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A Novel Biotechnological Approach to Phosphorus Removal from Wastewaters

STRIVE
Environmental Protection Agency Programme
2007-2013

SSTI
Strategy for Science, Technology and Innovation

Comhshaol, Oidhreacht agus Rialtas Áitiúil
Environment, Heritage and Local Government

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EPA STRIVE Programme 2007–2013

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(2005-ET-LS-10-M3)

STRIVE Report

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Prepared for the Environmental Protection Agency

by

The Queen's University of Belfast

Authors:

John W. McGrath and John P. Quinn

ENVIRONMENTAL PROTECTION AGENCY
An Ghníomhaireacht um Chaomhnú Comhshaoil
PO Box 3000, Johnstown Castle, Co.Wexford, Ireland

Telephone: +353 53 916 0600 Fax: +353 53 916 0699
Email: info@epa.ie Website: www.epa.ie

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The EPA STRIVE Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in relation to environmental protection. These reports are intended as contributions to the necessary debate on the protection of the environment.

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Details of Project Partners

John McGrath
School of Biological Sciences
Medical Biology Centre
Queen's University Belfast
97 Lisburn Road
Belfast
BT9 7BL
Tel.: +44 2890 972088
Email: j.mcgrath@qub.ac.uk

John Quinn
School of Biological Sciences
Medical Biology Centre
Queen's University Belfast
97 Lisburn Road
Belfast
BT9 7BL
Tel.: +44 2890 972287
Email: j.quinn@qub.ac.uk

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Executive Summary

The problem of eutrophication caused by the enrichment of water bodies through excessive nutrient inputs (particularly of phosphate) has become significant in Ireland and throughout the developed world. This project has attempted to develop an improved method to tackle the difficult problem of phosphorus (P) removal from treated municipal wastewaters, which constitute the major point source of phosphate discharge into inland lakes and rivers.

The project was designed to build on previous attempts by the project team to develop an improved alternative both to the use of chemical precipitants and to the enhanced biological phosphorus removal (EBPR) process – currently the major methods of achieving the levels of P reduction demanded by increasingly stringent European legislation. Each has significant drawbacks: the former is expensive and leads to increased levels of sludge production, while the latter can perform inconsistently in the low-strength, high-volume wastewaters characteristic of temperate latitudes.

Earlier work by the project team at laboratory and pilot-plant scale indicated that the maintenance of mildly acidic conditions (about pH 6.0) in a fully aerobic conventional activated sludge process can achieve levels of P removal comparable to those attainable under the aerobic/anaerobic cycling regime characteristic of the EBPR process (which has an optimum operationally pH of 7 to 8): this phenomenon has been termed the 'acid-stimulated biological phosphorus removal' (ASBPR). The major aims of the current study were to subject ASBPR to a full-scale trial under normal operational conditions (funded by the project's industrial partners Northern Ireland Water Ltd and the QUESTOR Centre), and to gain a better understanding of the biochemical mechanisms that underpin the phenomenon so that it might be exploited more fully.

Full-scale trialling of ASBPR took place over a 12-month period at an 185-person equivalent, two-lane activated sludge plant serving a rural hamlet near Lisburn, Northern Ireland. Work was hampered by a number of operational difficulties, including periodic sludge washout through stormwater overload, the fouling of acid-dosing equipment with unscreened materials such as rags which resulted in excessive pH shifts, and the effects of illegal toxic inputs from a local industry. Results obtained were in consequence inconsistent. Nevertheless, they indicated that under ideal conditions (fully aerobic and where the pH was maintained at approximately 6.0), levels of P removal were almost 30% greater than those in the untreated control lane (operated under standard aerobic-activated sludge conditions). It is recommended however that these results be verified in further on-site trials to assess the robustness of the ASBPR process. Additionally, these trials indicated that control of pH dosing is a key factor in the overall process and that a multipoint pH control system needs to be developed to allow maintenance of the activated sludge pH within the range 6–6.2.

Concurrent laboratory studies to better understand the nature of enhanced P accumulation by activated sludge microorganisms made very significant progress. A large number of process-relevant isolates were obtained and identified: the gene responsible for polymerisation of excess phosphate into the intracellular storage product, polyphosphate, was identified as polyphosphate kinase and its involvement demonstrated. In addition, new fluorimetric and enzymatic assay methods for the quantification of polyphosphate were developed. Finally, the transport mechanisms by which phosphate is accumulated under mildly acidic conditions were studied and the involvement of a novel, phosphate-insensitive, high-affinity transporter protein was shown.

Despite this progress, the failure to obtain consistent levels of acid-stimulated P uptake during routine-activated sludge sampling, even under laboratory conditions, led the project team to consider the possibility that other biochemical interactions (other than a response to mild acid stress) might be involved in the phenomenon. New reports from the scientific literature on the involvement of reduced sulphur species in the accumulation of polyphosphate by marine microorganisms were investigated, and it was possible to show that adding sulphite routinely produced modest increases in the levels of P uptake by activated sludge microorganisms, though not to economically exploitable levels.

Most encouragingly, however, the project team has very recently achieved a long-term goal – it has identified in the laboratory a physiological response triggered in the stationary-phase cells of activated sludge microorganisms, which leads to a consistent elevation in the rates of phosphate uptake and levels of intracellular polyphosphate accumulation. Its relationship to the acid-stimulated phenomenon remains unclear but its manipulation appears to offer real prospects for routinely achieving the levels of P removal from wastewaters that would meet legislative consents and for providing the feedstock for a possible P-recovery and recycling technology. This will be the focus of future studies. Success would offer the prospect of a simple P-removal technology, with a proven biochemical basis, that could effectively compete with EBPR in many situations.

