Urban Water Research Association of Australia

Microbiological Studies on Enhanced Removal of Phosphates from Sewage

R C Bayly, J W May
G Vasiliadis and G N Rees

Department of Microbiology
Monash University, Melbourne

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FOREWORD

This report is based on UWRAA Research Project No SS-5: '*Microbiological studies on enhanced removal of phosphorus*' which was current during the period April 1988 to December 1990. The project was a part of a longer program being conducted by Monash University in collaboration with CSIRO Division of Chemicals and Polymers. Organisational responsibility for the project was as follows:

Research Agency	:	Department of Microbiology Monash University, Clayton Victoria
Project Officer	:	Professor R C Bayly Department of Microbiology Monash University
Principal Researcher	:	Professor R C Bayly

SYNOPSIS

Strains of *Acinetobacter* were isolated from pilot- and laboratory-scale systems which were removing phosphate biologically from sewage. Several isolates accumulated phosphate as polyphosphate to levels of 15-20% of the dry weight of the cells. Selected *Acinetobacter* isolates were studied to determine how changes in growth conditions affected their ability to accumulate polyphosphate. Mutant strains which could not accumulate polyphosphate were obtained from some of the good polyphosphate-accumulating *Acinetobacter* isolates. From studies on these mutant strains and their parent strains a model has been proposed to explain how polyphosphate is accumulated by *Acinetobacter.*

The properties of the strains isolated has brought into question the accepted hypothesis that release of phosphate from polyphosphate in the anaerobic zone of plants is essential for removal of phosphate biologically.

The synthesis of poly-ß-hydroxybutyrate, which has been regarded as an essential prerequisite for phosphate removal, has also been studied in several *Acinetobacter* isolates. Not all isolates which synthesize polyphosphate can form poly-ß-hydroxybutyrate. This again raises some questions on the previous accepted hypotheses.

The information obtained from this study should prove to be applicable to improved operation and design of enhanced phosphate removal treatment systems.

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1 INTRODUCTION

The presence of phosphate in effluents from sewage treatment systems is one of the primary causes of the eutrophication of lakes and streams into which such effluents are discharged. In conventional activated sludge systems there is almost no biological removal of phosphate and effluents have to be treated chemically to obtain an acceptably low level of phosphate. Chemical precipitation has been used for many years but it has several disadvantages such as the difficulty in processing the precipitate, the cost of the treatment and the increased salinity of the waters into which the effluent is discharged. It has been estimated that the cost of chemical treatment of sewage effluent in a city such as Canberra would be approximately $3 million per year.

There have been numerous reports of the successful operation of activated sludge systems which have been modified to result in the biological removal of phosphate resulting in effluents which require no further treatment. Such systems have been designed empirically on the basis of several years of trial modifications, but many are still reported to fail to operate successfully on a long-term basis. Apart from the effects of mechanical failures, the biological reasons for failure have been understood only poorly and the systems have been the subject of extensive research in several countries (Ramdori, 1987).

Early experiences in Australia in the operation of systems designed to remove phosphate biologically were not encouraging. It was against this background that a research group in the Department of Microbiology, Monash University, began a collaboration with what is now the CSIRO Division of Chemicals and Polymers, to study the basic microbiology of the Biological Removal of Phosphate from Sewage.

It has been shown that *Acinetobacter*, a genus of bacteria commonly found in soil and water, play a major role in the removal of phosphate from sewage. They do this by polymerization of the phosphate in sewage to polyphosphate which accumulates in the bacterial cells and is removed from the system in the sludge. Effluent from successfully operating systems may contain, consistently, as little as 0.2 - 0.5 mg/litre of phosphate. There has been a considerable lack of understanding of the basic microbial mechanisms which result in the formation of polyphosphate

and how the variation in physiological conditions which occur in sewage treatment systems may affect this formation.

It was reported by Barnard (1984) that prefermentation of sewage in an Activated Primary Tank resulted in improved phosphate removal and results from a CSIRO 5m^3 pilot-plant at Lower Plenty, support Barnard's observation. The configuration of this plant is shown in Figure 1. It has been accepted generally that the biological removal of phosphate requires the intracellular formation of poly-ß-hydroxybutyrate by *Acinetobacter* in the anaerobic stage of the system (see Figure 1) and that this requires the formation, by fermentation, of small volatile fatty acids such as acetic acid in the anaerobic stage and the Activated Primary Tank. The poly-ß-hydroxybutyrate is subsequently used as a source of carbon and energy when the cells enter the aerobic stage. A scheme which has been proposed for the formation and degradation of poly-ß-hydroxybutyrate and phosphate by polyphosphate-accumulating microorganisms is shown in Figure 2.

