The effect of substrates enriched with protein on biological phosphate removal

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Abstract

The effect of substrates enriched with protein as compared to settled sewage on biological phosphate removal was studied in a laboratory-scale Bardenpho® unit, using the waste activated sludge phosphate concentration, P_w, calculated from the dynamic phosphate mass balance as performance criterium. A strong correspondence was found between the calculated P_w, and changes introduced in the feed composition. The easily biodegradable peptone and milk protein substrates were found to support enhanced phosphate removal significantly better than settled sewage. However, fresh protein substrate fed to sludge already adapted to it, temporarily decreased phosphate removal over the first day, followed by an increase over the next few days. The possible reasons for this phenomenon as well as the experimental findings are discussed.

Abbreviations:

F : Feed flow rate (ℓ/h)
F_w : Waste flow rate (ℓ/h)
V : Total unit volume (ℓ)
P_i : Influent phosphate concentration (mgP/ℓ)
P_a : Orthosphosphate concentration in the last anaerobic reactor (mgP/ℓ)
P_r : Phosphate actually released in the anaerobic stage (mgP/ℓ)
P_e : Effluent phosphate concentration (mgP/ℓ)
P_w : Waste mixed liquor phosphate concentration (mgP/ℓ)
P_{wss} : Steady state waste mixed liquor phosphate concentration (mgP/ℓ)
COD : Chemical oxygen demand (mgO_2/ℓ)
TKN : Total Kjeldahl nitrogen (mgN/ℓ)
MLSS : Mixed liquor suspended solids (mg/ℓ)
VSS : Volatile suspended solids (mg/ℓ)

Introduction

In the waste-water treatment field, biological phosphate removal is increasingly being considered as a viable alternative to chemical phosphate precipitation (Best, 1983; Raper, 1983). Its attraction lies in the utilisation of a biomass naturally growing on sewage in the form of activated sludge to remove a nutrient which is considered to be a major cause of eutrophication (i.e. extensive growth of undesirable microorganisms in rivers and lakes) (Slim, 1987). The efficient removal of phosphate from waste water presents the key strategy in controlling the eutrophication problem (Hansen, 1985). Removal of phosphate in excess of that necessary to sustain activated sludge growth has been attributed to biological accumulation (Levin and Shapiro, 1965), but this view was challenged by Menar and Jenkins (1970) who proposed the chemical precipitation of phosphate by calcium occurring in the influent as a major removal mechanism. Carberry and Tenney (1973) resolved the issue by showing that phosphate removal in activated sludge systems subjected to normal calcium influent values (<50 mgCa/ℓ), is a biological phenomenon, while Lan *et al.* (1983) demonstrated concurrent biological accumulation and precipitation at higher calcium concentrations. Recently published models satisfactorily explain some of the mechanisms underlying the observed events in biological phosphate removal (Comeau *et al.*, 1986; Wentzel *et al.*, 1986), although additional experimental evidence is required to validate and clarify some of the steps in the models. There is clearly a need to integrate theoretical and experimental approaches to develop a predictive model, not only in a qualitative sense, but also in quantitative detail.

Waste water feed composition is an important factor in the performance of a biological phosphate removal process (Marais *et al.*, 1983; Comeau *et al.*, 1986; Jones *et al.*, 1987; Somiya *et al.*, 1988). The poor controllability of the concentration and composition of a waste water such as domestic sewage impedes interpretation of results due to the masking of cause and effect relationships. To reduce variability, synthetic waste water or feedstock has been used in numerous laboratory-scale processes to investigate a variety of configurations and parameters. These include formulations containing milk solids (Hoover and Porges, 1952), castile soap and starch (Greenberg *et al.*, 1955), skimmed milk powder (Kountz and Forney, 1959), sucrose and yeast extract (Kucnerowicz and Verstraete, 1983), nutrient broth, glucose and yeast extract (Lan *et al.*, 1983), peptone and meat extract (Hashimoto and Furukawa, 1984), meat extract (Hascoet and Florentz, 1985), glucose, acetate and casein hydrolysate (Manning and Irvine, 1985) and glucose and peptone (Somiya *et al.*, 1988). In some cases the effect of organic feed composition on process performance was investigated with the inclusion of compounds such as sugars, fatty acids, amino acids and alcohols (Burkhead and Waddell, 1969), glucose and urea, sodium glutamate, ammonium sulphate or sodium nitrate (Enari and Matsumoto, 1982), skimmed milk powder, meat extract and peptone (Malnou *et al.*, 1984), and fatty acids and alcohols (Jones *et al.*, 1987). The higher process stability obtained by using a synthetic feedstock has contributed to the overall understanding of activated sludge processes.

Nitrogen-containing compounds, including protein, are required by activated sludge organisms to sustain growth (Symons and McKinney, 1958). Although Enari and Matsumoto (1982) concluded that organic nitrogen compounds have to be degraded to ammonia before its nitrogen could be utilised by the activated sludge, their results did not preclude the concurrent uptake of nitrogen compounds other than ammonia. The provision of amino acids to activated sludge rather than sugar and fatty acid substrates, resulted in a higher nitrogen content in the sludge

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(Burkhead and Waddell, 1969), which indicated the assimilation of the amino acids into cell material. Increased availability of amino acids or their degradation products (such as keto acids) in the feed, reduces the need for their *de novo* biosynthesis and therefore the requirement of large amounts of energy during protein synthesis in the aerobic stage. The uptake of these compounds is quite common and mechanisms for the transport of amino acids and keto acids across the plasma membrane have been demonstrated in many bacteria (Conn and Stumpf, 1976). In the event of protein being the only carbonaceous energy source provided, it is reasonable to suggest that the hydrolysis to amino acids and/or conversion to products such as keto acids, their transport over the membrane and incorporation into metabolic pathways, would result in a lag period before linking up with metabolic processes underlying biological phosphate removal. During such a lag period, biological phosphate removal may be temporarily suppressed or disturbed.

The purpose of the investigation reported in this paper was to study the effect of feed composition changes on biological phosphate removal and thus to find a feed composition that would make it possible to operate a stable laboratory process in which the underlying biochemical processes can be investigated. One of the objectives was to test the hypothesis of Heymann (1985) that protein does not contribute towards enhanced phosphate removal. A secondary objective was to establish whether protein as major or only carbonaceous and nitrogenous substrate is sufficient to support biomass growth. A new performance criterium, namely the waste mixed liquor phosphate concentration, P_w, based on the dynamic phosphate mass balance is proposed and applied to results obtained with a laboratory three-stage Bardenpho® process fed on protein-rich synthetic waste water and settled sewage.

Performance criterium

The most commonly used criterium to assess phosphate removal in waste-water treatment processes is the percentage of phosphate removed or phosphate removal efficiency (Riding *et al.*, 1979) in combination with the effluent phosphate concentration (Greenberg *et al.*, 1955; Levin *et al.*, 1972; Sherrard and Schroeder, 1972; Barnard, 1976; Arvin, 1984; Hashimoto and Furukawa, 1984; Malnou *et al.*, 1984; Hascoet and Florentz, 1985; Manning and Irvine, 1985; Jones *et al.*, 1987). The amount of phosphate removed (APR) has been used to assess performance (Lan *et al.*, 1983; Gerber and Winter, 1984), while Brar and Tollefson (1975) also used the amount of phosphate removed per unit sludge mass of specific phosphate removal (SPR). In a biological process the removal of phosphate is accomplished by incorporation into the sludge biomass, typically leading to values of 2 to 3% (based on VSS) in conventional activated sludge processes (Levin and Shapiro, 1965; Sekikawa *et al.*, 1966; Morgan and Fruh, 1974; Riding *et al.*, 1979; Wu and Okrutny, 1982), and 2 to 9% in nutrient removal processes (Menar and Jenkins, 1970; Rensink *et al.*, 1979; Hong *et al.*, 1984; Tetreault *et al.*, 1986). The importance attached to the sludge phosphate content in biological phosphate removal processes suggests it as a criterium to assess phosphate removal. In a study of Fukase *et al.* (1984), the sludge phosphate content reported closely corresponded with the changes introduced in the process parameters and did not display the high variability seen in the effluent phosphate concentration, indicating its potential use as performance criterium.