1 Introduction

Phosphorus (P) is an essential macronutrient for all organisms; it plays some part in almost all life processes. Despite this well-accepted central metabolic role, however, in excess, inorganic phosphate represents a potentially serious environmental and ecological problem (reviewed by McGrath and Quinn, 2003, 2004). In particular, the enrichment of water bodies with phosphate makes an important contribution to the process of eutrophication, which has developed into a serious water-management problem throughout the world. Eutrophication is a process that occurs when lakes or rivers are overloaded with nutrients that stimulate the excessive growth of algae or higher plants. As this material or organic matter decomposes, levels of oxygen in the water are reduced, causing fish and other aquatic organisms to die. Eutrophication can also lead to many other problems such as increased purification costs for drinking water, the possible harmful effects of algal toxins, and a decrease in the recreational value of lakes and rivers. In freshwaters, the main nutrient responsible for eutrophication is phosphate. Phosphate enters the environment from a variety of sources, for example, agricultural runoff, sewage discharges and soil erosion. Population growth and the intensification of farming have led to the collapse of the natural P cycle and its replacement with a system whereby phosphate is extracted from rock, passed through crops, animals and humans, to end up either in landfills or in the aquatic environment. Some 140 million tonnes of rock phosphate are cycled annually in this way (Yeoman *et al.*, 1988).

To put eutrophication into perspective, P concentrations in natural non-eutrophic freshwaters are usually below 25 µg/l. Concentrations above 50 µg/l are generally the result of anthropogenic inputs. Freshwater systems are considered eutrophic if the P concentration exceeds 25 µg/l. Increased licensing and more severe legislation now put strict limits on the levels of P that can be discharged from such point sources as municipal and industrial wastewater treatment plants. This has been reflected in the imposition of Statutory Instruments 258 of 1998, 684 of 2007 and 272 of 2009 within Ireland, and the imposition of the Urban Wastewater Treatment Directive 91/271 and 98/15 across Europe (Council of the European Community, 1991 and 1998). These articles set strict standards for P concentrations in both surface waters and in emissions from wastewater treatment plants, particularly for those discharging into areas designated as 'eutrophication sensitive'. Furthermore, implementation of the EU Water Framework Directive may necessitate the addition of P removal processes at many more wastewater treatment works to reduce external nutrient loading in order to achieve good ecological quality.

1.1 Phosphorus Removal from Wastewaters

1.1.1 Current state of the art

To meet P discharge emission limit values, two main processes are routinely employed by the wastewater treatment industry, either separately or in combination: (i) chemical precipitation; and (ii) enhanced biological phophorus removal (EBPR).

1.1.2 Chemical precipitation

Chemical precipitation involves the dosing of ferric, ferrous, aluminium or calcium salts. The technique is simple, reliable and capable of meeting P discharge limits. It is however expensive in terms of the cost of precipitants, their transportation, the requirement for further tertiary filtration due to heavy metal contamination and the necessity to dispose of the larger volumes of sludge produced (up to 25% more biomass may be generated). These cost considerations and sludge disposal issues suggest chemical precipitation may not be the most viable long-term solution to P removal from wastewaters.

1.1.3 Enhanced biological phosphorus removal

The recognition and systematic study of the phenomenon of EBPR by microorganisms dates back to 1955 when Greenberg *et al.* (1955) proposed that under certain circumstances activated sludge had the ability to accumulate P in excess of that required for balanced microbial growth. This hypothesis was subsequently demonstrated by Srinath *et al.* (1959) who, while studying the feasibility of using activated sludge liquor as a fertiliser for growing rice plants,

found that the plants suffered from the characteristic symptoms of phosphate deficiency exemplified by excessive vegetative growth and diminished grain formation. This lack of soluble P was traced to its excessive accumulation by the sludge biomass. Levin and Shapiro (1965) reported similarly enhanced P removal by activated sludge in the absence of added chemical precipitants; the addition of 2,4-dinitrophenol, a biocide, to the sludge biomass inhibited phosphate uptake, however, thus confirming a biological process. In later years this phenomenon was frequently referred to as 'luxury' phosphate uptake; its exploitation appeared to provide the basis of a possible biological alternative to chemical precipitation for P removal from waste streams and ultimately led to the development of the EBPR process.

The EBPR process is based upon the exposure of activated sludge to alternating periods in the absence and presence of oxygen – the anaerobic and aerobic phases. This is achieved by configuring the treatment system so that an anaerobic zone is added upstream of the traditional aerobic phase; influent wastewater is introduced into this anaerobic zone (reviewed in Yeoman *et al.*, 1988; Kortstee *et al.*, 1994; van Loosdrecht *et al.*, 1997; Brett *et al.*, 1997; Mino *et al.*, 1998; Seviour and McIlroy, 2008; Forbes *et al.*, 2010; Hirota *et al.*, 2010).

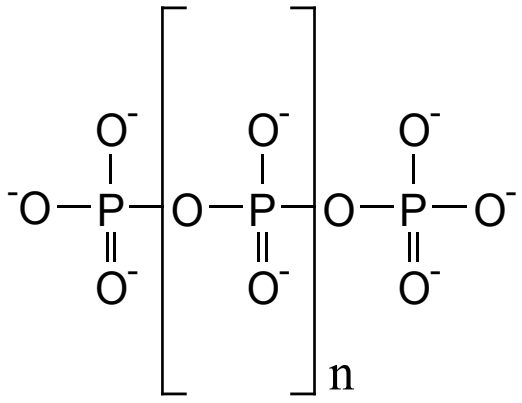

Figure 1.1. Structure of inorganic polyphosphate.