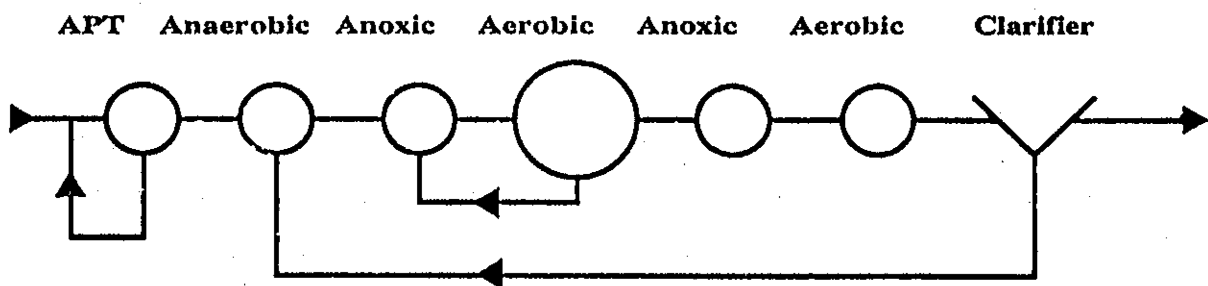

Figure 1. 5-Stage Bardenpho unit, incorporating an activated primary tank

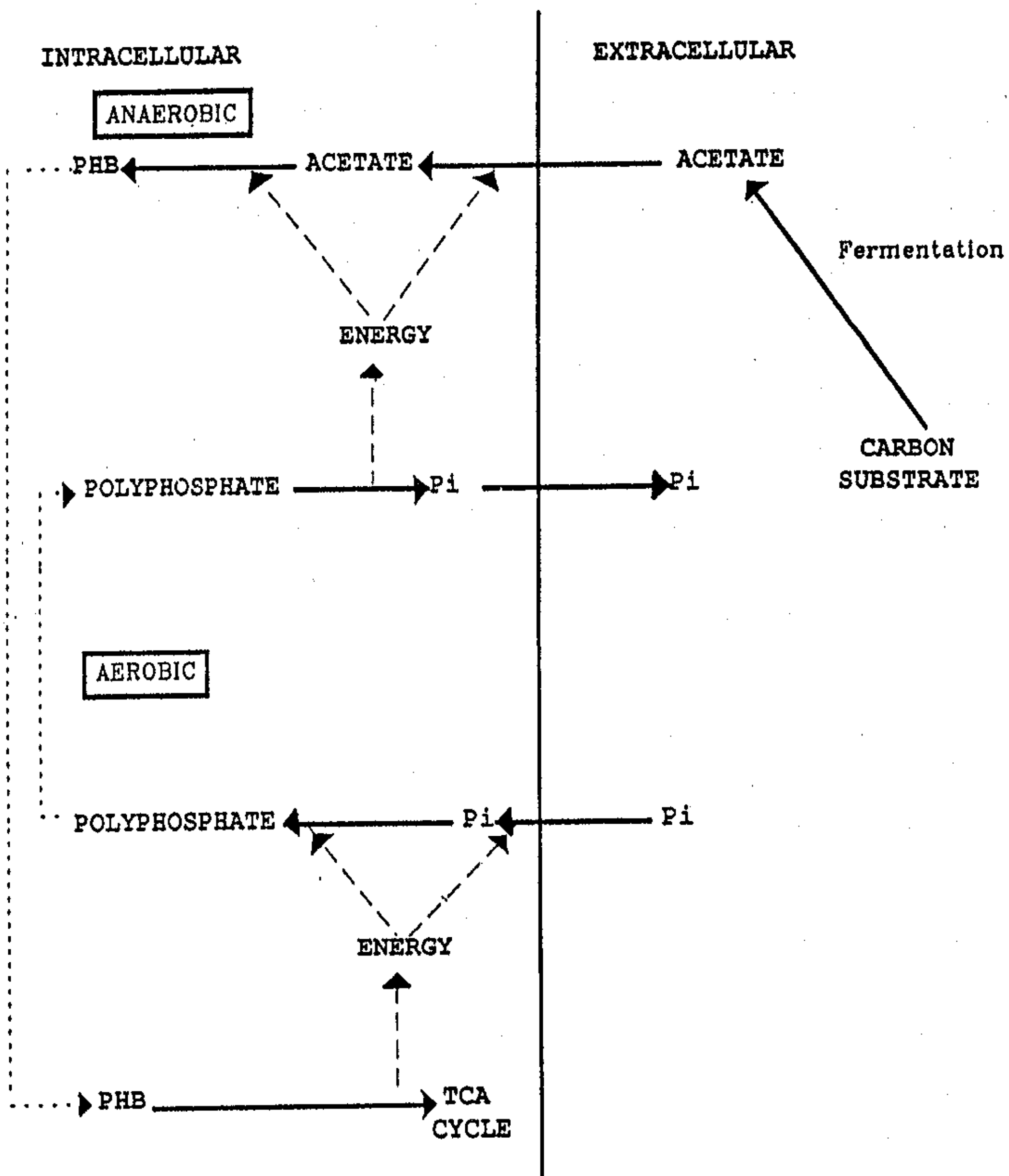

Fig. 2. Proposed Scheme for Formation and Degradation of Poly-ß-hydroxybutyrate and Polyphosphate by Polyphosphate-accumulating Organisms Grown in Alternating Aerobic and Anaerobic Conditions (Pi - orthophosphate)

The studies undertaken in this project have centred mainly on the microbial physiology of isolates of *Acinetobacter* which have been obtained from full-scale, pilot-scale and laboratory-scale treatment systems. The reason for this approach has been to gain an understanding of the basic mechanisms by which *Acinetobacter* accumulate phosphate and to assess how alterations in the conditions of the operation of sewage treatment systems may affect accumulation of polyphosphate, and therefore removal of phosphate. The parameters studied have been the type and concentration of carbon source, pH, dissolved oxygen tension, type of buffer system and level of other nutrients such as phosphate and nitrogen. An additional area of study has been in the genetics of *Acinetobacter* with the ultimate aims of understanding how the formation of phosphate and poly-ß-hydroxybutyrate is regulated in *Acinetobacter* species and of preparing a nucleic acid probe for the identification and enumeration of specific types of *Acinetobacter* in sewage treatment systems. This latter area has not come within the scope of work carried out by the Research Assistant supported by the Urban Water Research Association of Australia.

2 METHODS

2.1 Isolation of Strains of *Acinetobacter*

Strains were isolated from three types of experimental treatment plants.

i) a $5m^3$ pilot plant at Lower Plenty based on a five stage Bardenpho configuration with an Activated Primary Tank at the head of the plant. The plant was charged with $2m^3$ of return sludge from the Brushy Creek Treatment Plant.

ii) a pilot plant at Ballarat South Treatment plant which had alternating anaerobic/aerobic zones.

iii) laboratory-scale systems operated at the Division of Chemicals and Polymers, CSIRO; these systems were fed raw sewage from Lower Plenty and, in some instances, were supplemented by the addition of acetate (100 mg/l) as a carbon source.

Samples of sludge were plated onto casitone/glycerol/yeast extract agar medium (CYG) and colonies screened for their ability to accumulate phosphate when grown on a defined mineral salts

medium (ADM) which contained acetate as the sole source of carbon and inorganic phosphate (Duncan *et al.* 1988). Isolates were also obtained by micro-manipulation of clusters of cells in blended sewage samples as described by Duncan *et al.* (1988). Detection of phosphate was by microscopic examination after staining with Neisser's stain (Cowan, 1974).

A selection was made of strains with a range of ability to accumulate polyphosphate. Strains which were characterized as *Acinetobacter* spp. by biochemical tests (Bouvet and Grimont, 1987) and which were confirmed as *Acinetobacter* by the transformation test of Juni (1972) were given a laboratory number designation and retained for further studies. Table 1 shows the source and geno-species of strains studied in this project.

Table 1

STRAINS OF *ACINETOBACTER*

Strain	Origin	Geno-species
RA3053	Ballarat Pilot-plant	7
RA3114	Lower Plenty Pilot-plant	7
RA3116	Lower Plenty Pilot-plant	5
RA3739	Derivative of RA3116	5
RA3117	Lower Plenty Pilot-plant	7
RA3197	Derivative of RA3117	7
RA3203	Derivative of RA3117	7
RA3123	Lower Plenty Pilot-plant	7
RA3757	Laboratory-scale plant	-

2.2 Analytical Methods

Volatile fatty acids such as acetate, propionate and butyrate were analysed by Gas Liquid Chromatography using a Carbowax column. Poly-ß-hydroxybutyrate was analysed as described by Brandl *et al.* (1988). Ammonia was detected and determined using Nessler's Reagent.

Polyphosphate and phosphate were extracted from cells of *Acinetobacter* as described by Clark *et al.* (1986) and the concentration determined as reported by Bayly *et al.* (1991). The chain length profile of the polyphosphate was determined as described by Robinson *et al.* (1987).

2.3 Assay of Enzyme Activity

Assays for the determination of the activity of enzymes involved in the biosynthesis and degradation of phosphate and poly-ß-hydroxybutyrate were by the following methods: polyphosphate kinase (Robinson *et al.*, 1987); polyphosphate:AMP phosphotransferase (van Groenestijn *et al.*, 1987); polyphosphate glucokinase and 3-phosphoglycerate kinase (Wood and Goss, 1985); ß-ketothiolase and aceto-acetylCoA-reductase (Senior and Dawes, 1973).

