed by Pitman *et al.* (1988). Fractionation **Procedures A** and **B** (see below) were applied in parallel to the above-mentioned sludge sample.

Pure culture

Acinetobacter strain 210A was obtained by donation from the late Dr M H Deinema (Dept. of Microbiology, Wageningen Agricultural University, The Netherlands). It was transferred to agar plates of sodium acetate medium (Van Groenestijn, 1988) and grown for 48 h at 20°C before being transferred to sterile broth (two aliquots of 50 mℓ each) of the same sodium acetate medium. After 18 h growth in this broth, the resulting precultures were used to inoculate two batch reactors each containing ca. 1,45 ℓ sterile sodium acetate medium and each equipped with sintered glass diffusers for aeration as well as with magnetic stirrer bars. The culture in one reactor was allowed to grow under stirred, aerated conditions at 21°C for 9,5 h, while the other was grown under identical conditions for 25 h. Previous experiments with this strain had shown that the former culture would reach logarithmic phase, while the latter would have entered stationary phase. At the end of the relevant growth period, the pH of each culture was measured. Fractionation then commenced according to **Procedure B** (see below). In the case of the stationary phase culture, 400 mℓ served as starting material for the fractionation, while 1 200 mℓ of the log phase culture was used. For the pure culture fractionation, all centrifugations were carried out at 11 000 x g at ca. 10°C for 15 min. Dry weight determinations were performed gravimetrically: a cell pellet (obtained by centrifuging a suitable aliquot of culture) was washed twice with distilled water and dried in a crucible at 105°C, followed by ashing at 550°C to determine the volatile suspended solids (VSS) concentration.

Fractionation procedures

Procedure A

Unless otherwise specified, all centrifugations were performed at room temperature for 5 min with a maximum acceleration of 2 000 x g. Cold extractions took place in a water bath containing ethylene glycol as anti-freeze and equipped with a thermostat. The following steps were performed sequentially:

(a) Four aliquots of original sludge (200 mℓ each) were centrifuged. The VSS of a fifth aliquot was determined using Whatman GF/C in at least duplicate (Standard Methods, 1985). Portions (ca. 25 mℓ) of the supernatant from each aliquot were pooled, designated 'supernatant' and saved for analysis.

(b) The pellets from step (a) were evenly resuspended in 0,9% NaCl (25 mℓ per tube) to give a concentrated sludge and to wash it simultaneously. The four concentrated sludge samples were pooled and at least duplicate aliquots (5 mℓ) were withdrawn for VSS determination as before.

(c) Four aliquots (20 mℓ each) of concentrated sludge were transferred to extraction tubes (nominal volume 26 mℓ) and centrifuged. The supernatants were pooled and designated "NaCl wash".

(d) The pellets from step (c) were extracted five times for 5 min each at room temperature with 0,05 M EDTA, the extracts being pooled.

(e) The pellets from step (d) were extracted three times for 30 min each at 0°C with 61 mM TCA, the extracts being pooled.

(f) The pellets from step (e) were resuspended in cold distilled water. The pH was adjusted to between 9 and 10 with 1 M NaOH followed by extraction for 30 min at 0°C. This was followed by two further 30 min extractions with distilled water, the pH being checked to be between 9 and 10 with adjustment as before if necessary. The extracts from this step were pooled and designated "dilute NaOH".

(g) The pellets from step (f) were extracted for 60 min at 0°C with 1 M NaOH. This was followed by overnight extraction at room temperature with 1 M NaOH and, finally, again for 30 min the next morning. The 1 M NaOH extracts were pooled.

(h) The residues were each resuspended in distilled water (40 mℓ per extraction tube) and saved in the suspended state.

All extracts were stored at ca. 5°C until analysis. OrthoP analyses and gel chromatography of the acidic extract commenced as soon as possible after extraction.

Procedure B

This procedure differed from procedure A only in that step (d) was absent and step (e) was replaced with four extractions of 30 min each at 0°C with 0,5 M PCA, the extracts being pooled.

OrthoP analyses

Extracts were filtered through Whatman GF/C filters to remove particulate matter, and diluted with distilled water as necessary. For all extracts except those from the alkaline steps, the molybdate-ascorbic acid method for orthoP assay (Standard Methods, 1985, as modified by De Haas *et al.*, 1990a) was used with a suitable dilution (10 to 100-fold), so that only positive polyP interference was expected (De Haas *et al.*, 1990a) and that the linear range of the orthoP assay applied.