To compare phosphate removal and sludge phosphate content, the sludge phosphate content can be calculated from the amount of phosphate removed by using a phosphate mass balance model. Due to the biological nature of the process involving complex interactions between the biomass and its environment, the attainment of steady state is not easily realised or recognised. The utilisation of the dynamic sludge phosphate mass balance as opposed to a steady state approach should produce representative sludge phosphate values provided the assumptions and approximations made are valid.

The dynamic phosphate mass balance for an activated sludge process relates the changes in the phosphate content in the biological reactors, as represented by the waste mixed liquor concentration, P_w, with the difference between the amounts of phosphate entering and exiting the process:

$$V\frac{dP_w}{dt} = F.P_i - (F-F_w)P_e - F_w.P_w \qquad (1)$$

When the system has progressed to a steady state situation with regard to phosphate, the phosphate concentration in the process remains constant and Eq. 1 reduces to zero:

$$F_w.P_{wss} = F.P_i - (F-F_w)P_e \qquad (2)$$

Under non-steady state conditions, P_{wss} could only be a calculated parameter representing equilibrium, and the difference between it and the actual reactor phosphate concentration, P_w, is related to the change in phosphate concentration. This relationship is approximated by substituting Eq. 2 into 1:

$$V\frac{dP_w}{dt} = F_w(P_{wss} - P_w) \qquad (3)$$

Due to the slow-changing nature of the sludge phosphate content P_{wss} can be taken as constant over time t (an approximation improved by short time periods, in the order of one or two days) and Eq. 3 integrated:

$$\int_{P_{w1}}^{P_{w2}} \frac{dP_w}{P_{wss} - P_w} = \frac{F_w}{V}\int_0^t dt \qquad (4)$$

$$P_{w2} = P_{wss} - (P_{wss} - P_{w1})\, e^{\frac{-F_w}{V}t} \qquad (5)$$

P_{wss} is approximated as the average of n P_{wss} values available over time t (in practice only the values at the start and end of a one-day period were taken):

$$P_{wss} = \frac{1}{n.F_w}\sum_{j=1}^{n}(F.P_{ij} - (F-F_w).P_{ej}) \qquad (6)$$

Materials and methods

Activated sludge unit configuration and operation

The experimental side of the study involved the feeding of prepared feedstocks to a modified three-stage Bardenpho ® unit (Fig. 1), consisting of eleven, magnetically stirred, 1 000 mℓ glass reactors in series and a 500 mℓ conical glass clarifier. The

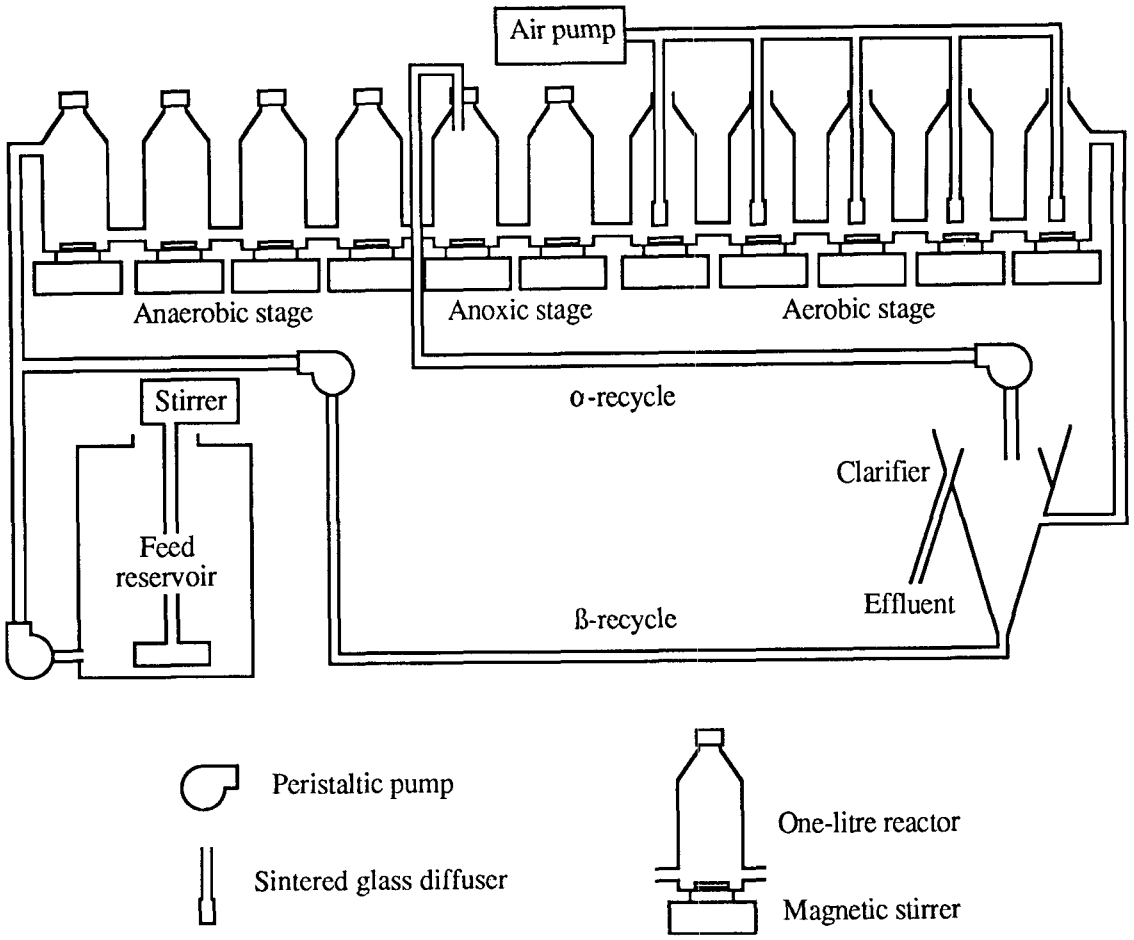

Figure 1
The modified three-stage Bardenpho® process.

TABLE 1
COMPOSITION OF TAP WATER AND DASPOORT SETTLED SEWAGE AFTER DILUTION TO OBTAIN A COD OF ABOUT 400 mgO_2/ℓ.

Determinant	Unit	Tap water	Settled sewage
Sodium	(mgNa/ℓ)	24	79
Potassium	(mgK/ℓ)	5	17
Magnesium	(mgMg/ℓ)	9	17
Calcium	(mgCa/ℓ)	23	29
Alkalinity	($mgCaCO_3/\ell$)	64	266
Total Kjeldahl nitrogen	(mgN/ℓ)	0,9	40
Ammonia	(mgN/ℓ)	0,7	29
Nitrate + nitrite	(mgN/ℓ)	0,8	0,3
Total phosphorus	(mgP/ℓ)	0,2	11
Orthophosphate	(mgP/ℓ)	0,1	9
Sulphate	($mgSO_4/\ell$)	49	65
Chloride	(mgCl/ℓ)	25	58
Chemical oxygen demand	(mgO_2/ℓ)	13	377
Total organic carbon	(mgC/ℓ)	5	103
Carbohydrate	(mg/ℓ)	0	20
Protein	(mg/ℓ)	5	110
Lipid	(mg/ℓ)	5	99
Detergent	(mg/ℓ)	0	3
Total dissolved solids	(mg/ℓ)	214	586
Total suspended solids	(mg/ℓ)	0	120

anaerobic, anoxic and aerobic stages comprised four, two and five reactors respectively. The feed rate (Q) was maintained around 500 mℓ/h, the β-recycle at Q and the α-recycle at 2Q. Sludge age was maintained at 25 d by manually removing 600 mℓ of the mixed liquor in a single amount from the last aerobic reactor per day. The α-recycle was taken from the clarifier supernatant to avoid mixing sludges in different metabolic states (from anaerobic and aerobic conditions) in the anoxic stage, which resulted in the anaerobic stage having a MLSS roughly double that of the anoxic and aerobic stages. The actual retention times for the three stages and the clarifier were: Anaerobic = 4 h; Anoxic = 1 h; Aerobic = 2,5 h; Clarifier = 0,5 h. The system temperature remained constant at 27°C due to the operating temperature of the magnetic stirrers. Air was introduced into the aerobic stage by an air pump and sintered glass diffusers (porosity no. 2). The feed reservoir contained up to 45 ℓ of feed and was stirred continuously by an overhead stirrer. Plastic balls (10 mm diameter) were used to cover the liquid surface to avoid air contact.