3 RESULTS

3.1 Operation of the Lower Plenty Pilot Plant

Over the 5.5 months of operation after start-up, raw sewage entering the plant usually contained 8-10 mg phosphate-P 1^{-1}. The concentrations of particular nutrients in selected stages of the plant are shown in Table 2. Clearly, at 4 and 5.5 months after start-up, the plant was showing enhanced removal of phosphate (0.1-0.2 mg phosphate-P 1^{-1} effluent).

Table 2

NUTRIENT CONCENTRATIONS (mgl^{-1}) IN SAMPLES FROM SELECTED STAGES OF THE LOWER PLENTY PILOT PLANT. VALUES ARE RANGES OVER 3 DAYS

Time after start up (months)	Phosphate-P at end of anaerobic stage	Phosphate-P in effluent from clarifier	Nitrate/nitrite-N in effluent from clarifier
0	8 - 10	8 - 11	< 3
4	12 - 20	0.1 - 0.2	1.5 - 2.5
5.5	7 - 14	0.1	1.5

At start-up, filamentous bacteria were the most noticeable component of the sludge; whereas 4 and 5.5 months later, although filamentous bacteria were still present, the most noticeable component was clusters (diameter 5-50 µm; mean approximately 20 µm) of coccoid cells (diameter up to 2 µm) densely packed with polyphosphate.

At start-up, of 100 isolates obtained from sludge by colony-picking using CGY agar, 34 were Gram-negative and oxidase-negative. Of these 34 isolates, 5 were assigned to *Acinetobacter* using the Microbact 24E system, but only 1 of the 5 was identified as an *Acinetobacter* by the transformation test. Four months later, when the plant was exhibiting enhanced removal of phosphate, of 100 isolates obtained by colony-picking using CGY agar, 22 were Gram-negative and oxidase-negative. Of these 22 isolates, 8 were assigned to *Acinetobacter* using the Microbact 24E system, but only 4 of the 8 were identified as *Acinetobacter* by the transformation test. Clearly, identification of an isolate as an *Acinetobacter* using a limited number of conventional biochemical tests (as in the Microbact 24E system) is unreliable compared with the transformation test. All 5 of the isolates confirmed as *Acinetobacter* belonged to genospecies 7.

Since it was not known whether colonies of *Acinetobacter* arose from single bacterial cells or the large clusters of polyphosphate-containing cells, a further set of isolates was obtained by using both conventional colony-picking and micro-manipulation to separate the clusters of coccoid cells from the other bacteria in the preparation. In order to increase the proportion of *Acinetobacter* recovered, isolates were made on ADM agar as well as CGY agar. Furthermore, in order to

overcome the problem of identifying isolates as acinetobacters, all Gram-negative, oxidase-negative isolates were subjected to the transformation test.

Details of the isolates obtained by colony-picking and by micro-manipulation of clusters from sludge at 5.5 months after start-up are shown in Table 3. With colony picking, the proportion of isolates identified as *Acinetobacter* was higher on ADM agar (16%) compared with CGY agar (4%). With both media, however, the dominant genospecies was 7 with a lesser number of genospecies 8 or 9. With micro-manipulation, only about a third of the isolated clusters yielded viable progeny. This may have been a consequence of the use of inadequate media for recovery or to the intrinsic nonviability of the cells perhaps due to over-accumulation of polyphosphate.

Table 3

GENOSPECIES OF *ACINETOBACTER* ISOLATED FROM ACTIVATED SLUDGE AT 5.5 MONTHS AFTER START-UP OF THE PLANT

Isolation method	Medium	Number of colonies or clusters of cells:		Number of isolates identified as *Acinetobacter* assigned to genospecies										
		selected	viable	1	2	3	4	5	6	7	8/9	10	11	12
Isolation method	CGY	100	100	0	0	0	0	0	0	3	1	0	0	0
Colony-picking	ADM	100	100	0	0	0	0	0	0	14	2	0	0	0
Micro-manipulation	CGY	10	3	0	0	0	1	0	0	2	0	0	0	0
Micro-manipulation	ADM	50	18	0	0	0	0	2	0	6	1	0	0	0

In a numerical study of strains of *Acinetobacter* isolated from diverse sources, Schutte *et al.* (1987) found that 4 strains isolated from activated sludge were included in their cluster A1, which they equated with genospecies 8 of Bouvet and Grimont (1987). However, the validity of their extrapolation is very doubtful since: i) the transformation test of Juni (1972) was not used to confirm membership of the genus; and ii) the results of API tests were used initially to cluster

the strains and then equate these clusters to the subgroups of Baumann *et al.* (1968) which were then related to the genospecies of Bouvet and Grimont (1987).

The application of appropriate identification procedures should reveal whether genospecies 7 of *Acinetobacter* is generally dominant in plants removing phosphate or whether the nature of the dominant genospecies depends upon the particular conditions existing in each plant.

3.2 Detection of *Acinetobacter* in Sewage Treatment Plants

A method which is commonly used for the detection of specific genera of microorganisms in natural habitats is to prepare antisera to the microbial genus to be detected, label it with a fluorescent dye and detect members of the genus by reaction of the fluorescent antibody with the microbial cells; the reaction is detected microscopically.

Antisera to three isolates of *Acinetobacter* were prepared in rabbits but application of these antisera to detection of *Acinetobacter* in samples of sludge from treatment plants was unsuccessful due to non-specific binding of the antisera to components in the sludge

3.3 Polyphosphate in *Acinetobacter* Strains Isolated from Sewage Treatment Systems

3.3.1 Accumulation of polyphosphate

Table 4 shows the intracellular concentration of long-chain and long-chain-granular polyphosphate in good and poor polyphosphate-accumulating isolates. The acid-soluble fraction has been shown to consist almost entirely of orthophosphate (Vasiliadis *et al.*, 1990). The analytical data showing high levels of long-chain polyphosphate in strains RA3053, 3116 and 3117 is consistent with the microscopic observations. The reason for the increase in the level of orthophosphate has not been investigated.

Table 4

INTRACELLULAR POLYPHOSPHATE CONCENTRATIONS IN *ACINETOBACTER* STRAINS

Strain	Polyphosphate (μgPO_4-P/mg protein)		
	Acid-soluble	Acid-insoluble	
		LCPP	LCGPP
RA3053	132	220	27
RA3114	30	21	8
RA3116	52	336	38
RA3739	15	10	6
RA3117	118	476	26
RA3197	28	25	6
RA3757	6	60	14

LCPP - long-chain polyphosphate
LCGPP - long-chain granular polyphosphate

3.3.2 Activities of polyphosphate synthesizing enzymes

Several enzymes have been reported to play a role in the synthesis/metabolism of polyphosphate. Four of these, and the reactions they catalyze, are shown in Fig. 3.