The orthoP content of the alkaline extracts could not be determined by the above-mentioned method since the strongly acidic molybdate reagent resulted in turbidity due to proteins precipitated from the extracts. Accordingly, the method of Chifflet *et al.* (1988), which tolerates up to 1 000 mg/ℓ bovine serum albumin, was used for these extracts. Neutralisation of the extracts to a pH of 7 (± 1) was found to be of importance in this method.

In expressing the results of the orthoP analyses, compensation was made for the estimated maximum polyP interference in the orthoP assay and polyP hydrolysis to orthoP in the acidic extracts at 0°C. These estimates were based upon *in vitro* tests using Graham's salt and have been detailed by De Haas (1989b).

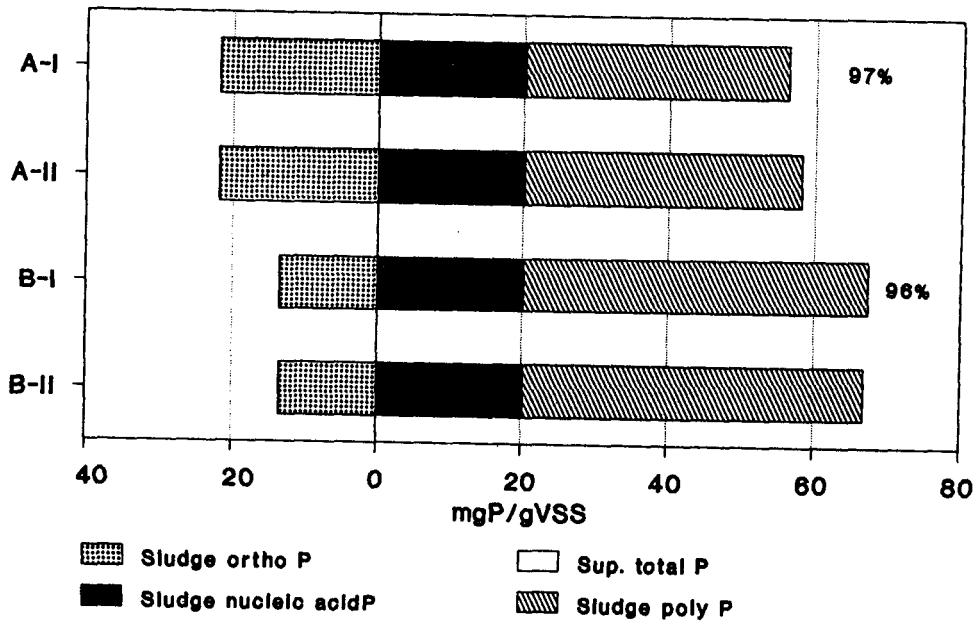

Figure 1

Fractionation pattern of Goudkoppies activated sludge sample (25/4/88). A: ***Procedure A;*** *B:* ***Procedure B;*** *I : 7-min polyP determination; II: polyP determination by nucleic acid difference (see text for details). Per cent recovery of totalP in extracts relative to original whole sludge given beside bars. Supernatant (Sup.) totalP = 0,14 mgP/g VSS in each case.*

PolyP and nucleic acid analyses

PolyP was estimated by two methods. The first made use of 7-min hydrolysis in 1 M HCl at 95°C (boiling water) as described by De Haas *et al.* (1990b). Filtration (Whatman 41) after hydrolysis was necessary for most samples. By this method:

$$\text{PolyP} = \text{7minP} - \text{orthoP}$$

The second method involved RNA and DNA determination by means of colorimetric methods using orcinol and diphenylamine respectively (De Haas, 1989b). By this method:

$$\text{PolyP} = \text{totalP} - (\text{orthoP} + \text{RNA P} + \text{DNA P})$$

since the phospholipid content of the extracts was found to be negligible (De Haas, 1989b).

TotalP and metal cation analyses

The totalP content of the original sludge, extracts and residues was determined after persulphate digestion in 1 M H_2SO_4 and 5% $(NH_4)_2S_2O_8$ (De Haas *et al.,* 1990a). Filtration (Whatman 41) after digestion was necessary for most samples.

Metal cations were determined by atomic absorption spectrometry after digestion in the same manner as for totalP except that the time of digestion was extended from 30 min to 120 min. Filtration (as for totalP) was still necessary after digestion for some samples.

Gel chromatography

A Sephadex G-25 or Sephadex G-25:G-50(1:1) column (1,6 cm x 98 cm) was used at room temperature with 50 mM Tris buffer (containing 0,1 M KCl and 0,2 g/ℓ sodium azide) as eluent at a flow rate of 30 mℓ) of extract (after filtration through Whatman no. 41) was applied using a sample applicator (Pharmacia) with glycerol (ca. 0,4 mℓ) added for density. One hundred fractions (2 mℓ each) were collected and subjected to the 7-min hydrolysis for polyP (see above) before being diluted to 8 mℓ with distilled water and analysed for orthoP (Standard Methods, 1985 -see above). Polyphosphate glasses (Sigma) were used for column calibration.