Feed preparation and composition

The settled sewage feed (collected twice a week from Daspoort Municipal Sewage Treatment Works) was diluted with tap water to a COD value of approximately 400 mgO_2/ℓ (Table 1), while the synthetic feed was prepared by dissolving the ap-

propriate compounds in tap water also to yield a COD of approximately 400 mgO_2/ℓ. Feed pH varied between 6,7 and 7,0, and therefore needed no adjustment. The plastic balls and feed reservoir were washed thouroughly with tap water before a new batch of feed was introduced. Each 45 ℓ batch of feed was freshly prepared twice a week and kept at room temperature while fed to the unit, thus not precluding possible microbial action in the feed reservoir.

As a feeding medium, the tap water showed no apparent deficiency other than organics, nitrogen and phosphate (Table 1). The proteinaceous substrates used were peptone (trypsin digested casein from Merck) and milk protein, the latter obtained by ultrafiltration of skimmed milk followed by low pressure spray drying, yielding about 60% protein and 24% lactose (the rest being moisture and salt). α-Ketoglutaric acid was chosen as a representative keto acid which can readily be produced from glutamic acid by deamination and transamination enzymes (Metzler, 1977) and expected to be found among the biological degradation products of peptone and milk protein which contains a large amount of glutamic acid (Table 5). α-Ketoglutaric acid can also be metabolically derived from glutamine, proline, arginine and histidine (Metzler, 1977; Stryer, 1981).

The activated sludge unit, which was filled initially with mixed liquor obtained from a full-scale plant, received a succession of feedstock formulations during each run as indicated in Table 2. The sequential mode of the introduction of different feedstock compositions was adopted to reduce the initial adaptation period that would otherwise be necessary for each batch of fresh sludge obtained from a full-scale plant. The periods during which specific feed formulations were used, were short because experience had shown that the reaction of the activated sludge to feed changes usually occurred within one to two days. The COD was maintained between 300 and 400 mgO_2/ℓ, and the TKN between 35 and 45 mgN/ℓ. The total phosphate concentration was monitored in the influent, while the orthophosphate concentration in the effluent was taken as representative of the total phosphate concentration, substantiated by own experience and literature (Hashimoto and Furukawa, 1984; Malnou *et al.*, 1984).

TABLE 2
CORRELATION BETWEEN DETERMINED AND CALCULATED WASTE MIXED LIQUOR PHOSPHATE CONCENTRATION VALUES.

Run	Number of values	Correlation coefficient
A	16	0,922
B	15	0,680
C	98	0,926
D	76	0,914

Analytical methods

Solids, COD, TKN, detergents and inorganic compounds were determined according to Standard Methods (1985). Technicon Autoanalysers were used for the determination of orthophosphate, nitrate and ammonia. Protein was determined by the Folin-Lowry method (Lowry *et al.*, 1951), carbohydrate by the phenol-sulphuric acid method (Benefield and Randall, 1976), lipids by extraction-gravitation (Standard Methods, 1985) and by the hydroxylamine method (Snyder and Stephens, 1959). Amino acid composition was determined by hydrolysing lyophilised settled sewage, milk protein and peptone with 6 M hydrochloric acid under nitrogen for 24 h at 105°C and dissolving the amino acids in 0,2 mol dm^{-3} sodium citrate hydrochloric acid buffer (pH 2,20). The amino acids were chromatographically separated in a Beckman 121M Amino Acid Analyser on AA20 resin with sodium citrate hydrochloric acid buffers and monitored with ninhydrin post-reaction. The sodium citrate hydrochloric acid buffers concentrations used were 0,35 mol dm^{-3} (pH 5,26) for the separation of the basic amino acids, and 0,2 mol dm^{-3} (pH 3,28) and 0,4 mol dm^{-3} (pH 4,10) applied sequentially for the separation of the other amino acids.

Experimental approach

To evaluate the role of protein in biological phosphate removal, the laboratory three-stage Bardenpho ® process (Fig. 1) was fed on either settled sewage, protein-enriched settled sewage, or protein-rich synthetic waste water. The study consisted of four runs, subsequently designated as runs A, B, C and D respectively. Run A was aimed at establishing peptone as a substrate able to support the biomass in the process as well as exploring its influence on phosphate removal. Runs B and D were aimed at evaluating the effect of changeover between protein-rich and sewage feedstocks on phosphate removal. Run C consisted of three parts, the first to evaluate peptone as organic substrate for phosphate removal, the second to study phosphate removal during the changeover to sewage-based feedstock, and the third to determine the effect of α-ketoglutaric acid added to sewage feedstock on phosphate removal. Activated sludge to inoculate the unit was obtained from the Daspoort Municipal Sewage Treatment Works near Pretoria (a conventional activated sludge plant showing normal phosphate removal) for runs A and B, and from Goudkoppies Sewage Treatment Works in Johannesburg (a five-stage Bardenpho ® process showing enhanced phosphate removal) for runs C and D. It was not expected that the different inoculums would show different behaviour after an acclimatisation period.

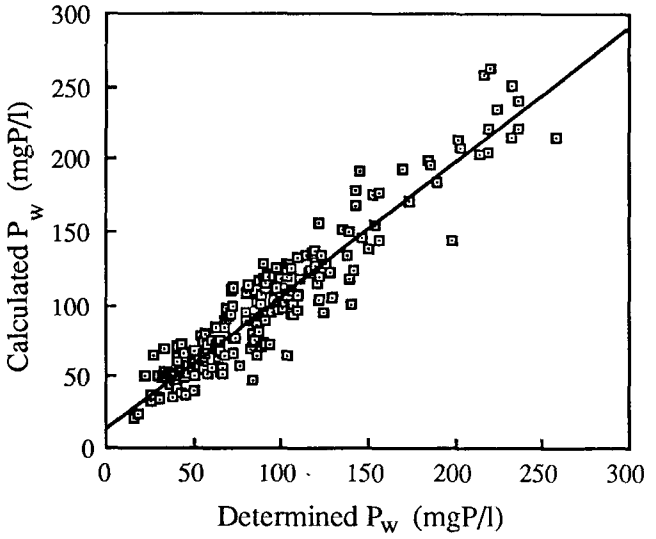

Figure 2
Correlation of the determined and calculated sludge phosphate concentration (P_w) values.