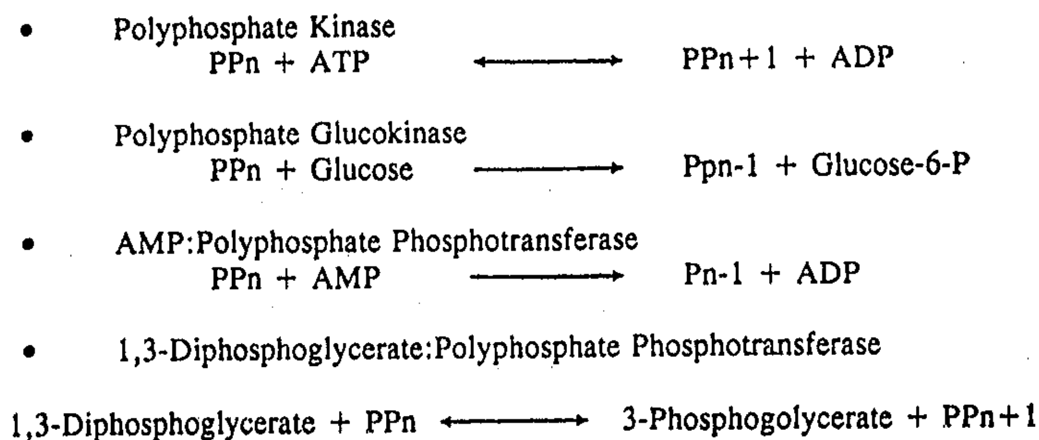

Fig. 3. Enzymes Involved in Polyphosphate Metabolism

The specific activities of polyphosphate kinase, polyphosphate AMP-phosphotransferase and 3-phosphoglycerate kinase were determined for several of the *Acinetobacter* isolates grown on ADM. As no polyphosphate glucokinase activity was detected in the good polyphosphate accumulating strains RA3116 and 3117 this enzyme was not investigated further.

Table 5

SPECIFIC ACTIVITIES OF POLYPHOSPHATE SYNTHESIZING ENZYMES IN *ACINETOBACTER* STRAINS

Strain	Enzymes		
	PK	PPT	PGK
RA3114	20	<10	55
RA3116	20	<10	71
RA3117	18	<10	57
RA3123	36	ND	62
RA3197	32	<10	92
RA3739	43	<10	68

Activities expressed as nmol/min/mg protein

Abbreviations: ND - not detected; PK - polyphosphate kinase;
PPT - polyphosphate AMP-phosphotransferase; PGK - 3-phosphoglycerate kinase.

The activities of polyphosphate kinase in all six strains were similar and those of polyphosphate AMP-phosphotransferase were very low. The high levels of 3-phosphoglycerate kinase were probably due to the cells undertaking gluconeogenesis since acetate was the carbon source.

3.3.3 Synthesis of polyphosphate in the presence of an ATP synthase inhibitor, N,N′-dicyclohexylcarbodiimide.

The growth characteristics of strain RA3117 and its variant strain RA3197 in ADM are similar but the patterns of accumulation of polyphosphate are significantly different (Fig. 4a and b); the variant failed to show any significant increase in polyphosphate throughout the growth cycle. When N,N′-dicyclohexylcarbodiimide (10 μM) was added to the culture medium, RA3117 showed

an increase in its lag phase from 2 h to 8 h and its doubling time increased to 2.6 h from 1.6 h. However, polyphosphate accumulation continued throughout the growth cycle and the final concentration was similar to that attained in the absence of N,N'-dicyclohexylcarbodiimide. (Fig. 4c).

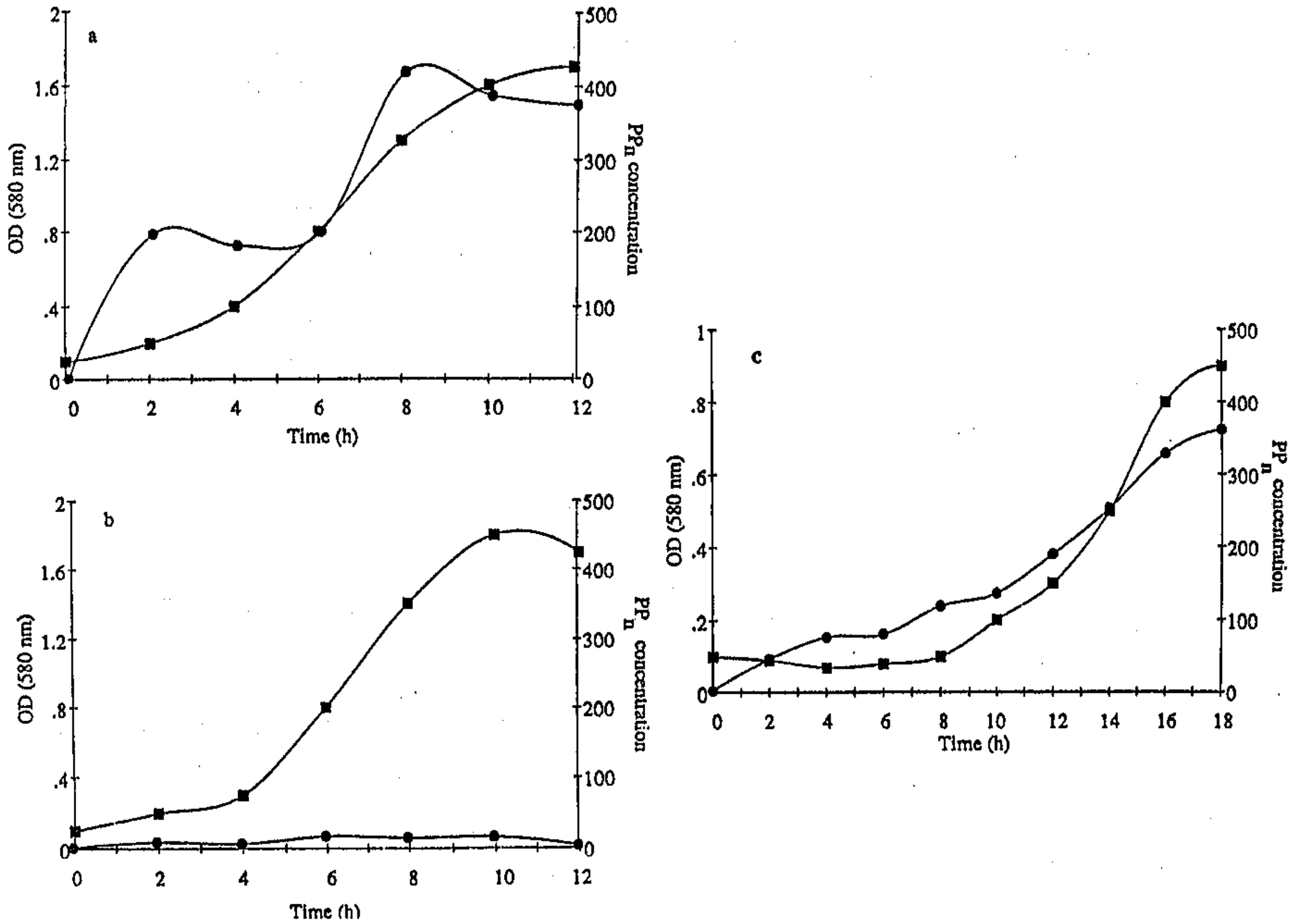

Fig. 4. Accumulation of polyphosphate by *Acinetobacter* spp. grown on acetate
a) RA3117; b) RA3197; c) RA3117 + 10µM N,N'-dicyclohexylcarbodiimide.
• PP_n - expressed as µg PO_4-Pmg^{-1} protein ■ Optical density - 580nm

A comparison of the effect of N,N'-dicyclohexylcarbodiimide on the independently-isolated good polyphosphate accumulating strain RA3116 and its variant RA3739, with RA3117 and RA3197, given in Table 6, shows that the strains reacted similarly.