Plate counts

Viable bacterial plate counts of the Goudkoppies activated sludge sample were performed using GCY agar as described by Lötter and Murphy (1985) and Venter *et al.* (1989).

Results and discussion

Activated sludge fractionation

Fig. 1 depicts the results of fractionation **Procedures A** and **B** applied in parallel to the activated sludge sample from Goudkoppies. Table 1 shows the effect of compensating for potential polyP interference from two sources in the sludge orthoP result: from direct interference and from polyP hydrolysis. In the case of direct interference, the polyP concentration in a given extract was maximised by assuming the sludge orthoP contribution to be zero. The orthoP contributed by that level of polyP to the orthoP assay was then estimated (after taking dilution factors into account) on the basis of 4% positive interference in the orthoP assay (De Haas *et al.,* 1990a). In the case of polyP hydrolysis to orthoP during extraction, a maximum of 5% of the sludge orthoP measured was assumed to have originated from this source (De Haas, 1989b). The orthoP content of the NaCl wash was regarded as part of the supernatant fraction in these results.

TABLE 1
NET CHANGE IN EXTRACT ORTHO P LEVELS REGISTERED FOR GOUDKOPPKIES (25 APRIL 1988) ACTIVATED SLUDGE UPON COMPENSATION FOR MAXIMUM EXPECTED POLY P INTERFERENCE IN THE ORTHO P ASSAY (BOTH DIRECT AND DUE TO HYDROLYSIS DURING EXTRACTION). ALL FIGURES IN mgP/gVSS.

Procedure	Extract	Uncompensated orthoP	Net change due to maximum expected polyP interference in orthoP assay		Compensated orthoP
			Direct	Acid hydrolysis	
A	EDTA	12,796	-0,714	0	12,080
	TCA	6,983	-0,566	-0,345	5,982
	dilute NaOH	0,824	-1,005	0	-0,181
	NaOH	1,273	0	0	1,273
B	PCA	11,270	-1,877	-0,564	8,829
	dilute NaOH	1,410	-0,422	0	0,988
	NaOH	0,756	0	0	0,756

From Table 1 it is clear that polyP interference could account for an estimated maximum 12% of the sludge orthoP extracted by **Procedure A** and 21% of that extracted by **Procedure B.** It follows that the major part of the sludge orthoP (as shown in Fig. 1) originates from a source other than polyP. Fig. 1 also indicates that, relative to **Procedure B,** a larger portion of the sludge total P was extracted as sludge orthoP using **Procedure A** and a smaller portion as polyP. It is significant that the two methods for polyP estimation were in close agreement for both fractionation procedures. Thus it would appear that the sludge orthoP fraction is overestimated using **Procedure A.** From Table 1 it is evident that the EDTA extract contained the most orthoP in **Procedure A.** Since the EDTA step was carried out at pH 4,5 and, along with the intervening centrifugations, took close to 1 h at room temperature to complete, it is conceivable that some orthoP release from the bacterial cells occurred due to enzyme-catalysed polyP degradation under anaerobic conditions (Wentzel *et al.,* 1986). It is unclear whether EDTA itself or an endogenous compound served as substrate in this release.

Intracellular orthoP

From **Procedure B** (Fig. 1 and Table 1) at least 14% to 18% of the sludge totalP in the Goudkoppies sample was orthoP which could not be ascribed to polyP hydrolysis. The contribution of intracellular orthoP levels to the sludge totalP can be estimated using predictions from the bacterial cell counts for the same Goudkoppies sample:

The viable cell count recorded on GCY agar for this sample was 5,3 x 10^9 cells/g VSS. Cloete and Steyn (1988) reported that total cell counts of activated sludge (by direct microscopy using 0,22 μm filters and acridine orange staining) were at least two orders of magnitude greater than viable cell counts on GCY agar for the same samples. Hence, the total cell count for the Goudkoppies sample used in this study was probably close to 2,76 x 10^{11} cell/g VSS. From the results of Rae and Strickland (1975) in which an electrophoretic method was applied to *Escherichia coli* whole cells, intracellular orthoP may be accepted to be of the order of 5,6 nmol P/10^9 cells. Therefore, it may be concluded that the intracellular orthoP content of the above-mentioned Goudkoppies sample was 0,048 mgP/g VSS (or 0,06% of the sludge totalP). This estimate is valid, provided the intracellular orthoP concentration in the living (bacterial) world is fairly constant. Kulaev and Vagabov (1983) suggested that part of the function of polyP metabolism in microorganisms is to keep the intracellular orthoP levels low and stable. It is interesting to note that using estimates of intracellular orthoP concentration of human erythrocytes (Lehninger, 1975) and of typical bacterial cell volumes (Cloete *et al.,* 1985), an estimate of 2 nmol P/10^9 cells is obtained which agrees fairly well with the *E.coli* measurement by Rae and Strickland (1975).