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Results

The calculated waste phosphate concentration, P_w, showed exceptional correlation with the determined parameter (Table 2; Fig. 2), given the multitude of variables involved in the process. The most influential variable was found to be the mixed liquor wastage rate, F_w, emphasising the importance of accurate sludge wastage recording. The P_w incorporates the amount of phosphate removed in a cumulative manner, resulting in a smoothing of the variability seen in the amount of phosphate removed, and strongly indicates the trend of phosphate incorporation into the sludge mass. Within a given set of conditions, the process as a whole progresses towards a steady state at an exponentially decreasing rate, until all parameters essentially remain constant, including the phosphate content of the waste activated sludge (Fig. 3). The steady state value, P_{wss}, was determined for each feed composition (Table 3) by fitting the P_w values to an exponential curve, even though steady state had not been reached in many instances. Any change in a condition influencing process performance (such as the feed composition) would result in a new steady state towards which the process tends. Thus, if the feed composition has an important influence on the accumulation of phosphate by the activated sludge, then changes in feed composition would cause significant changes in the P_w and the P_{wss}. Changes in feed composition were indeed strongly reflected in the P_{wss}, and in the trends shown by the P_w, leading to the conclusion that these parameters can serve as useful performance criteria (Table 3 and Figs. 4 to 7).

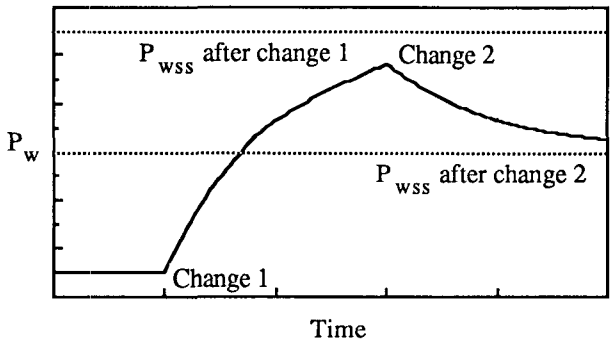

Figure 3
Curves to illustrate the progression of the waste phosphate concentration, P_w, towards its steady state value, P_{wss}, after changes in conditions influencing process performance.

TABLE 3
AVERAGE PERFORMANCE PARAMETERS OF A BARDENPHO® UNIT WITH DIFFERENT FEED COMPOSITIONS.

Run	Days	Organic feed composition	P_i	P_r	P_e	P_{wss}	NO_3	MLSS	VSS
A	1-42	Peptone	15,9	46,6	6,7	195,6	7,4	1517	994
B 1	1-16	Peptone	22,8	49,6	12,1	203,3	7,1	1007	628
2	17-55	Sewage	8,7	17,6	7,7	49,4	8,0	1089	852
3	56-80	Peptone: Sewage (1:2)	15,1	19,6	9,8	120,4	10,1	1377	1011
C 1	1-63	Peptone (~ 9 mgP/ℓ)	9,0	32,1	1,8	144,1	9,3	978	730
2	64-77	Peptone (~ 12 mgP/ℓ)	11,8	49,0	1,5	205,1	11,2	1434	1184
3	78-105	Peptone (~ 20 mgP/ℓ)	20,0	55,1	8,2	227,9	11,4	1747	1260
4	106-119	Peptone (~ 30 mgP/ℓ)	29,5	67,6	13,6	304,7	10,8	2389	1584
5	120-133	Peptone: Sewage (1:2)	11,2	24,5	7,0	78,5	14,3	2059	1342
6	134-147	Sewage	12,5	14,7	10,2	33,9	14,1	2108	1404
7	148-161	Sewage + 20 mg α-kga/ℓ	11,9	14,0	7,3	85,4	13,8	1572	1133
8	162-175	Sewage + 50 mg α-kga/ℓ	12,4	24,8	7,6	86,9	11,9	1359	1011
D 1	1-28	Sewage	9,9	15,8	5,4	65,2	6,9	1218	970
2	29-56	Milk protein: Sewage (1:2)	10,4	18,0	2,7	125,2	8,8	1493	1125
3	57-63	Milk protein: Sewage (1:1)	8,3	28,6	1,2	137,4	8,4	1758	1357
4	64-91	Sewage	11,9	20,6	7,5	98,1	11,6	1577	1129
5	92-105	Milk protein: Sewage (1:2)	12,3	37,2	4,2	128,5	12,3	1824	1265
6	106-119	Sewage	13,1	15,5	10,1	75,0	13,1	1341	1106

P_i : Influent total phosphorus concentration (mgP/ℓ)
P_r : Actual amount of phosphate released in the anaerobic stage (mgP/ℓ)
P_e : Effluent orthophosphate concentration (mgP/ℓ)
P_{wss} : Calculated steady state waste mixed liquor phosphorus concentration (mgP/ℓ)
NO_3 : Effluent nitrate concentration (mgN/ℓ)
MLSS : Mixed liquor suspended solids (mg/ℓ)
VSS : Volatile suspended solids (mg/ℓ)
α-kga : α-ketoglutaric acid

The average performance of the activated sludge unit during the four experimental runs in terms of P_{wss} is summarised in Table 3 and time courses for P_w given in Figs. 4 to 7. Removal of organic and nitrogenous matter varied between 81 and 94%, and 97 and 99% respectively (data from all four experimental runs). Nitrification was not influenced by the proteinaceous and sewage feedstocks used, and no significant correspondence between effluent nitrate concentration and phosphate removal was observed. The relatively long anaerobic and anoxic retention times (actual retention times 4 and 1 h respectively) were found to be adequate to reduce the recycled nitrate concentration to undetectable values (< 0,2 mgN/ℓ).

Peptone as sole source of carbon, nitrogen and energy was shown in Run A (Fig. 4) to be able to support enhanced phosphate removal in a Bardenpho ® process, and sustained performance on peptone feedstock over more than two months was demonstrated in Run C (Fig. 6a: Days 0 to 63). In Run B (Fig. 5: Days 17 to 55), P_w was found to decrease after the peptone-adjusted unit was subjected to settled sewage feedstock. The change-over from peptone to sewage via a mixture in Run C (Fig. 6a: Days 120 to 175) similarly caused a dramatic decrease in P_w. In Run B (Fig. 5: Days 56 to 80) an increase in enhanced phosphate removal was obtained by the substitution of a third of the sewage COD with peptone. In Run D (Fig. 7: Days 29 to 63)

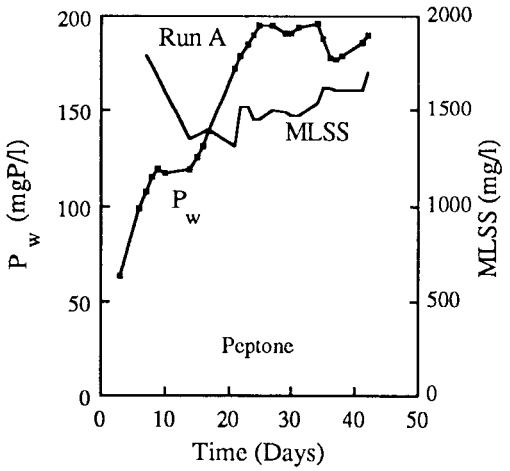

Figure 4
Waste phosphorus concentration and MLSS during Run A.

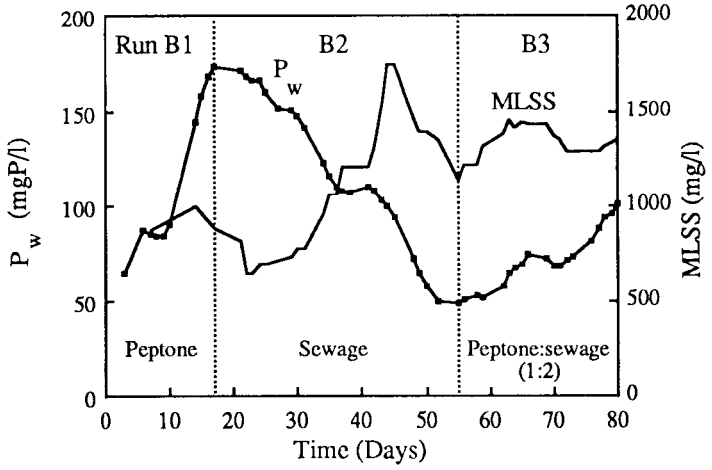

Figure 5
Waste phosphorus concentration and MLSS during Run B.