Table 6

POLYPHOSPHATE ACCUMULATION IN *ACINETOBACTER* STRAINS GROWN IN THE PRESENCE OF N,N'-DICYCLOHEXYLCARBODIIMIDE

Strain		N,N'-dicyclohexylcarbodiimide (µM)		
		0	10	20
RA3116	Growth	+++	+++	+++
	Polyphosphate	+++	+++	+
RA3739	Growth	+++	+++	+/-
	Polyphosphate	-	-	-
RA3117	Growth	+++	+++	+++
	Polyphosphate	+++	+++	+
RA3197	Growth	+++	+/-	-
	Polyphosphate	-	-	-

The data which has accumulated from studies on Bardenpho-type treatment plants all emphasize the requirement for an alternating anaerobic/aerobic phase if the plants are to remove phosphate biologically. Common to all reports are: i) that acetate or other volatile fatty acids are formed in the anaerobic phase and are converted to poly-ß-hydroxybutyrate; ii) there is a release of orthophosphate from polyphosphate in the anaerobic phase and this provides the energy for formation of poly-ß-hydroxybutyrate; iii) in the aerobic phase, poly-ß-hydroxybutyrate utilization acts as an energy source for the synthesis of polyphosphate; iv) recycling of sludge containing polyphosphate to the anaerobic phase is essential for good operation of the plant. From these observations Comeau *et al.* (1986) proposed a biochemical model to explain how bacteria accumulate polyphosphate. This model, however, cannot explain some of the data which have been presented here. It must be emphasized that the results obtained from this study with pure cultures of *Acinetobacter* do not all agree with the observations from operating plants. The main difference is that inorganic phosphate release is not found under anaerobic incubation of the *Acinetobacter* isolates so far examined and this brings into question as to whether the so-called 'recycling of phosphate' is necessary for satisfactory operation of a plant.

The most important requirement to obtain an understanding of how 'biological removal of phosphate' plants should be operated is to elucidate the basic physiological conditions which control the synthesis of polyphosphate.

The enzyme most commonly implicated in the biosynthesis of polyphosphate is polyphosphate kinase, but there have been conflicting reports as to the importance of the role of this enzyme (T'Seyen *et al.*, 1985; van Groenestjin *et al.*, 1989). The results from a range of *Acinetobacter* examined in this study show that the activity of polyphosphate kinase did not vary significantly between those strains which do and do not accumulate polyphosphate to high levels and the same finding applied to the other enzymes assayed. This suggests that the synthesis of polyphosphate in *Acinetobacter* does not involve any of the enzymes examined.

From the results of experiments carried out with low levels of N,N′-dicyclohexylcarbodiimide in the growth medium we have been able to propose modifications to the Comeau model for the synthesis of polyphosphate by *Acinetobacter*. The model which we have proposed (Fig. 5) is discussed in detail in May *et al.* (submitted for publication) and is summarized here. When bacteria use acetate as a source of carbon they cannot produce ATP, which they require for energy, by fermentation under anaerobic conditions, but must produce ATP by respiration under aerobic conditions. The latter involves extrusion of protons from the cell in order to form a proton gradient across the cell membrane, followed by a proton influx through the membrane-associated ATP synthase complex to produce ATP.

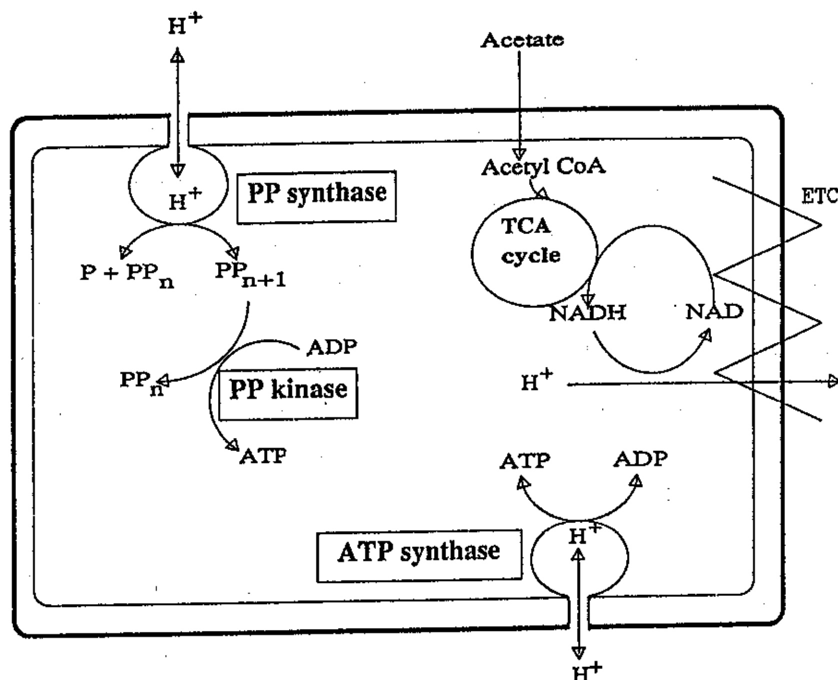

Fig. 5. Proposed model for polyphosphate formation by *Acinetobacter* spp. growing on acetate as sole source of carbon and energy (May *et al.* submitted)

P - Phosphate ETC - Electron transport chain PP_n - Polyphosphate

The inability of the variant strain, RA3197, to show significant growth in the presence of N,N′-dicyclohexylcarbodiimide was due presumably to inhibition of its ability to form ATP required for biosynthesis and growth. RA3117, a good polyphosphate accumulating strain and the parent strain of RA3197, both grew in the presence of N,N′-dicyclohexylcarbodiimide and accumulated polyphosphate under these conditions of growth. This may have been due to its ATP synthase being more resistant to N,N′-dicyclohexylcarbodiimide than that of its variant, but such an explanation is inconsistent with its ability to form high levels of polyphosphate.

It has been proposed that polyphosphate may act as a phosphoryl donor for production of ATP when ATP synthase was inhibited by N,N′-dicyclohexylcarbodiimide (Suresh *et al.* 1984), but an organism could only maintain growth under these conditions if polyphosphate was produced without the use of ATP as a phosphoryl donor. In the model now proposed, formation of polyphosphate in RA3117 would be by a proton-gradient driven system - a ‘polyphosphate synthase’ - analogous to ATP synthase. The polyphosphate kinase activity in the cell could produce ATP from polyphosphate and ADP and this would enable growth to continue. It is suggested that the variant, RA3197, lacks the proposed ‘polyphosphate synthase’ and that the low

level of polyphosphate formed in this strain in the absence of N,N′-dicyclohexylcarbodiimide is synthesized by polyphosphate kinase using ATP as a phosphoryl donor.