In terms of the microbial biochemistry that underpins it, the EBPR process can be described as a tale of two biopolymers: (i) poly-β-hydroxybutyrate (PHB) and (ii) polyphosphate (polyP) (Fig. 1.1). In the initial anaerobic phase it appears that a specialised group (or groups) of microorganisms is able to gain a selective advantage through its ability to take up the short-chain fatty-acid molecules, such as acetate, which are present in the liquor as fermentation by-products, and to convert these to a carbon-storage polymer. This is normally PHB, although poly-β-hydroxyvalerate (PHV) has also been observed. The energy required for the uptake and synthesis of PHB comes at the expense of another biopolymer, polyP, whose synthesis is a second defining characteristic of the microbial groups responsible for EBPR. Hydrolysis of the intracellular polyP reserves stored by EBPR microorganisms provides the energy required for their accumulation of PHB under anaerobic conditions. As a result, phosphate release to the extracellular medium is characteristic of this phase of the EBPR process.

In the subsequent, aerobic, stage of EBPR, those microorganisms that contain stored PHB/PHV are able to replenish their internal polyP reserves; in doing so they remove not only the phosphate released during the anaerobic phase of the process but also almost all the available phosphate from the surrounding oxygen-rich environment. As much as 30% of the PHB/PHV previously formed during the anaerobic phase is thus consumed by EBPR microorganisms under aerobic conditions as they take up phosphate and convert it to polyP. The exact physiological or environmental factors that trigger this 'sacrifice' of stored PHB/PHV by the members of the EBPR microflora in order to replenish their polyP reserves is at present unknown. However, it seems clear that the catabolic and anabolic interplay between polyP and PHB is central to microbial phosphate removal during the EBPR process (Oehmen *et al.*, 2007; Seviour and McIlroy, 2008).

When operated under favourable conditions EBPR plants are often able to remove 80–90% of influent P (Water Environment Federation, 1998), compared to the 20–40% removal typical of conventional activated sludge treatment (Streichan *et al.*, 1990). The ultimate elimination of P from the system is then achieved by the wastage of P-rich excess sludge. (It must be noted, however, that the absence of nitrate in the anaerobic zone is essential for polyP utilisation during PHB formation [and thus the effectiveness of the EBPR process]; nitric oxide exhibits an inhibitory effect on the enzyme adenylate kinase. Adenylate kinase plays a key role in energy generation from accumulated polyP [van Niel *et al.*, 1998]). Anoxic denitrification stages are

therefore often built into EBPR systems, thus ensuring nitrate removal prior to anaerobiosis.

The selective enrichment of microorganisms capable of polyP accumulation through the cycling of activated sludge between anaerobic and aerobic zones is therefore the basis of conventional EBPR technology; P removal occurs only during the aerobic phase. Recent investigations have however demonstrated that phosphate uptake and polyP formation may not necessarily require an aerobic zone and that under anoxic conditions nitrate can provide an alternative electron acceptor (Kuba *et al.*, 1993; Jorgensen and Pauli, 1995; Barker and Dold, 1996; Barak and van Rijn, 2000; Egli and Zehnder, 2002; Barnard and Abraham, 2006). Nitrification-denitrification biological enhanced phosphorus removal (NDEBPR) may in fact have a number of advantages over traditional EBPR – including considerable energy savings, reduced biomass production and a maximisation of the amount of chemical oxygen demand (COD) available for both nitrogen and P removal (Kuba *et al.*, 1993). Various laboratory-scale studies have demonstrated that NDEBPR sequence batch reactors can achieve results comparable to those of the conventional EBPR process, albeit at lower biomass levels and with different P release and uptake rates (Barnard and Abraham, 2006). Microbial community analysis has revealed that those microorganisms capable of conventional EBPR, using oxygen as the terminal electron acceptor, may also be those responsible for the use of nitrate in an NDEBPR system (Dabert *et al.*, 2001).

An essential prerequisite for efficient EBPR is the presence of a substantial concentration of readily available organic carbon in the anaerobic phase. This allows the microorganisms responsible to accumulate reserves of PHB/PHV, at the expense of their stored polyP, and with the concomitant release of phosphate. Much of the work reported on EBPR has been conducted in North America and South Africa where stronger sewages (i.e. having biological oxygen demand [BOD]:P ratios greater than 20) facilitate the anaerobic formation of PHB/PHV (Upton *et al.*, 1996). In countries like Ireland and the UK such BOD:P ratios are found infrequently: therefore, several commercial processes have been developed to overcome these differing sewage characteristics. Such modified biological P removal systems may take the form of either a sidestream process (treating only a percentage of the flow, e.g. The Phostrip Process) or a mainstream system (treating the entire flow, e.g. The University of Cape Town Process). Many other process configurations exist for EBPR, including the Modified Bardenpho Process, The Three Stage Phoredox Process, The Anaerobic/ Oxic (A/O) Process, The Rotanox Process and the Modified University of Capetown Process (reviewed in Yeoman *et al.*, 1988; Brett *et al.*, 1997). Irrespective of their individual features, however, the success of each is necessarily dependent on the ability of the microflora of the system to take up and store phosphate intracellularly in the form of polyP.