Hence it would appear that intracellular orthoP levels of activated sludge are negligible in comparison with the total P content. It can therefore be concluded that, in contrast to the results of Murphy and Lötter (1986), the orthoP content of activated sludge extracts is due chiefly to chemical precipitation.

Sludge cation content

The concentration of selected metal cations present in the Goudkoppies activated sludge sample is shown in Table 2. Although the metals considered were largely extracted in the EDTA and TCA steps **(Procedure A)** or PCA step **(Procedure B),** it was not possible to deduce a simple formula for the chemical precipitate of orthoP present in the sludge, nor to discern the principal polyP counter-ion.

PolyP extraction

Table 3 shows that, for the same sludge sample, the major polyP fraction in **Procedure B** was extracted in PCA with a smaller fraction in dilute NaOH, whereas in **Procedure A** polyP was mainly extracted in dilute NaOH, with the preceding EDTA and TCA steps containing smaller polyP fractions. This strongly suggests that polyP extractability is primarily related to the nature of the extractant rather than to the cytological location or physiological function of the polyP.

TABLE 2
EXTRACTED METAL CATION OF GOUDKOPPIES ACTIVATED SLUDGE (25 APRIL 1988). FIGURES IN mg/mg VSS UNLESS OTHERWISE STATED (NA = NOT APPLICABLE)

Procedure	Extract		Metal	Ratio mol (+) / mol orthoP	Ratio mol (+) / mol polyP
A	EDTA	K	8,247	0,51	0,92
		Mg	4,124	0,82	2,24
		Ca	10,309	1,25	2,24
		Fe	21,650	2,82	5,07
	TCA	K	12,990	1,49	1,71
		Mg	8,041	2,97	0,16
		Ca	0,619	0,14	0,16
		Fe	3,169	0,87	1,00
	dilute NaOH	K	1,127	1,08	0,04
		Mg	2,818	8,75	0,04
		Ca	0,564	1,06	0,04
		Fe	1,348	2,72	0,11
B	PCA	K	18,364	1,29	0,43
		Mg	16,069	3,63	0,47
		Ca	10,330	1,42	0,47
		Fe	21,268	3,14	1,04
	dilute NaOH	K	1,182	0,66	0,08
		Mg	1,182	2,13	0,08
		Ca	0,591	0,65	0,08
		Fe	4,658	5,50	0,67
	Whole sludge	K	32,017	NA	NA
		Mg	23,285	NA	NA
		Ca	26,195	NA	NA
		Fe	38,046	NA	NA

To investigate this aspect further, the nature of the polyP in the various extracts was examined using gel chromatography. In Fig. 2, chromatograms for TCA **(Procedure A),** PCA **(Procedure B)** and dilute NaOH **(Procedure B)** extracts, from the same Goudkoppies activated sludge sample are shown; in Fig. 3 a calibration chromatogram is shown. From Fig. 2, two distinct polyP peaks are evident in the chromatogram for the PCA extract **(Procedure B),** while only one major peak is evident for the TCA extract **(Procedure A);** these results are in agreement with those of Clark *et al.* (1986). Furthermore, comparing Figs. 2 and 3, the polyP extracted by TCA **(Procedure A)** had a longer mean chain length than the polyP extracted by PCA **(Procedure B).** From these observations it can be concluded that since the extracts were obtained from the same sludge sample, polyP was hydrolysed to shorter fragments in the PCA extraction. Thus the label "low molecular polyP" attached to PCA-extracted polyP (Mino *et al.,* 1985) may have been based on an artifact, namely, the hydrolysis of polyP in the PCA extraction. Further clarification of this aspect can be obtained by comparing the chromatograms for the PCA extract with that for the dilute NaOH extract of **Procedure B** (Fig. 2) - the polyP in the dilute NaOH extract had a mainly fragmented chain length of similar chain length to that of the PCA extracts. Accordingly, it would seem largely futile to label the PCA-extracted poly P "low molecular polyP" and the alkaline-extracted polyP "high molecular polyP" as was done by Mino *et al.,* 1985 (the alkaline extraction in **Procedure B** being equivalent to the hot acid extraction of Mino *et*