Studies are continuing with these strains to determine if the mechanism proposed for the formation of polyphosphate can be confirmed. Elucidation of this mechanism could result in establishing the best conditions for formation of polyphosphate in treatment plants so enabling a more consistent operation in relation to phosphate removal.

3.3.4 Synthesis of polyphosphate under limiting-nutrient growth conditions

Synthesis of storage materials, such as polyphosphate, by bacteria is often initiated when growth is under conditions where a specific nutrient is limiting. Three isolates of *Acinetobacter*, RA3117, 3197 and 3757 have been studied under growth conditions where one of either sulphur, phosphorus or nitrogen was limiting.

The experiments were carried out in Braun Biolab fermenters where pH and oxygen levels were controlled. In relation to the normal defined medium (see Methods), the concentrations of sulphate, phosphate and ammonium ions were reduced, respectively, to 1/30, 1/50 and 1/10 of the concentrations normally used. The results are summarized in Table 7.

The significant finding from these experiments is that the mutant strain RA3197, derived from RA3117, which will not accumulate polyphosphate under 'normal' growth conditions, can accumulate significant amounts of polyphosphate when grown under limiting sulphate concentrations.

This finding requires further investigation as it indicates that synthesis of polyphosphate may be regulated, at least in part, by the level of sulphate in a system. Alternatively, it may be that there are two separate mechanisms whereby *Acinetobacter* synthesize polyphosphate.

The polyphosphate accumulated by strain RA3117 grown in non-limited sulphate conditions and that accumulated by strain RA3197 grown under sulphate-limitation, was extracted from the cells and examined to determine if the chain-length profile of the polyphosphate synthesized in the two strains varied. Preliminary results from this experiment have shown no difference in chain-length of the polyphosphate from the two strains. Experiments to compare the activities of the polyphosphate kinase synthesized under sulphate-limiting growth conditions have yet to be determined.

Table 7

POLYPHOSPHATE ACCUMULATION BY *ACINETOBACTER* STRAINS GROWN AT 100% DISSOLVED OXYGEN WITH NUTRIENT LIMITATION

Strain	ADM	Limiting Sulfate	Limiting Phosphate	Limiting Ammonia
3117	+++	++++	-	++++
3197	-	++	-	-
3757	++	++	-	+++

3.4 Synthesis of Poly-ß-Hydroxybutyrate in *Acinetobacter* Isolated from Sewage Treatment Systems

As outlined in the Introduction, one of the models for the biological removal of phosphate from sewage is that the polyphosphate accumulating microorganisms synthesize poly-ß-hydroxybutyrate in the anaerobic zone of the plant. Three isolates of *Acinetobacter*, RA3117, 3197 and 3757 (see Table 1) were grown under varying nutrient conditions in the Biolab fermenters and the cells examined for poly-ß-hydroxybutyrate and for the enzymes required for synthesis of that compound. The results are shown in Tables 8 and 9, respectively.

Table 8

POLY-ß-HYDROXYBUTYRATE PRODUCTION BY *ACINETOBACTER* STRAINS GROWN AT 100% DISSOLVED OXYGEN WITH NUTRIENT LIMITATION

Strain	ADM	Limiting Sulfate	Limiting Phosphate	Limiting Ammonia
3117	-	-	Trace	-
3197	-	-	Trace	-
3757	-	++	++	++

Table 9

SPECIFIC ACTIVITIES OF POLY-ß-HYDROXYBUTYRATE BIOSYNTHETIC ENZYMES IN *ACINETOBACTER* STRAINS

	Sudanophilic material		Specific Activity[a]			
			ß-Ketothiolase		AcetoacetylCoA reductase	
Strain	ADM	Limiting PO_4	ADM	Limiting PO_4	ADM	Limiting PO_4
RA3117	-	++	ND[b]	ND	ND	ND
RA3197	-	++	ND	ND	ND	ND
RA3757	+/-	+++	4.7	7.7	ND	23.5

[a] Specific activity expressed as nmoles/min/mg protein
[b] Not detected

The results show that strain RA3757 synthesizes a significant amount of poly-ß-hydroxybutyrate when one of either sulphate, phosphate and ammonia are limiting on the growth medium. The analytical data for this strain (Table 8) is consistent with the enzyme data for the biosynthetic poly-ß-hydroxybutyrate enzymes (Table 9). RA3757 synthesized up to 10% poly-ß-hydroxybutyrate dry weight (data not shown).

The analytical data for strains RA3117 and 3197 are also consistent with the enzyme data. However, both RA3117 and 3197 accumulated material which stained with sudan black, a dye used to detect compounds such as poly-ß-hydroxybutyrate. Clearly this material is not poly-ß-

hydroxybutyrate as the latter compound was not detected analytically. Further investigations will be required to identify the sudanophilic material.

4 CONCLUSION

The results presented show that conditions of growth control the synthesis of the polyphosphate and reinforce the view that an understanding of the basic microbiological processes of polyphosphate formation is necessary before the full potential of plants designed to remove phosphate biologically can be achieved. Translation of results obtained by studies with pure cultures to mixed culture systems such as are found in sewage treatment plants can be difficult, and even misleading, but without the knowledge of the basic mechanisms taking place, such systems will always remain a 'black-box'.

The results obtained have laid a solid foundation to determine how best to operate sewage treatment plants designed to remove phosphorus biologically. In systems which are required to carry out several functions such as treatment of carbonaceous compounds, nitrogenous compounds as well as phosphate removal, it may always be necessary to compromise some requirements to achieve acceptable results in others.

In the many reports on processes designed to remove phosphorus biologically, it is generally observed that polyphosphate is released from sludge during the anaerobic phase and poly-ß-hydroxybutyrate is formed, but it has not been shown that this phenomenon occurs within the same cells in the sludge. The results so far obtained with strain RA3757 indicate that this strain may act more closely to the manner observed in activated sludge. It forms significant amounts of both polyphosphate and poly-ß-hydroxybutyrate when grown under some nutrient-limiting conditions and polyphosphate is degraded, albeit under aerobic conditions. Further studies will be necessary to determine the mechanisms acting in this strain.

In conjunction with the above program, studies on the Activated Primary Tank at the Lower Plenty pilot-plant have been taking place at the CSIRO Division of Chemical and Polymers to

determine what compounds are formed in this tank. This work has been funded by an Australian Water Research Association grant (Project No 88/10) and shows that significant levels of volatile fatty acids such as acetate and propionate are formed, presumably by fermentation of complex carbohydrates, proteins and lipids present in the sewage. Microbiological studies carried out in collaboration with Project 88/10 have been funded by an AWRAC Grant (Project No 88/09) and results from these projects will be reported elsewhere.