1.2 Background to this Proposal and its Relationship to Previous Work

The cornerstone of EBPR, however, is the microbial synthesis, intracellular storage and ultimate hydrolysis of polyP. Intracellular granules that stain metachromatically with basic dyes have been described in the microbiological literature since the early 1900s. It was not however until 1947 that Wiame (1947; 1948) demonstrated that these 'volutin' granules corresponded to deposits of polyP, a molecule that is today recognised as one of the most widely distributed natural biopolymers, having been detected in many bacteria, fungi, yeasts, plants and animals (Dawes and Senior, 1973; Kulaev and Vagabov, 1983; Kulaev *et al.*, 1999). PolyP consists of a linear chain of phosphate residues linked together by high-energy phosphoanhydride bonds, and ranges in length from 3 to greater than 1000 orthophosphate residues (Kulaev, 1979) (Fig. 1.1). Intracellular polyP inclusions appear as dark granules of very variable size and number when examined by electron microscopy (Fig. 1.2). Under optimal conditions, polyP may amount to 10–20% of the cellular dry weight and as such greatly exceeds the P requirements of the cell, which suggests that it may fulfil a metabolic role(s) other than simply that of a phosphate reserve (Pick *et al.*, 1990). Reviews by Harold (1966), Dawes and Senior (1973), Kortstee *et al.* (1994), Kulaev *et al.* (1999), Kornberg and Fraley (2000) and Rao *et al.* (2009) detail many of the physical and chemical properties of polyP as well as its predicted role in prebiotic evolution.

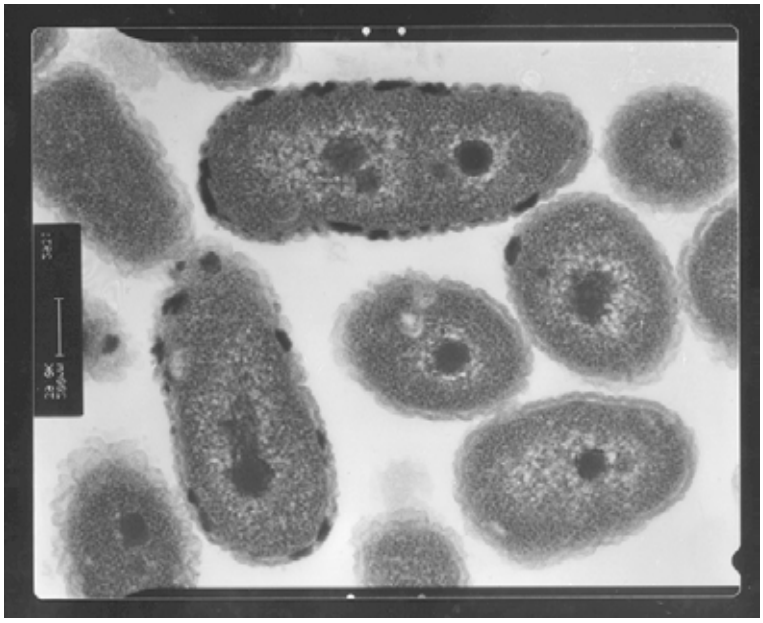

Figure 1.2. Cells of *Burkholderia cepacia* AM19 showing intracellular polyphosphate granules as black inclusion bodies.

Microbial biosynthesis of polyP is primarily catalysed by the enzyme polyphosphate kinase 1 (polyphosphate: adenosine-5'-diphosphate [ADP] phosphotransferase; PPK1; EC 2.7.4.1). PPK has been characterised extensively in a number of prokaryotes, including *Vibrio cholerae*, *Pseudomonas aeruginosa* 8830, *Propionibacterium shermanii*, *Acinetobacter* sp. Strain ADP1, *Neisseria meningitidis*, *Arthrobacter atrocyaneus*, *Corynebacterium xerosis*, *Salmonella minnesota*, *Burkholderia cepacia* AM19, *Cryptococcus humicolus*, *Sulfolobus acidocaldarius* and, most extensively, in *Escherichia coli* (Kulaev *et al.*, 1999; McGrath and Quinn, 2000; Kornberg and Fraley, 2000; Mullan *et al.*, 2002; Rao *et al.*, 2009). The enzyme catalyses the progressive synthesis of the polyP chain through the reversible transfer of the gamma phosphate from adenosine-5'-triphosphate (ATP) to polyP. In addition to PPK1, most bacterial species have PPK2. PPK2 activity differs from PPK1 in that it uses both ATP and guanosine-5'-triphosphate (GTP) for polyP synthesis: PPK1 has a strict requirement for ATP. Additionally, the equilibrium of the PPK2 reaction favours synthesis of GTP from guanosine-5'-diphosphate (GDP) and polyP. PPK2 is therefore considered to be a polyP-driven nucleoside diphosphate kinase used by cells to synthesise GTP, especially upon entry into the stationary phase. PPK1 can also act as a polyP-driven nucleoside diphosphate kinase synthesising ATP although the equilibrium of this reaction lies more towards polyP synthesis (Rao *et al.*, 2009).

Over the last number of years the project team's work has focused on microbial polyP metabolism, and in particular its role in the physiological adaptation of microbial cells during growth and development, and in their response to nutritional and environmental stimuli. Of particular significance has been the discovery of ASBPR, whereby certain microorganisms show greatly enhanced levels of intracellular polyP accumulation, under fully aerobic conditions, if the growth pH is adjusted to the range 5.0–6.5 (McGrath *et al.*, 2001).

Under these conditions, an increase of up to 4-fold in P uptake and a concomitant increase of up to 10.5-fold in sludge polyP content have been demonstrated (McGrath *et al.*, 2001). Together with the project team's industrial collaborators, the potential of this process as a novel treatment technology at pilot plant scale (removing 64% of influent total P) has also been shown (Figs 1.3 and 1.4) (Mullan *et al.*, 2006). Such a single-stage P-removal system might have considerable advantages over EBPR technology in terms of: (i)

Figure 1.3. Pilot plant facility.

ease of retrofitting; (ii) rate of throughput; (iii) tolerance of high nitrate levels (the anaerobic phase of EBPR is sensitive to nitrate); (iv) reduced dependence upon wastewater strength; and (v) operation at low volatile fatty acid (VFA) concentrations – ASBPR is not VFA dependent.