TABLE 3
COMPARISON OF POLY P EXTRACTION PATTERNS FROM GOUDKOPPIES (25 APRIL 1988) ACTIVATED SLUDGE SAMPLE USING TWO DIFFERENT PROCEDURES (SEE TEXT). FIGURES IN mg P/g VSS

Procedure	Extract	polyP 7-min method
A	EDTA	7,109
	TCA	6,031
	dilute NaOH	21,133
	NaOH	1,479
B	PCA	34,081
	dilute NaOH	11,633
	NaOH	1,135

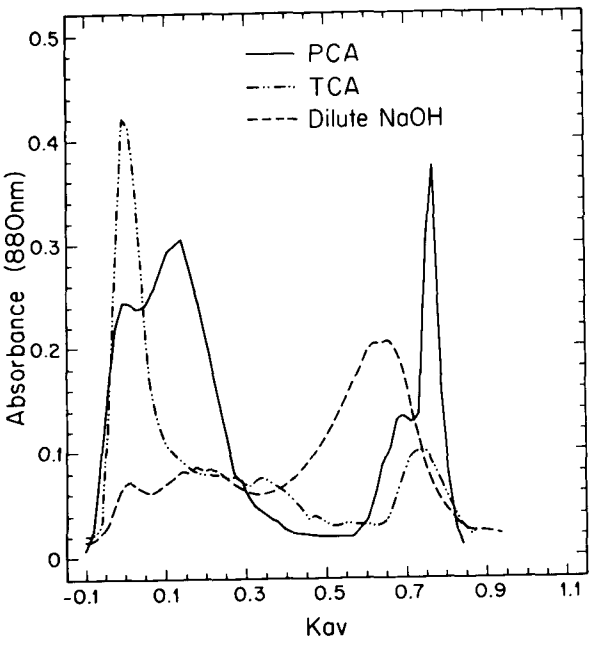

Figure 2
*Sephadex G-25 gel chromatogram of extracts of Goudkoppies activated sludge (25/4/88). TCA extract : **Procedure A**; PCA and dilute NaOH extracts : **Procedure B***

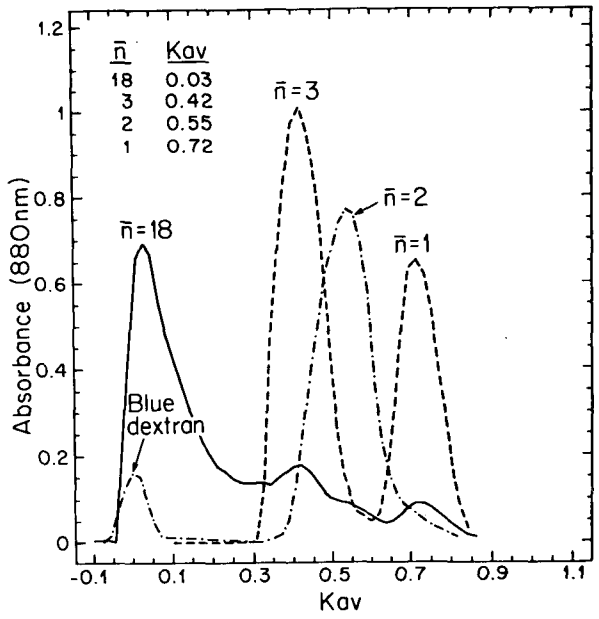

Figure 3
Sephadex G-25 gel calibration chromatogram. n : mean chain length of linear polyP species, where n = 1 for orthoP

al., 1985). However, it remains that two polyP fractions were obtained in the fractionation procedures, one fraction in the PCA (or TCA) extract (acid-soluble) and the other in the alkaline extract (acid-insoluble). As noted earlier, the distribution of polyP between these two fractions probably depends to a large degree on the nature of the extractant. Also, the distribution probably results to some extent from partial hydrolysis of the cells' polyP pool during extraction. Thus, ascribing physiological significance to the amount of polyP in either fraction would appear not to be justified for the present. Clearly this aspect of the fractionation procedure requires further investigation.