The original proposal to Urban Water Research Association of Australia, indicated that some aspects of the genetics of *Acinetobacter* would be studied with the ultimate aim of producing gene probes which could be used to monitor plants designed to remove phosphorus biologically. It has been necessary to undertake basic research into the genetics of *Acinetobacter* and this has been done by a postgraduate candidate funded by a Monash University Graduate Scholarship. There is still a considerable amount of work to be carried out before any of the findings so far obtained can be related to phosphate removal by *Acinetobacter*.

The studies to date show that what have been the commonly accepted mechanisms of biological removal of phosphate may have to be modified, especially that aspect which requires release of polyphosphate in an anaerobic zone before polyphosphate can be synthesized in the aerobic zone. Future work will be directed towards obtaining an understanding of this finding.

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6 REFERENCES

Barnard, J.L. (1984). Activated primary tanks for phosphate removal. *Water SA. 10*, 121-126.

Baumann, P., M. Doudoroff and R.Y. Stanier (1968). A study of the *Moraxella* Group II Oxidative-negative species (Genus *Acinetobacter*). *J. Bacteriol. 95*, 1520-1541.

Bayly, R.C., A. Duncan, J.W. May, M. Schembri, A. Semertjis, G. Vasiliadis and W.G.C. Raper (1991). Microbiological and genetic aspects of the synthesis of polyphosphate by species of *Acinetobacter*. *Wat. Sci. Tech. 23*, 747-754.

Bouvet, P.J.M. and P.A.D. Grimont (1987). Identification and biotyping of clinical isolates of *Acinetobacter*. *Ann. Inst. Pasteur/Microbiol. 138*, 569-578.

Brandl, H., R.A. Gross, R.W. Lenz and R.C. Fuller (1988). *Pseudomonas oleovorans* as a source of poly(ß-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl. Environ. Microbiol. 54*, 1977-1982.

Clarke, J.E., H. Beegan and H.G. Wood (1986). Isolation of intact chains of polyphosphate from *Propionibacterium shermanii* grown on glucose and lactate. *J. Bacteriol. 168*, 1212-1219.

Comeau, Y., K.J. Hall, R.E.W. Hancock and W.K. Oldham (1986). Biochemical model for enhanced biological phosphorus removal. *Wat. Res. 20*, 1511-1521.

Cowan, S.T. (1974). Cowan and Steele's Manual for the Identification of Medical Bacterial. Second Ed. London, Cambridge University Press.

Duncan, A., G.E. Vasiliadis, R.C. Bayly and J.W. May (1988). Genospecies of *Acinetobacter* isolated from activated sludge showing enhanced removal of phosphate during pilot-scale treatment of sewage. *Biotechnol Lett. 10*, 881-886.

Juni, E. (1972). Interspecies transformation of *Acinetobacter*. Genetic evidence for a Ubiquitous Genus *112*, 917-931.

May, J.W., A. Duncan, G.N. Rees, M. Schembri, G.E. Vasiliadis and R.C. Bayly. A model for the biosynthesis of polyphosphate in *Acinetobacter*. Submitted to *Arch. Microbiol* (1991).

Ramadori, R. (1987). Biological phosphate removal from wastewaters. Proceedings of an IAWPRC Specialized Conference held in Rome, 28-30 September. Pergamon Press, Oxford.

Robinson, H.A., J.E. Clark and H.G. Wood (1987). Polyphosphate kinase from *Propionibacterium shermanii.* Demonstration that polyphosphates are primers and determination of the size of the synthesized polyphosphate. *J. Biol. Chem. 262*, 5216-5222.

Schutte, C.E., T.S. Britz and W.A. Joubert (1987). The isolation and characterisation of *Acinetobacter* strains present in the liquid phase of an anaerobic fixed bed digester while treating a petrochemical effluent. *Water SA. 13*, 57-62.

Senior, P.J. and E.A. Dawes (1973). The regulation of poly-ß-hydroxybutyrate metabolism in *Azotobacter beijerinckii. Biochem. J. 134*, 225-238.

Suresh, N., R. Warburg, M. Timmerman, J. Wells, M. Coccia, M.F. Roberts and H.O. Halvorson (1984). New strategies for the isolation of microorganisms responsible for phosphate accumulation. In Post-Conference Seminar on Enhanced Biological Phosphorus Removal from Wastewaters, Paris pp 132-144.

T'Seyen, J., D. Malnou, J.C. Block and G. Faup (1985). Polyphosphate kinase activity during phosphate uptake by bacteria. *Wat. Sci. Tech. 17*, 43-56.

van Groenestijn, J.W., M.M.A. Bentvelsen, M.H. Deinema and A.J.B. Zehnder (1989). Polyphosphate degrading enzymes in *Acinetobacter* spp. and activated sludge. *Appl. Environ. Microbiol. 54*, 219-223.

van Groenestijn, J.W., M.H. Deinema and A.J.B. Zehnder (1987). ATP production from polyphosphate in *Acinetobacter* strain 210A. *Arch. Microbiol. 148*, 14-19.

Vasiliadis, G., A. Duncan, R.C. Bayly and J.W. May (1990). Polyphosphate production by strains of *Acinetobacter. FEMS Micriobiol. Lett. 70*, 37-40.

Wood, H.G. and N.H. Goss (1985). Phosphorylation enzymes of the propionic acid bacteria and the roles of ATP, inorganic pyrophosphate and polyphosphates. *Proc. Natl Acad. Sci.* USA. *82*, 312-315.

APPENDIX A

PUBLICATIONS ARISING FROM RESEARCH ON THE BIOLOGICAL REMOVAL OF PHOSPHATE PROGRAMME

Note: In addition to support from the UWRAA there has been support from other bodies.

Journals and Books

1. Vasiliadis, G., R.C. Bayly, J.W. May and W.G.C. Raper (1987). Changes in concentrations of polyphosphate-containing bacteria and *Acinetobacter* spp. during establishment of enhanced phosphate removal. IAWPRC Newsletter No 1.

2. Vasiliadis, G., A. Duncan, R.C. Bayly, J.W. May and W.G.C. Raper (1987). *Acinetobacter* genospecies isolated from activated sludge showing enhanced removal of phosphate. Poster paper presented at IAWPRC Intl. Spec. Conf. on Biological Phosphate Removal from Waste Waters, Rome, 28-30 September.

3. Duncan, A., G. Vasiliadis, R.C. Bayly, J.W. May and W.G.C. Raper (1988). Genospecies of *Acinetobacter* isolated from activated sludge showing enhanced removal of phosphate during pilot-scale treatment of sewage. *Biotechnology Letts. 10,* 831-836.

4. Vasiliadis, G., A. Duncan, R.C. Bayly and J.W. May (1990). Polyphosphate production by strains of *Acinetobacter. FEMS Microbiol. Letts. 70,* 37-40.

5. Duncan, A., R.C. Bayly, J.W. May, W.G.C. Raper and G. Vasiliadis (1990). Enhanced biological removal of phosphorus from Wastewater. In Proceedings of the Symposium on 'The Role of Surface and Colloid Chemistry in Natural Waters and Water Treatment'. Plenum Press.