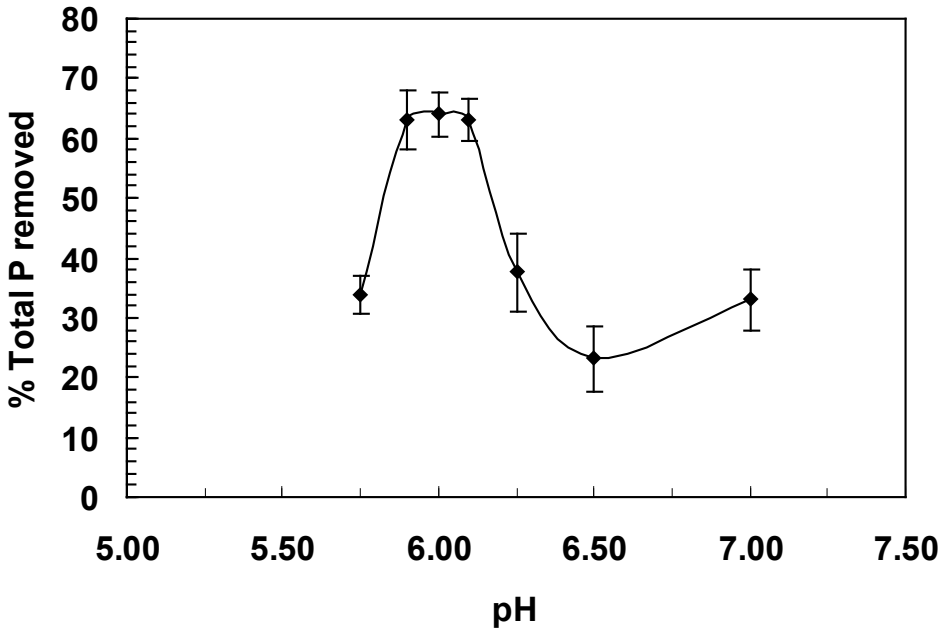

Figure 1.4. Phosphorus removal in pilot plant.

2 Objectives

Acid-stimulated P removal and polyP accumulation is a previously unrecognised aspect of microbial P cycling that may have considerable environmental and economic benefits as an alternative biological P-removal technology. The project team's previous studies have necessarily focused on successfully establishing the potential of this phenomenon in laboratory and pilot plant trials (Mullan et al., 2006). Encouraged by these results a full-scale trial of the system was initiated in association with project partners Northern Ireland (NI) Water Ltd and the QUESTOR Centre, with support from both the Environmental Protection Agency of Ireland (EPA) and the UK Engineering and Physical Sciences Research Council (EPSRC). Four main questions were asked in this research project:

1 What microorganisms are responsible for ASBPR?

2 How is phosphate transported into the microbial cells at acidic pH?

3 How is this transported phosphate polymerised into polyP?

4 Are there any other 'triggers' for microbial polyP accumulation which could be exploited as either novel treatment technologies or that would augment the acid-stimulated process?

2.1 Summary of Conclusions and Recommendations

Full-scale trialling of ASBPR took place over a 12-month period at an 185-person equivalent, two-lane activated sludge plant serving a rural hamlet near Lisburn, Northern Ireland. Although the results obtained were inconsistent, they nevertheless indicated that P removal could be increased by up to 30%, compared with the unamended control lane, under fully aerobic conditions when the pH was maintained at approximately 6.0. Concurrent laboratory studies obtained and identified a large number of process-relevant isolates in which the gene responsible for intracellular polyphosphate formation was identified as polyphosphate kinase and its involvement demonstrated in ASBPR. New fluorimetric and enzymatic assay methods for the quantification of polyphosphate were also developed. In addition, the transport mechanisms by which phosphate is accumulated under mildly acidic conditions were studied and the involvement of a novel, phosphate-insensitive, high-affinity transporter protein was demonstrated. Two additional triggers, involving either supplementation with sulphite or stationary phase stress, were shown to induce enhanced P uptake and polyphosphate accumulation in microbial cells. It is recommended that these should form the basis of future studies along with further trialling of the ASBPR system with more robust pH control hardware.

3 Full-scale Trial of a Novel Phosphorus Removal

3.1 Process

At the onset of the project the industrial partners – NI Water Ltd and the QUESTOR Centre – invited the project team to initiate a full-scale trial of the ASBPR system. The team's collaborators agreed to provide site rental, routine supervision, plant technical back-up, plant parts, power and other ancillary facilities. The collaborators had identified an 185-person equivalent (30,000 L) aerated activated sludge plant located at Mullaghglass outside Belfast (Fig. 3.1). The plant features two lanes receiving the same primary domestic influent, which is split using a flow splitter between the two channels, each with a 15,000 l capacity. Influent enters the inlet channel where it passes through a macerator before entering the interstage pumping wet well: this consists of a baffled chamber with v-notches to allow balancing between lanes. The aeration and clarification stages of the plant are integrated, with the influent entering the aeration reactor, mixing with activated sludge and passing over a weir into the settlement compartment. Sludge is returned to the aeration basin through an opening at the base of the aeration and settlement compartments. Desludging (mixed liquor suspended solids [MLSS] removal) is therefore completed manually via a tanker. Control of pH was provided in one lane only using 50% sulfuric acid for pH adjustment. The pH controller was fitted with a datalogger which measured the pH every 5 minutes. Prior to the onset of the trial, the plant was cleaned out and subsequently filled with activated sludge biomass from Hilden Sewage Treatment Works (Lisburn, Co. Antrim), which was the site of the project team's previous pilot scale trials. This was carried out to ensure a similar microbial population existed in the full-scale trial as had previously been used for the pilot plant studies. The trial was conducted over a 12-month period during which time the pH was lowered in increments of 0.2 pH units from pH 7.4 to pH 6.0 (Fig. 3.3) to assess the influence of operational pH on plant performance. Effluent parameters (soluble reactive P, total P, ammonia, COD, MLSS, nitrate, nitrite and effluent suspended solids) were routinely measured both by the project team and NI Water Ltd.