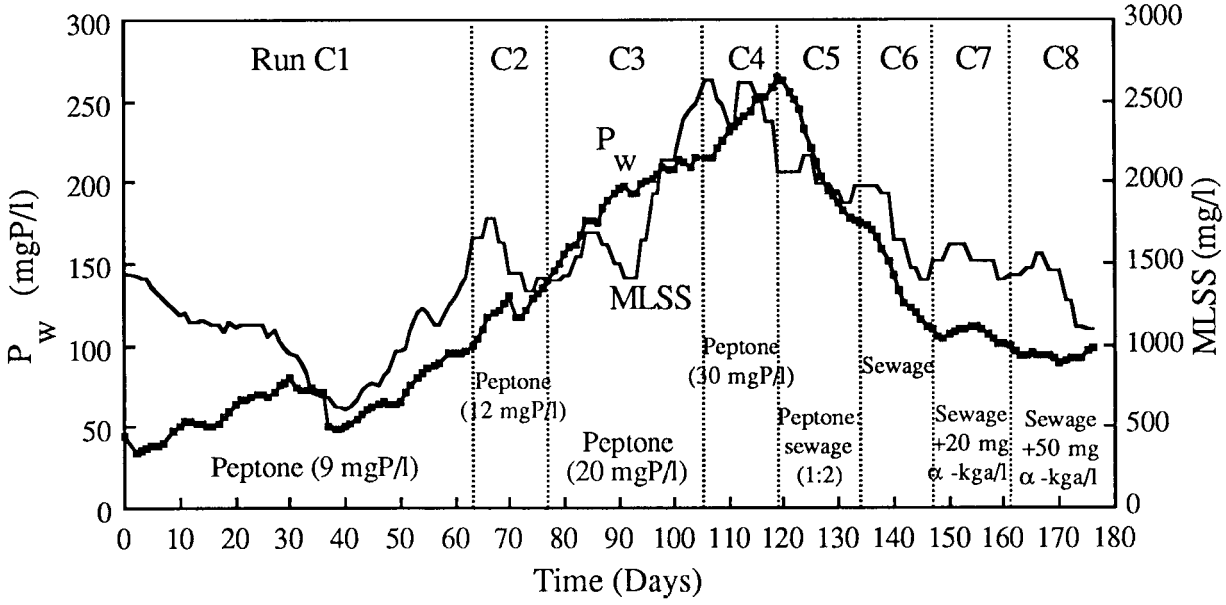

Figure 6a
Waste phosphorus concentration and MLSS during Run C.

the substitution of a part of the sewage feed COD with milk protein also caused a very significant increase in the P_w, with subsequent sewage feeding (Fig. 7: Days 64 to 91) having a detrimental effect. The repetition of this cycle (Fig. 7: Days 92 to 120) resulted in the same pattern of consequences. It is thus concluded that peptone can be used as a sufficient source of carbon, nitrogen and energy to support enhanced phosphate removal. The proteinaceous substrates were also more conducive to phosphate removal than settled sewage in the same unit.

The actual amount of phosphate released averaged for each feed composition is given in Table 3, and was calculated with the following formula:

$$P_r = P_a - \frac{P_i + P_e}{2}$$

where P_a is the orthophosphate concentration in the last anaerobic reactor. The phosphate release was found to be the highest for the peptone feedstocks (32 to 68 mgP/ℓ released), followed by the peptone or milk protein and sewage mixtures (18 to 37 mgP/ℓ released), with sewage feedstock resulting in the lowest values (14 to 21 mgP/ℓ). Phosphate release did not vary with nitrate levels, but increased with higher influent and sludge phosphate concentrations (Fig. 6b). The anaerobic phosphate release corresponded to the trends in phosphate removal and accumulation in the biomass, although the relationship is not strong enough to suggest a direct connection.

Increasing the influent phosphate concentration through three steps during Run C (Table 3; Fig. 6a: Days 64 to 119) led to an increase in the P_w as well as the MLSS and the phosphate released in the anaerobic stage. This increase in MLSS was also

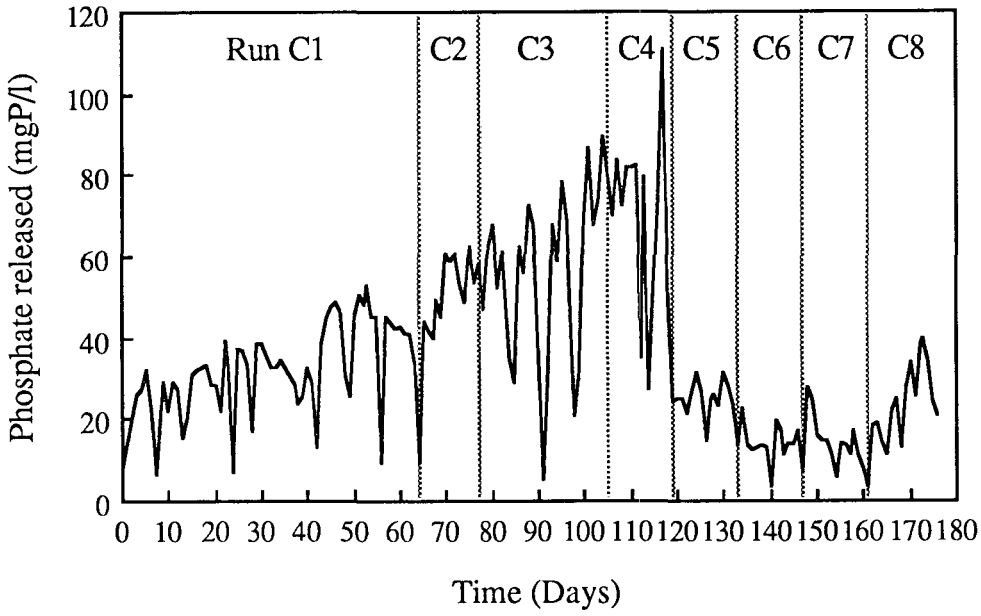

Figure 6b
Actual phosphate released in the anaerobic stage during Run C.

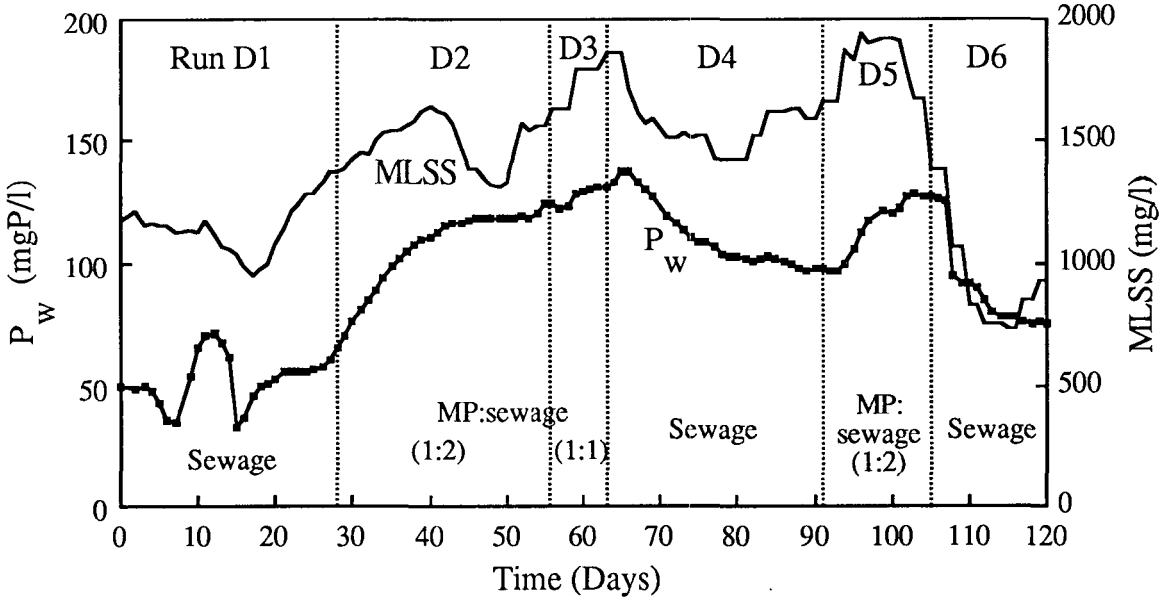

Figure 7
Waste phosphorus concentration and MLSS during Run D.