6. Rees, G.N., A. Duncan, G. Vasiliadis, J.W. May, W.G.C. Raper and R.C. Bayly (1990). Biological removal of phosphate from wastewater. *Aust. J. Biotechnol. 4,* 186-187.

7. Bayly, R.C., A. Duncan, J.W. May, M. Schembri, A. Semertjis, G. Vasiliadis and W.G.C. Raper (1991). Microbiological and genetic aspects of the synthesis of polyphosphate by species of *Acinetobacter. Wat. Sci. Tech. 23*, 747-754.

8. May, J.W., A. Duncan, M. Schembri, G. Vasiliadis and R. Bayly. A model for the biosynthesis of polyphosphate in *Acinetobacter.* Submitted to *Arch. Microbiol.*

Conference Papers

1. Duncan, A., R.C. Bayly, J.W. May, G. Vasiliadis and W.G.C. Raper (1988). Characteristics of *Acinetobacter* genospecies from activated sludge showing enhanced removal of phosphate. Presented as a Poster Paper at ASM Annual Scientific Meeting, May, Sydney, and also presented at an NZMS Meeting, May, Dunedin.

2. Vasiliadis, G., A. Duncan, R.C. Bayly, J.W. May and W.G.C. Raper (1988). Characteristics of *Acinetobacter* associated with enhanced removal of phosphate by activated sludge. Presented to a meeting of representatives of Australian Water Boards at Ballarat, October 1988.

3. Bayly, R.C. *et al.* (1989). The role of *Acinetobacter* spp. in the removal of phosphate from waste waters. In Proceedings of the 8th Australian Biotechnology Conference, University of NSW, 6-9 February, 279-282.

4. Bayly, R.C., A. Duncan, J.W. May, N.H. Pilkington, W.G.C. Raper and G.E. Vasiliadis (1989). Effect of primary fermentation on biological nutrient removal. Paper presented at the 13th Federal Convention of the Australian Water and Wastewater Convention, Canberra, March. Paper presented by A. Duncan and W. Raper.

5. May, J.W., A. Duncan, R.C. Bayly, G. Vasiliadis and W.G. Raper (1989). *Acinetobacter* species involved in the removal of phosphate from sewage. Poster presented at the Australian Society for Microbiology Conference, Adelaide, July. Poster presented by J.W. May.

6.-8. Presented at Australian Society for Microbiology, Launceston, July 1990.

6. Duncan, A., R.C. Bayly, J.W. May and G. Vasiliadis. A proposed mechanism for polyphosphate accumulation by *Acinetobacter.*

7. Rees, G.N., R.C. Bayly, J.W. May and W.G.C. Raper. Anaerobic pretreatment of sewage can improve phosphate removal in Bardenpho plants.

8. Vasiliadis, G., R.C. Bayly and J.W. May. Polyphosphate synthesis in *Acinetobacter* spp. from activated sludge.

9. Bayly, R.C., J.W. May, A. Duncan, G. Vasiliadis, M. Schembri (1990). Physiology of polyphosphate accumulating *Acinetobacter* strains. In Proceedings of the 1st Australian Conference on Biological Nutrient Removal from Wastewater, pp 107-115, Bendigo, 9-12 July.

10. Duncan, A., R.C. Bayly, J.W. May, G. Vasiliadis, A. Semertjis (1990). Analysis of plasmids in *Acinetobacter* strains isolated from activated sludge. In Proceedings of the 1st Australian Conference on Biological Nutrient Removal from Wastewater, pp 117-123, Bendigo, 9-12 July.

11. May, J.W., A. Duncan, R.C. Bayly, M. Schembri and G. Vasiliadis (1990). Presented at a FEMS *Acinetobacter* Workshop, Paris, 5-7 September.

12. Bayly, R.C., J.W. May, G.N. Rees and G. Vasiliadis (1991). The role of *Acinetobacter* in the biological removal of phosphate from wastewaters. American Society of Microbiology Conference on Biotechnology, New York, 27-30 June.

UWRAA RESEARCH REPORTS

Report No.	Title	Author
1	Trickling filter - solids contact process: Pilot plant studies.	M. Laginestra
2	A model of water pricing for Melbourne, Sydney and Perth	P.B. Dixon P.M. Norman
3	Taste generation associated with chloramination	M. Kerslake
4	Bacterial regrowth in water supplies	K. Power L.A. Nagy
5	Leakage management: Assessing the effect of pressure reduction on losses from water distribution systems	B. Horvath
6	Improving communication with the public on water industry policy issues	B.E. Nancarrow G.J. Syme
7	Water use efficiency of domestic appliances	I.J. Beith D.J. Horton
8	Pilot plant study of fermentation units to increase degradable COD fraction in sewage.	P.J. Bliss D. Barnes P.R. Evans I. Law
9	Artificial destratification of water storages in Australia.	T.F. McAuliffe R.S. Rosich
10	Taste thresholds of monochloramine and chlorine in water	R. O'Halloran C. Veres
11	Chromatographic analysis of chloramines using electro-chemical detection	R. O'Halloran Hai Lin Ge P. Spizziri
12	Glass reinforced plastic bore casing for large diameter and deep bores	R. Bowyer
13	A guide to improving communication with the public on water industry policy issues	B.E. Nancarrow G.J. Syme
14	Fouling and cleaning of fine bubble ceramic dome diffusers	K.J. Hartley
15	Chloramination of Water Supplies	P.M. Thomas (ed)
16	The 1988 Australian Winter Storms Experiment: Report on aircraft observations	J.B. Jensen
17	Pipeline assets: Life cycle management and economic life	R. Vass M. Anderson R. Lewis D. Samson
18	Development of empirical model for tradewaste discharges to small treatment plants	Camp Scott Furphy
19	PRELIM users guide (Amended): Australian Version	Camp Scott Furphy
20	Chemical regeneration of activated carbon: Preliminary studies	G. Newcombe
21	Management and display of dam surveillance data	D.M. Stirling G.L. Benwell A.B. Murnane
22	Evaluation and demonstration facilities for primary sensors	J.A. Lanaway M. Cavey
23	Modelling and design of reservoir aeration destratification systems	D.P. Lewis J.C. Patterson J. Imberger R.P. Wright S.G. Schadlow
24	Modelling optimum conditions for reservoir destratification using mechanical mixers	R.M.A. Velzeboer J.A. Cugley J.C. Patterson
25	Methods for detection of *Giardia* and *Cryptosporidium* in water: A preliminary assessment	C.A. Bee P.E. Christy B.E. Robinson
26	Toxic cyanobacteria in water supplies: Analytical techniques	D.J. Flett B.C. Nicholson
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34	Stratification, mixing and water quality in Darwin water supply reservoirs	R. Lukatelich D. Robertson K. Boland J. Imberger J. Patterson
35	Performance auditing in the Australian urban water industry	S. O'Kane I. Parry D. Blunden D. Herring
36	Microbiological studies on enhanced removal of phosphates from sewage	R.C. Bayly J.W. May G. Vasiliadis G.N. Rees