The chromatograms also provide information on hydrolysis of polyP in **Procedure A.** Previously (in the section **Activated sludge fractionation)** it was proposed that some enzyme-catalysed hydrolysis of polyP to orthoP occurred during extraction with EDTA in **Procedure A.** In this regard, the difference in the chromatograms (Fig. 2) for the PCA and TCA extracts (PCA extract polyP peaks having a mean chain length less than that of the TCA extract) suggest that, if enzyme-catalysed polyP degradation did occur during the EDTA extraction **(Procedure A),** it apparently was less random than the process of hydrolysis that occurred during PCA extraction **(Procedure B).** Less random hydrolysis results in cleaving off of terminal orthoP residues - this may explain why a higher orthoP content for the sludge was measured with **Procedure A,** than with **Procedure B.**

Finally, the chromatograms also provide information on the orthoP extracted from the sludge. Comparing Figs. 2 and 3, it is clear that variability occurred in the peaks eluted in the orthoP region of the chromatogram (K_{av} = 0,6 to 0,8). Allowing for an experimental error of 0,03 in K_{av}, this suggests that at least three orthoP complexes were present in the extracts (K_{av} = 0,62; 0,96 and 0,74 respectively). This and other details of such chromatograms have been discussed by De Haas (1989b).

Acinetobacter fractionation

At the time of harvesting, the early log phase culture of *Acinetobacter* had a pH of 7,1 while the stationary phase culture had a pH of 9,1. The log phase biomass had taken up 4,9 mg P/ℓ from the medium biomass, while the stationary phase biomass had taken up 82,1 mg P/ℓ. The log phase culture attained a cell concentration of 60 mg VSS/ℓ, while the stationary phase culture reached 843 mg VSS/ℓ. This gives 0,082 mgP/mg VSS for the log phase culture and 0,097 mg P/mg VSS for the stationary phase culture.

Since the log phase culture contained a large proportion of its total P in the supernatant, the supernatant and NaCl fractions were neglected in a comparison of the fractionation patterns of the two cultures. The results for P fractionation using **Procedure B** for the log phase and stationary phase cultures are shown in Fig. 4. From Fig. 4, comparing the fractionation patterns for the two cultures, it is evident that although the various fractions accounted for similar levels of totalP (P in biomass) in the two cultures, the fractionation patterns differed markedly: Relative to the log phase culture, the stationary phase culture contained a larger orthoP fraction and smaller polyP and nucleic acid fractions. The distribution of polyP in the various extracts is shown in Fig. 5 for the log and stationary phase cultures. From Fig. 5, it is evident that, for the stationary phase culture, only a small portion of the polyP (<2%) was extracted with PCA, while most of the orthoP (98%) was located in this extract; this implies that for the stationary phase culture, polyP interference in the orthoP assay (directly or due to hydrolysis during extraction) was negligible. For the stationary phase culture, the polyP was mainly extracted in the alkaline fraction (Fig. 5); this is in contrast to the results ob-

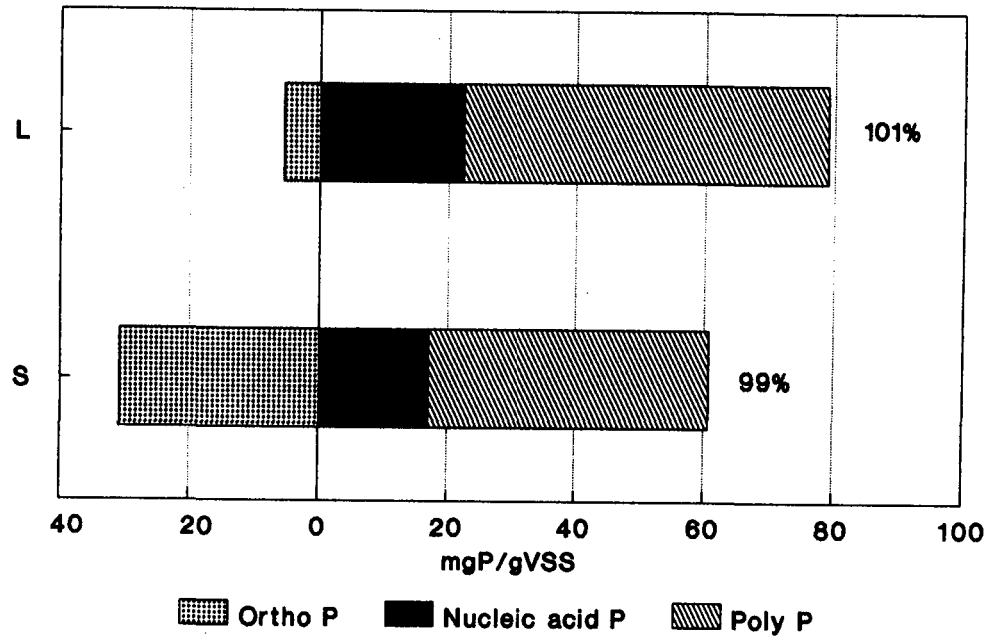

Figure 4
Fractionation pattern of Acinetobacter *210A cell cultures. L:Log phase culture; S : Stationary phase culture*