observed with the peptone substrate in Run A and with the milk protein: sewage mixtures in Run D (Table 3: Figs. 4 and 7). The increase in biomass indicates some beneficial effect that the higher influent phosphate concentrations in combination with the protein substrates have on sludge mass formation. The VSS and protein contents of the solilds decreased at higher MLSS values (Fig. 8), and the anaerobic phosphate release increased (Table 3: Run C), indicating an increase in the inorganic content of the soldis (mainly consisting of phosphate compounds and metal ions) and the possible precipitation of phosphate compounds in the sludge floc. The pH in the reactors varied between 7,1 in the anaerobic stage and 7,3 in the aerobic stage, indicating little opportunity for the precipitation of phosphate compounds due to pH elevation. Calcium and magnesium levels in the protein-enriched feedstocks were not expected to exceed 30 mg/ℓ (based on calculations from known ash contents), while the levels in the sewage feedstocks were also too low (< 50 mg/ℓ) (Table 1) to result in significant precipitation of phosphate (Carberry and Tenney, 1973; Lan *et al.*, 1983). Apart from these considerations, the biological incorporation of phosphorus in the form of an inorganic compound (such as polyphosphate and its associated cations) into the sludge mass, necessarily increases the sludge's inorganic fraction. It is therefore concluded that no significant chemical phosphate precipitation took place.

The phosphorus was added to the peptone feed solution as the potassium dihydrogen phosphate salt, leading to potassium concentrations ranging from 15 to 45 mgK/ℓ with increasing phosphate concentrations in the feed during Run C (about 5 mgK/ℓ originating from the make-up tap water), a factor that may have contributed to the increased biomass production. Smith *et al.* (1954) found that polyphosphate synthesis in *Klebsiella pneumoniae (Aerobacter aerogenes)* depends upon the presence of potassium, with the formation of volutin granules (polyphosphate-containing structures) only becoming limited at potassium concentrations below 10 mgK/ℓ, which is well below the concentrations used in this study. Gerber and Winter (1984) reported the concurrent release of phosphate, potassium and magnesium in the anaerobic stage of a nutrient removal laboratory unit (the phosphate released to an extent similar to our findings), followed by concurrent uptake of these substances in the

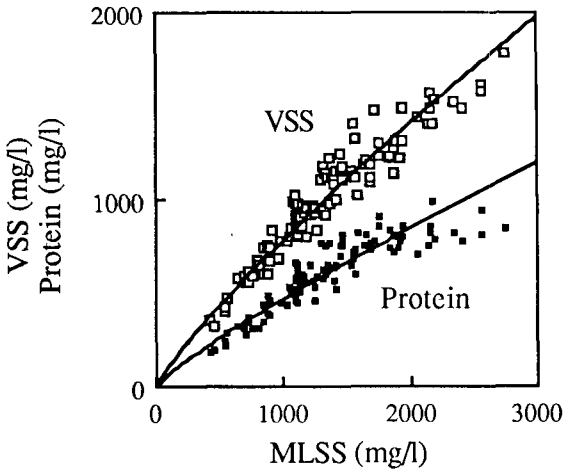

Figure 8
Relationship between VSS, protein and MLSS during Run C.

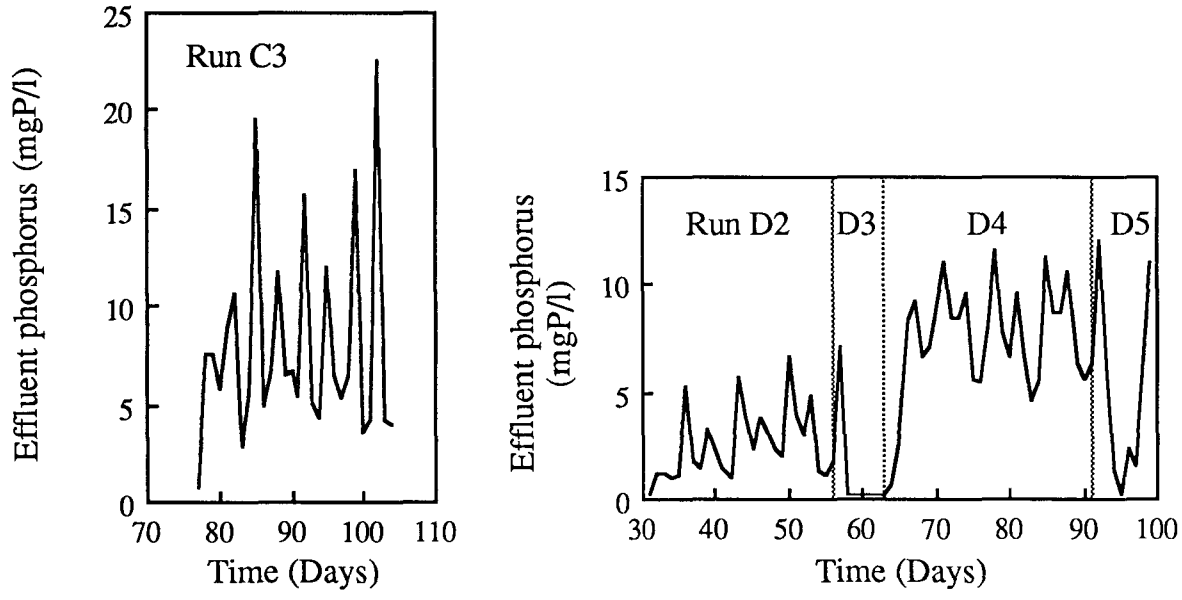

Figure 9
Variation in the effluent phosphorus concentration during Runs C and D with the twice weekly feed cycle. All maxima represent effluent phosphate concentrations obtained 24 h after the introduction of fresh batches of feedstock.

aerobic stage. The net uptake of potassium was found to be about 8 mgK/ℓ with about 13 mgK/ℓ remaining in the effluent, while more than 25 mgP/ℓ was removed. It seems from these two studies that only about 10 mgK/ℓ is needed to sustain sludge growth and phosphate removal, and that the provision of higher amounts will not significantly influence the functioning of the process. It is concluded that the increase in biomass production could not have been caused by the increased influent potassium concentration, but rather by the increased influent phosphate concentration possibly enhanced by the availability of a source of amino acids in the form of peptone.