Figure 3.1. Full-scale trial site.

Figure 3.2. Rag build-up on probes.

During the 12 months of operation the full-scale trial suffered a number of engineering difficulties, including: (i) continual biomass washout due to heavy rain; (ii) clogging of pH probes with rags leading to acid overdosing (Figs 3.2 and 3.3) and (iii) illegal toxic inputs from a local industry.

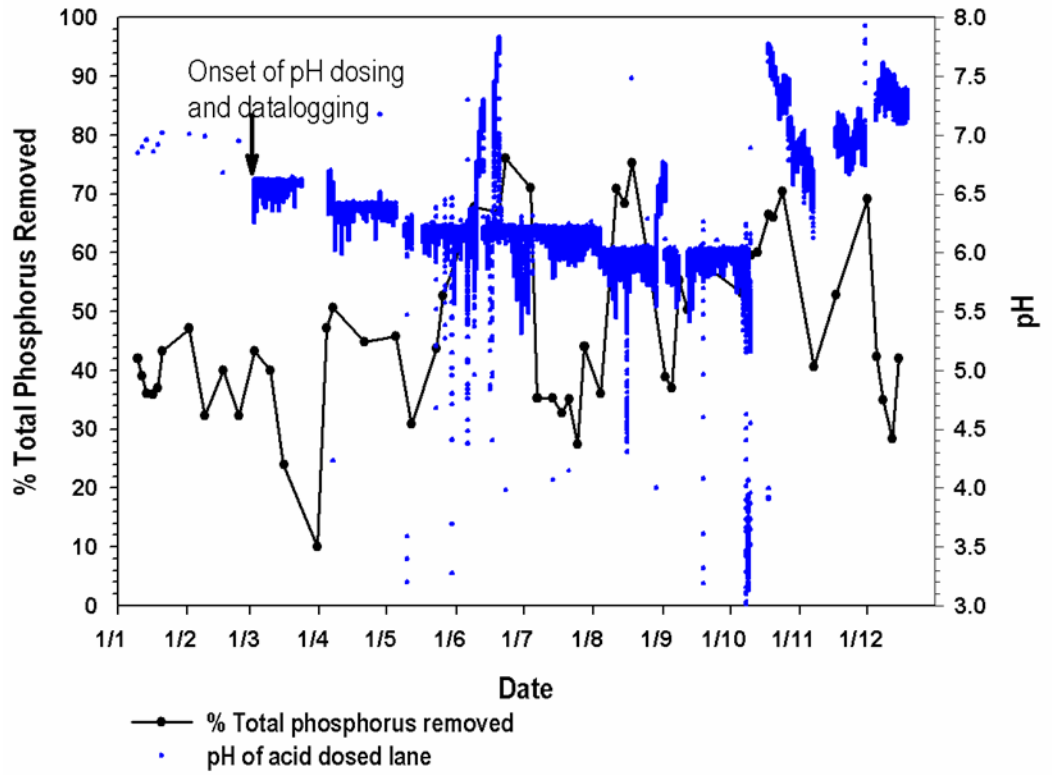

Figure 3.3. Effect of pH on % total phosphorus removed during full-scale trial. *x*-axis data represent the months of the year.

Nevertheless, the results obtained, although inconclusive, did to some extent mirror those obtained in the previous pilot plant study, despite the frequent engineering breakdowns, washouts and pH overdosing issues. The P removal performance of the Mullaghglass acid-dosed lane was monitored for 3 months prior to the onset of acid dosing. Total P removal from the works varied between 32 and 48% within the pH range 6.7–7.1 (Fig. 3.3). Upon pH reduction, this increased to a maximum of 80% at pH 6.2 (Fig. 3.3; at midway point between June and July) before a pH dosing failure and subsequent overdose severely affected plant performance (Fig. 3.3; just prior to the start of July). However, upon reestablishment of the pH 6.0 dosing regime the percentage of P removal once again increased to a maximum of 73% (midway between August and September) before a second dosing error occurred at the beginning of September (Fig. 3.3). Importantly, acid dosing could be achieved without any compromise in COD removal or sludge and final effluent quality: final effluent pH, after settlement, was routinely 0.2 units higher than in the activated sludge tank, i.e. when dosed at pH 6, final effluent pH was 6.2. Particularly significant however was the failure of the pH dosing to inhibit nitrification (Fig. 3.4).

A number of studies have demonstrated that nitrification is particularly sensitive to changes in pH, with the rate of nitrification decreasing as pH falls (Eckenfelder and Grau, 1992). It would appear however that significant nitrification can continue at somewhat lower pH values (this study; Tarre *et al.*, 2004; Gieseke *et al.*, 2006), possibly since the process is now thought to be carried out by a wider range of sludge microorganisms (Seviour and McIlroy, 2008). No change in floc formation, sludge volume index, sludge settleability or MLSS levels were observed upon pH reduction to 6.0 in the full-scale system. In the pilot plant trial, MLSS concentrations did however decrease by up to 20%: this had no effect on final effluent quality with respect to COD or effluent suspended solid levels. In both trials a significant deterioration in floc structure occurred at pH values below 6.0 with decreased settleability and an unacceptable increase in the suspended solids present in the effluent.