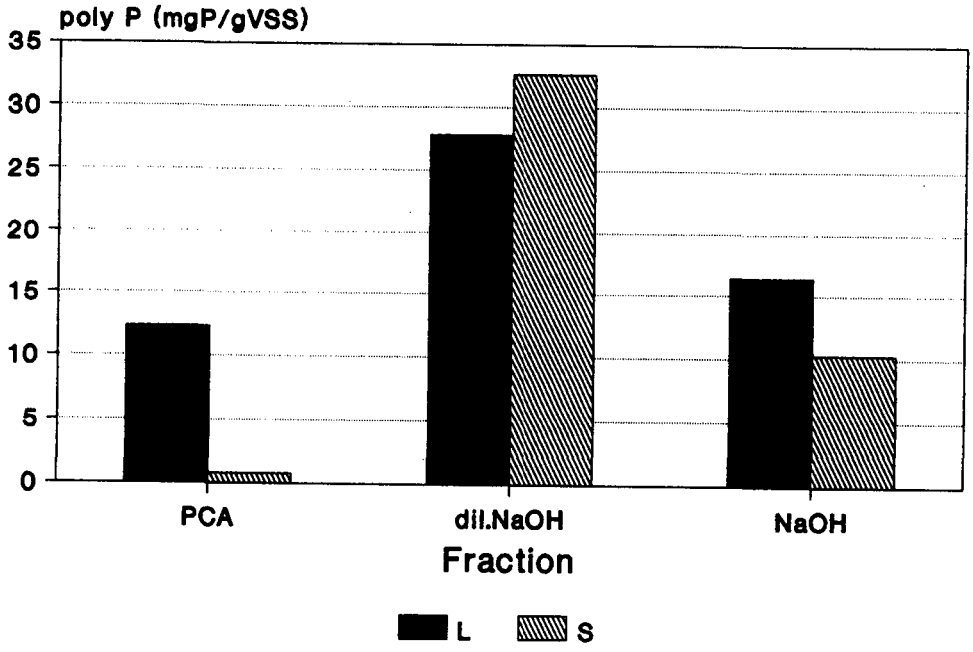

Figure 5
Distribution of polyP in extracts of Acinetobacter *210A cultures. L:Log phase culture; S:Stationary phase culture. Per cent recovery of totalP in extracts (relative to original culture) given beside bars*

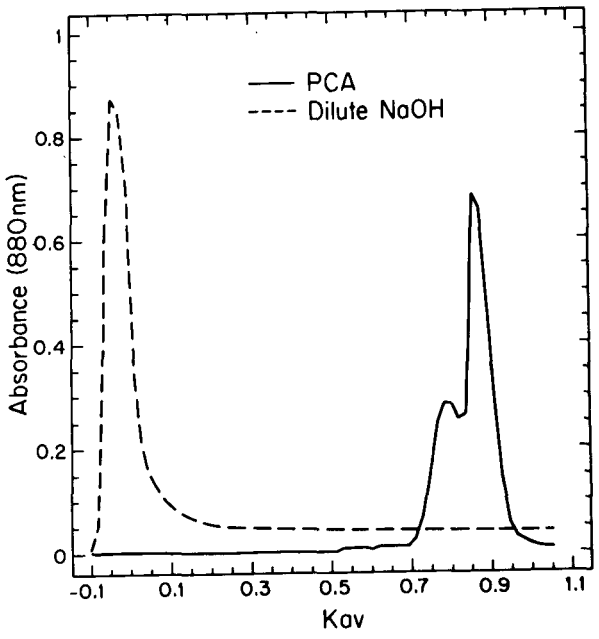

Figure 6
Sephadex G-25:50 gel chromatogram of extracts of Acinetobacter *210A stationary phase culture*

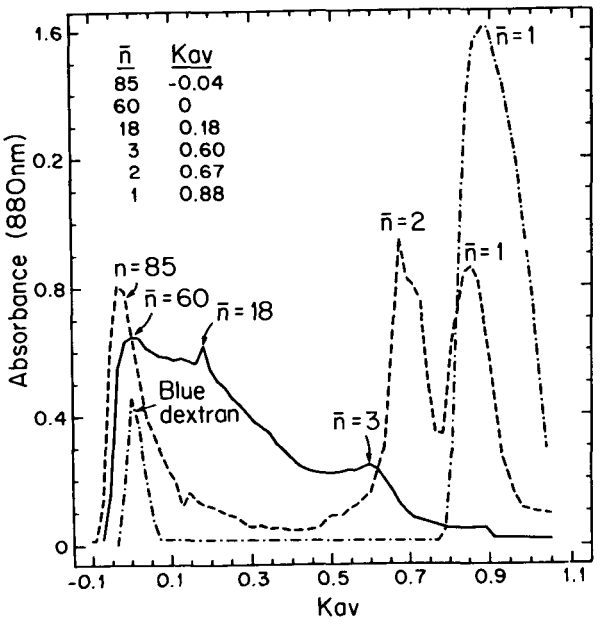

Figure 7
Sephadex G-25:50 gel calibration chromatogram. n = mean chain length of linear polyP species, where n = 1 for orthoP