Variability in the effluent phosphate concentrations of the unit fed on peptone, milk protein: sewage mixture or sewage corresponded closely with the twice-weekly preparation cycle of the feedstock (Fig. 9). During the first 24 h after the introduction of a fresh batch of feedstock, the effluent phosphate concentration usually increased, followed by a decrease similar in magnitude over the next one or two days. The COD and protein concentration of the protein-rich feedstocks were found to decrease, while the free ammonia concentration increased (Table 4). The slow degradation of the COD and protein in the feed container, as well as the generation of ammonia indicated microbial action producing degradation products from protein in the feed (the container was kept at room temperature, about 23°C), although sewage gave rise to similar phenomenon, but at higher effluent phosphate values. The appearance of free ammonia in the feed container indicates the presence of deaminases, which remove the amine groups from amino acids to form ammonia and α-keto acids (Metzler, 1977). In order to investigate the influence of such a degradation product on phosphate removal, α-ketoglutaric acid was added to sewage feedstock during Run C (Fig. 6a: Days 148 to 175) in concentrations in a range expected from the peptone feedstocks. α-Ketoglutaric acid can be derived from glutamic acid, glutamine, proline, histidine and arginine (Fig. 10), representing 35% of the peptone solids and 20% of the milk protein solids (Table 5). The rate of P_w change decreased and the P_{wss} doubled to a value similar to that for the peptone: sewage mixture (Table 3), indicating an enhancing trend in phosphate removal. In comparison with protein or amino acids, this keto acid can be transported without prior biochemical conversion to contribute for instance to the Krebs cycle. Thus the time required for its utilisation would decrease, compared to lower fatty acids which have to be activated first before entering into the general metabolism. The incorporation of α-ketoglutaric acid into the Krebs cycle would give rise to the formation of NADH and oxaloacetate, promoting the utilisation of acetate and providing precursors for the synthesis of a variety of compounds including PHB, amino acids and proteins. However, one would expect the addition if a single amino acid derivative to sewage to have only a limited effect compared to peptone or milk protein.

Discussion

Since Comeau *et al.* (1986) and Wentzel *et al.* (1986) proposed biochemical models aimed at explaining some of the biochemical processes underlying the main events in the anaerobic, anoxic and aerobic stages of nutrient removal systems, we felt obliged to at least attempt to discuss our findings in relation to these models. Such an exercise is appropriate since both the above-mentioned models were developed for acetate as the principle substrate while our results were obtained with protein (amino acids) as a component or only substrate in the feedstock.

TABLE 4
AVERAGE CHANGES IN THE FEED RESERVOIR OVER THE FOUR DAYS WHILE A BATCH OF FEEDSTOCK WAS FED TO THE UNIT.

Feed composition	COD ($mg O_2/ℓ$)		Protein (mg/ℓ)		Ammonia (mgN/ℓ)	
	Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
Peptone	406	303	338	202	5,7	29,2
Peptone: Sewage (1:2)	453	388	129	98	31,8	41,6
Milk protein: Sewage (1:2)	423	248	146	89	20,0	29,1
Sewage	428	351	110	106	34,8	36,4

TABLE 5
AMINO ACID COMPOSITION OF PEPTONE, MILK PROTEIN AND LYOPHILISED SEWAGE (EXPRESSED AS A PERCENTAGE OF THE TOTAL SOLIDS).

Amino acid	Peptone	Milk protein	Sewage
Alanine	2,56	3,15	0,43
Valine	5,82	3,15	0,25
Leucine	8,77	6,54	0,32
Isoleucine	4,40	2,49	0,17
Phenylalanine	4,18	2,98	0,16
Methionine	2,47	1,87	0,08
Proline	11,79	7,00	0,18
Glycine	1,75	1,28	0,28
Serine	4,83	1,88	0,21
Threonine	3,62	2,11	0,24
Tyrosine	1,94	3,18	0,12
Aspartic acid	6,52	4,69	0,59
Glutamic acid	17,28	9,90	0,46
Lysine	6,94	4,83	0,32
Histidine	2,47	1,52	0,07
Arginine	2,98	1,90	0,21
Total	88,32	58,49	4,10
Protein †	88,20	59,50	17,30
Organic nitrogen *	12,80	10,00	1,24
Crude protein •	80,00	62,50	7,77
Carbohydrate	0	23,80	2,80

† Determined by the Folin-Lowry method (Lowry *et al.*, 1951)
* Difference between TKN and ammonia determined
• Organic nitrogen multiplied by 6,25

For the sake of clarity the main findings of our research are summarised first. As regards the main objectives of this investigation the results provided evidence that protein in the form of peptone as the only carbonaceous and nitrogenous substrate is sufficient to support biomass growth in a modified three-stage Bardenpho® laboratory unit (Runs A and C: Table 3; Figs. 4 and 6a), corroborating the work of Enari and Matsumoto (1982), Hashimoto and Furukawa (1984), Malnou *et al.* (1984) and Hascoet and Florentz (1985) among others. Evidence was also provided with sewage and protein-rich feedstocks that protein or degradation products thereof did indeed contribute towards enhanced phosphate removal. However, since the milk protein preparation contained approximately 24% lactose, it is reasonable to conclude that the latter contributed meaningfully to the response obtained.

While increasing the influent phosphate concentration with peptone feedstock led to a marked increase in MLSS (Run C: Table 3) as well as VSS and protein (Fig. 8), the protein and VSS contents of the sludge tended to decrease.

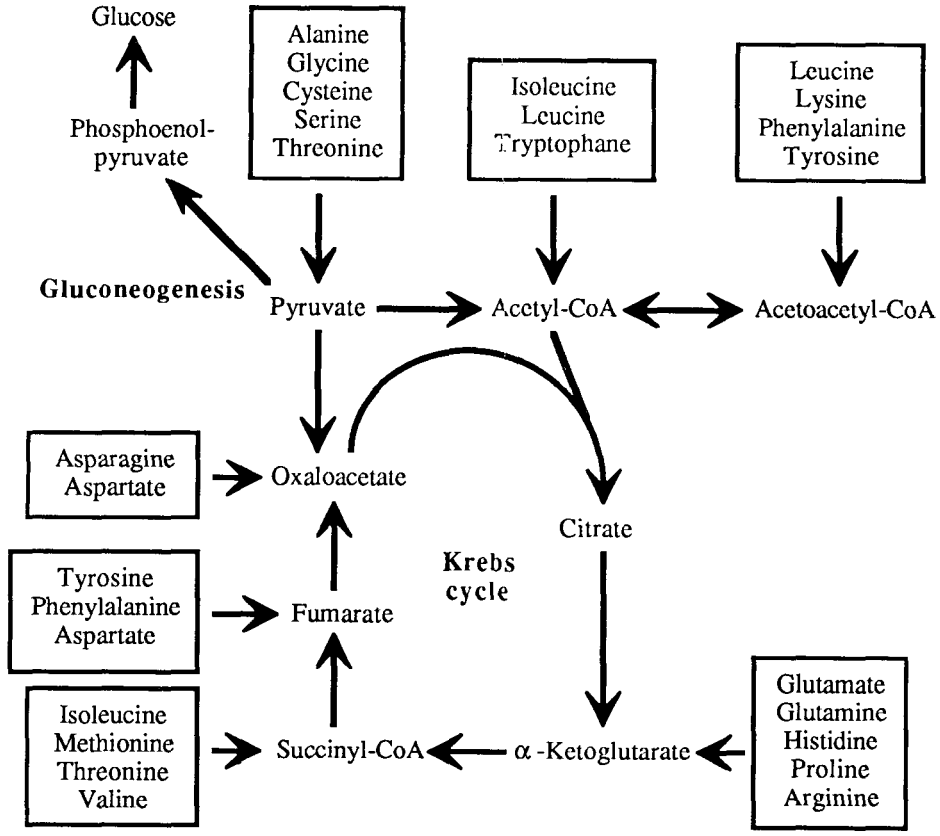

Figure 10
The entry points of amino acids into the general metabolism and production of metaholic intermediates from them (Stryer, 1981).

Especially at higher influent phosphate concentration, the effluent phosphate concentration peaked 24 h after the introduction of fresh peptone feedstock, and dropped again to previous values over the next one or two days (Run C: Fig. 9). Similar observations were made with a mixture of milk protein and sewage or with sewage alone (Run D: Fig. 9), although the variation was much less than obtained with peptone. Apparently the influent phosphate concentration as well as the nature of the organic substrate influenced the degree of variability.