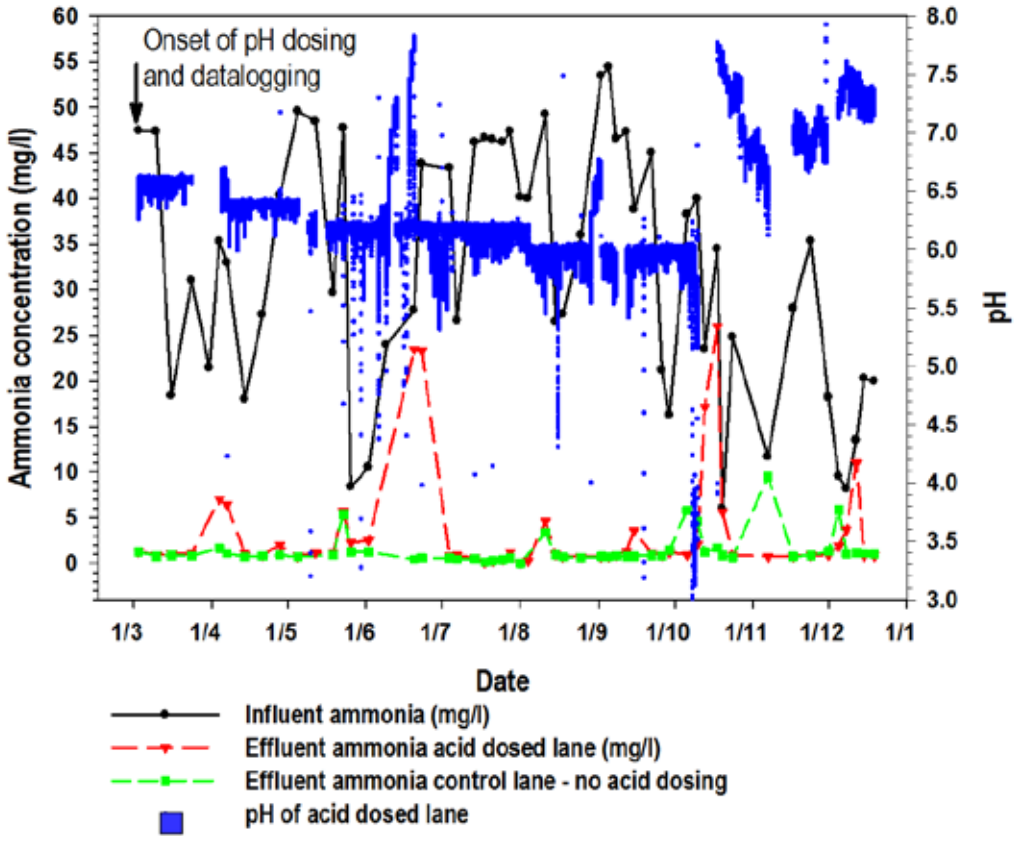

Figure 3.4. Effect of pH on nitrification activity during pilot plant trial. *x*-axis data represent the months of the year.

This also correlated with a failure in nitrification and decreased P removal performance. Severe overdosing led to the complete failure of the treatment process requiring re-seeding of the plant: this was accompanied by a major change in the activated sludge microbial community as determined by denaturing gradient gel electrophoresis (DGGE) (see Section 3.1.2 below).

The perceived failure of the full-scale trial to demonstrate ASBPR unequivocally must be tempered with an appreciation of the engineering issues. However, these cannot be ignored and do suggest that a potential limitation of applying ASBPR is in controlling the pH dosing system used in this trial (Fig. 3.3): pH control is however successfully practised in many other industries. Nevertheless, frequent over-/under-dosing was a key problem encountered in this full-scale trial, mainly because of the failure of the dosing equipment employed under field conditions. This has practical implications in terms of process implementation and would require upgrading the pH management hardware to those units employed for example in the fermentation industry, where such pH control is routine.

There also seems to be an issue with regard to the system's stability. Despite a period of good weather and correct dosing, no significant increase in P removal was observed during July and August (Fig. 3.3). During this summer period, influent COD levels were higher. A similar pattern of varying P removal performance was observed with axenic cultures tested during this study (results not shown) in that sometimes ASBPR worked satisfactorily while at others no increase in P removal was observed.

3.2 Identification and Isolation of Polyphosphate Accumulating Organisms from Activated Sludge

A prerequisite to a better understanding of the ASBPR process is a description of the microbiology pertinent to the system and the subsequent isolation of process-relevant microorganisms. A total of 90 wastewater microorganisms from New Holland Sewage Treatment Works activated sludge, whch was used to commission the full-scale plant, were isolated on a synthetic sewage medium at pH 7.5. These were chosen on the basis of apparent differences in colony morphology and colouration to ensure a diversity of organisms. For each isolate, phosphate removal from the culture medium (assayed in duplicate) was determined at pH 7.5 and pH 6.0. Phosphate as KH_2PO_4 was added to a concentration of 1.0 mM: 47 isolates grew well at both pH 7.5 and pH 6.0, enabling assays of both growth and phosphate removal; 43 isolates either failed to grow at all or had extremely poor growth at pH 6.0 and were not included in this study. Of the 47 isolates studied, 21 (45%) showed an increase in P removal at pH 6.0 of between 15 and 173%. However, 13 isolates showed improved P removal at pH 7.5 of between 15 and 76%. The remainder either showed no difference in P removal or a difference of less than 10% between the test pHs (Fig. 3.5). Of the 21 isolates displaying ASBPR, all had visible intracellular polyP inclusions (e.g. *B. cepacia* AM19; Fig. 1.2).