tained with activated sludge (Fig. 2) where polyP mainly extracted in the PCA fraction. These observations are reflected in gel chromatograms of the extracts (Fig. 6, calibration chromatogram Fig. 7). Gel chromatography of the log phase cell extracts was not possible since the concentrations of orthoP and polyP in these extracts were too low. However, it was estimated that polyP interference accounted for 40% of the orthoP content of the log phase cells reported in Fig. 4.

The large orthoP content of the stationary phase culture strongly supports the finding by Gerber *et al.* (1987) that chemical precipitate can form in batch cultures of *Acinetobacter* in which the pH is uncontrolled. Since most of the P-uptake accompanies the transition from log phase to stationary phase growth during which the pH of the medium rises sharply, metabolic studies based on observations under these conditions *(inter alia* Deinema *et al.*, 1980; Brodisch, 1985; Ohtake *et al.*, 1985; Murphy and Lötter, 1986) are in error if chemical precipitate is ignored. This also applies to "enhanced cultures" fed pure fatty acid in laboratory-scale modified activated sludge systems (De Haas, 1989a; Wentzel *et al.*, 1988). PolyP formation (as described in the biochemical model of Wentzel *et al.*, 1986) will be measureable against orthoP uptake from the culture medium only if continuous pH control close to neutrality is applied (Van Groenestjin, 1988; Wentzel *et al.*, 1988). With regard to the precipitate formed in the stationary phase culture, the PCA extract contained 1,2 mol Mg/mol P. This suggests that $MgNH_4PO_4$ (struvite) was formed in stationary batch cultures of *Acinetobacter,* an observation also made by Eagle (1987).

Differences in the polyP extraction patterns between the log and stationary phase cultures (Fig. 4) and between these cultures and activated sludge (Table 3) support the argument presented previously that undue physiological significance should not be ascribed to the magnitude of the polyP in the various fractions extracted from a given sample. Quantification of the polyP in the various fractions may be justified if polyP hydrolysis can be shown to be absent during extraction (Clark *et al.*, 1986). For PCA-based procedures, which are superior to TCA-based procedures for solubilising chemical precipitate (De Haas, 1989b), the absence of such hydrolysis could not be demonstrated in this study using activated sludge samples.

Conclusions

- Although the major phosphorus fraction in a sample of activated sludge from a plant with a history of excellent P-removal was found to be polyphosphate, a minor fraction (18% of sludge total phosphate) was extracted as orthophosphate. Taking maximum polyP interference into account, the orthophoshate fraction represented at least 14% of the sludge total phosphate.
- The contribution of intracellular orthophosphate to the sludge total phosphate was estimated to be negligible (0,06% of the sludge total phosphate). Therefore it appears that chemical precipitates of orthophosphate form naturally in modified activated sludge systems not dosed with chemical precipitants, and therefore make a minor contribution to overall P-removal. The extent of that contribution may depend partly on the relative dominance of polyphosphate formation by the biological mechanism, and partly on chemical factors such as cation composition of the waste water and pH in the reactors.
- The mechanism of natural chemical precipitate formation in activated sludge was not investigated, but it may be biologically mediated (Arvin, 1985). It was not possible to deduce a simple formula for the precipitate (or for polyphosphate charge

neutralisation) on the basis of the metal cation content of the activated sludge extracts.

- Chemical precipitate of orthophosphate (possibly as struvite) formed readily in batch pure cultures of *Acinetobacter* when the pH was not controlled and rose to ca. 9. Metabolic studies based on observations under these conditions are in error if chemical precipitate is ignored. This also applies to "enhanced cultures" fed pure fatty acid in laboratory-scale modified activated sludge systems. Thus, pH control in such cultures is essential.
- Classification of acid-soluble polyphosphate as having a lower molecular weight than acid-insoluble polyphosphate could not be validated for cold perchloric acid-based extraction procedures applied to activated sludge. For the present, quantification of acid-soluble and acid-insoluble polyP would not seem justified as in this study such quantification was found to be influenced by the nature of the extractant and by polyP hydrolysis during extraction.

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