The addition of α-ketoglutaric acid to sewage in the feedstock resulted in a fair increase in phosphate removal concurrently with a decrease in MLSS (Run C: Table 3). However, the decrease in MLSS was probably a consequence of the switch-over from peptone to settled sewage in the feedstock, because a similar change was observed after the switch-over from milk protein: sewage to sewage alone (Run D: Table 3).

The biochemical model of Comeau *et al.* (1986) was based on experimental data obtained in batch experiments with sludge originating from nutrient removal plants (Comeau *et al.*, 1987). This model is applicable to the so-called bio-P bacteria which is defined as bacteria that can store both polyphosphate and carbon, for instance in the form of PHB (poly-β-hydroxybutyrate). The model of Wentzel *et al.* (1986) attempts to explain the behaviour of *Acinetobacter* spp. in enhanced biological phosphate removal by activated sludge systems. A common feature of both models is the proposal that polyphosphate serves mainly as a source of energy under anaerobic conditions for the activation of acetic acid to acetyl CoA, which is then incorporated into PHB with the utilisation of NADH generated in the Krebs (tricarboxylic acid) cycle. In the Comeau *et al.* (1986) model, the energy in polyphosphate is also used to maintain the pmf (proton motive force) which is required to drive acetic acid uptake and regulate phosphate and metal release across the plasma membrane. The acetic acid diffuses passively over the membrane in the Wentzel *et al.* (1986) model, and phosphate and metal cations are released through hydroxyl and proton antiport systems respectively.

In order to relate our findings to these models, certain assumptions have to be made, viz.:

- The protein substrate was subjected to extracellular proteolytic enzymes and taken up as short peptides, amino acids and keto acids, the latter derived from the amino acids by deamination. This is a reasonable assumption since it is well-known that many bacteria produce extracellular proteases; even a small number of *Acinetobacter* strains produce gelatinase, a representative protease (Juni, 1978). Extracellular deamination of amino acids is common in bacteria (Metzler, 1977) and was indicated by the elevated values of ammonia in the feed container. Amino acids can be transported over the plasma membrane of bacteria by facilitated diffusion as well as by respiratory linked active transport, even under anaerobic conditions (Konings and Poolman, 1987). In the latter case a suitable

electron donor such as lactate, L-malate or NADH, and a suitable electron acceptor such as nitrate, are required (Conn and Stumpf, 1976).

- Amino acids can be converted to acetyl CoA and other compounds that can enter into the Krebs cycle at different points to yield NADH (Fig. 10). Thus all requirements for PHB synthesis and subsequent polyphosphate synthesis were satisfied when protein was fed as the only source of carbon and nitrogen. Since the milk protein contained lactose one would expect an even better supply of acetate and NADH for PHB synthesis from this source as a result of anaerobic fermentation.

The uptake of amino acids is as a rule driven by the pmf or energy obtained directly from ATP (Konings and Poolman, 1987). In principle this is consistent with the transport of acetic acid in the model of Comeau *et al.* (1986). The high phosphate release observed with peptone feedstock (average above 30 mgP/ℓ) (Table 3) strongly suggests the involvement of polyphosphate as a source of energy for transport of amino and keto acids across the plasma membrane. Based on the well-known catabolic pathways for amino acids and the incorporation of the end-products into the Krebs cycle, it is reasonable to suggest that in the experiments with peptone an adequate supply of precursors and NADH were available for PHB synthesis by bio-P bacteria. In both the models of Comeau *et al.* (1986) and Wentzel *et al.* (1986) ATP is required to activate acetate to acetyl CoA for PHB synthesis, the ATP probably derived from polyphosphate. However, less energy would be required from polyphosphate for the conversion of the protein substrate to metabolically usable forms because 12 of the 22 naturally occurring amino acids can enter the Krebs cycle without energy input from ATP and/or polyphosphate (those entering via pyruvate, oxaloacetate and α-ketoglutarate (Fig. 10)). The observed phosphate release was similar in magnitude and range to that obtained in both continuous-flow and batch studies (Malnou *et al.*, 1984; Gerber *et al.*, 1986; Jones *et al.*, 1987). The question now arises whether some bio-P bacteria, excluding *Acinetobacter* spp., when supplied under anaerobic conditions with substrates other than acetate, and which do not require ATP for conversion to PHB, would utilise some of the available energy in polyphosphate for the synthesis of other cell components. The synthesis of proteins and lipids, as well as gluconeogenesis would require ATP, NADH or NADPH, other cofactors and suitable precursors. The variety of carbon skeletons provided by amino acids and their readily interconvertable nature coupled to the availability of energy in the form of polyphosphate sets the scene for many synthetic activities, in spite of the anaerobic conditions. This line of thought is corroborated by the findings of Fukase *et al.* (1982) and Comeau *et al.* (1987) that only a part of the acetate supplied in the feed (44% and 8 to 13% respectively) is used in the synthesis of PHB.

The sudden and transient increases in effluent phosphate peaking 24 h after the introduction of fresh feedstocks, whether it was sewage, protein enriched sewage or peptone (Fig. 9), probably originated as a result of microbial activity in the feed container. It is conceivable that such activity might for instance have included hydrolytic, deaminating and fermentative reactions. These conversions would have influenced the chemical nature of the compounds present in the fresh feedstocks. It is suggested that the change in composition also influenced those biochemical processes associated with enhanced phosphate removal in the different reactors. In fact, the results in Table 3 clearly show that peptone in particular promoted phosphate release in the anaerobic stage and uptake in the aerobic stage, with an increase in influent phosphate concentration enhancing both processes. Furthermore, the fluctuations in effluent phosphate were the largest in the case of peptone (Fig. 9), which also showed the most change in composition in the feed container (Table 4). Since peptone is derived from casein, it is reasonable to conclude that the transient changes in effluent phosphate had their origin in the effect of amino acids and/or their degradation products, on processes such as the synthesis of PHB and polyphosphate. Microbial activity in the feed container probably made the amino acids more amenable for PHB synthesis, and according to the models of Comeau *et al.* (1986) and Wentzel *et al.* (1986), resulting in increased polyphosphate synthesis. In contrast, fresh feedstocks required that conversion of peptides and amino acids had to take place in the anaerobic stage, with the consequence that less PHB could be synthesised. However, the possibility that some of the products of the peptides and amino acids were carried over into the aerobic stage, cannot be excluded. In such an event these substrates might have influenced the selective utilisation of ATP for polyphosphate and cell component synthesis. Increased utilisation of ATP for the synthesis of cell components such as proteins, lipids and nucleic acids would have resulted in increased effluent phosphate concentrations.

The proposals regarding the utilisation of amino acids for PHB synthesis and synthesis of other cell components in the anaerobic stage and the possible influence of these events on the regulation and utilisation of ATP for polyphosphate and cell component synthesis in the aerobic stage need to be taken into account in future investigations.

Conclusion

The dynamic phosphorus mass balance provides a means by which steady state can be estimated using non-steady state data. The waste mixed liquor phosphate concentration, P_w, and its steady state value, P_{wss}, can be used as criteria to evaluate performance under experimental conditions, as they represent the phosphorus physically removed from the system under non-steady and steady state conditions respectively.

The fact that protein-rich substrates in the feed of a nutrient removal process can support the biomass and can initiate and enhance phosphate removal is in general consistent with the biochemical models proposed by Comeau *et al.* (1986) and Wentzel *et al.* (1986). However, extension of these models is required to satisfactorily explain the results obtained with protein-rich substrates. Among others provision must be made for the utilisation of amino or keto acids by some bio-P bacteria for PHB synthesis without energy input from polyphosphate in the anaerobic stage, and a certain amount of selectivity regarding the utilisation of ATP for polyphosphate or cell material synthesis in the aerobic stage.

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