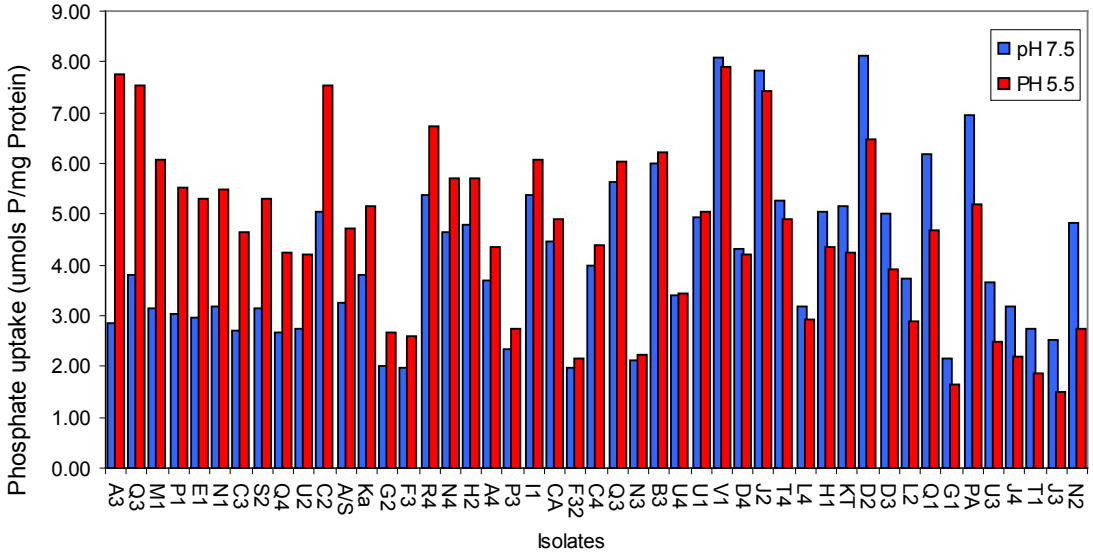

Figure 3.5. Phosphorus removal of activated sludge isolates at pH 7.5 and pH 6.0.

Enhanced P uptake by these strains was accompanied by increases of between 2- and 10.5-fold in their polyP content. Further microscopic examination of activated sludge samples exposed to the acid pH indicated that some 34% of *total* microbial cells contained visible polyP inclusions under acidic conditions as opposed to 7% at neutral pH values. Of those microorganisms displaying the acid stimulated phenomenon, positive identities were obtained for 17 using community-level physiological profiling. This was carried out using the Biolog system (Hayward, CA, USA) according to the method of Victorio *et al.* (1996). Although the Biolog system was unable to give positive identifications for many of the isolates, the profiles of carbon source utilisation on the microtitre plates could be compared visually and it was established that there were around 40 unique isolates. Those isolates displaying ASBPR belonged to the genera *Acinetobacter*, *Burkholderia*, *Pseudomonas*, *Cytophaga*, *Agrobacterium*, *Sphingobacterium*, *Aeromonas*, *Klebsiella* and *Edwardsiella.* Each displayed enhanced P uptake and polyphosphate accumulation in response to acid stress (results not shown).

3.2.1 *Screening of sequenced bacterial isolates for ASBPR*

A number of environmental isolates whose genomes have been fully sequenced and annotated (available to the project team through commercial culture collections) were screened for the ASBPR phenomenon. Those isolates chosen for study were *Corynebacterium glutamicum*, *Erwinia carotovora*, *Deinococcus radiodurans*, *Pseudomonas putida* KT2440, *Pseudomonas aeruginosa* PAO1 and *Acinetobacter calcoaceticus*. Of these, only *Acinetobacter calcoaceticus* displayed enhanced P uptake and polyphosphate accumulation under acid conditions, albeit inconsistently (with an optimum pH of 5.5). Increases in P removal varied from 0–30% at acid pH compared with pH 7.5: in each case, glucose was supplied as the sole carbon source at a concentration of 4 mM. More intracellular polyphosphate inclusions were however always observed under 4',6-diamidino-2-phenylindole (DAPI) staining (which stains polyP inclusions yellow under UV illumination) when at pH 5.5 compared with pH 7.5 (data not shown). Intriguingly, *C. glutamicum* removed the largest amount of P from the mineral-salts medium – 21.5 mg/L P at pH 7.5. No growth was observed at acid pH. Large polyphosphate inclusions were observed during and after growth at pH 7.5. The reason why *C. glutamicum* should accumulate such substantial quantities of polyphosphate at pH 7.5 is at present unknown and warrants further investigation.

3.2.2 *Evaluation of activated sludge community changes with pH*

Denaturing gradient gel electrophoresis (DGGE) is a molecular ecological tool which allows shifts in microbial community structure to be monitored. This method uses the 16S rDNA molecule present in all bacteria as a molecular marker for species identification. Denaturing gradient gel electrophoresis analysis of the bacterial community structure was first carried out on curated grab samples obtained from the pilot plant. These samples were obtained from the project team's pilot plant facility run under a conventional activated sludge regime for a period of 62 days at either pH 6.0 or 7.0 (Fig. 3.6). For comparison, samples were also taken from the main Hilden Sewage Treatment Works aeration basins, from which the pilot plant and the full-scale trial were seeded; samples were also taken for the acid-dosed lane in the full-scale trial during a period of increased P removal performance. In the pilot plant, upon adjustment of the pH to 6.0, the P content of the microbial biomass increased (Fig. 3.6). This was accompanied by a concomitant increase in P removal: the P removal results from this extended run have been published (Mullan *et al.*, 2006). After 62 days the pH of the plant was changed to pH 5.75 to investigate the limits of ASBPR. At this lower pH, the P removal capacity of the microbial biomass decreased rapidly. This was accompanied by a loss in nitrification and a breakdown in sludge floc structure (data not shown), resulting in poor sludge settling.

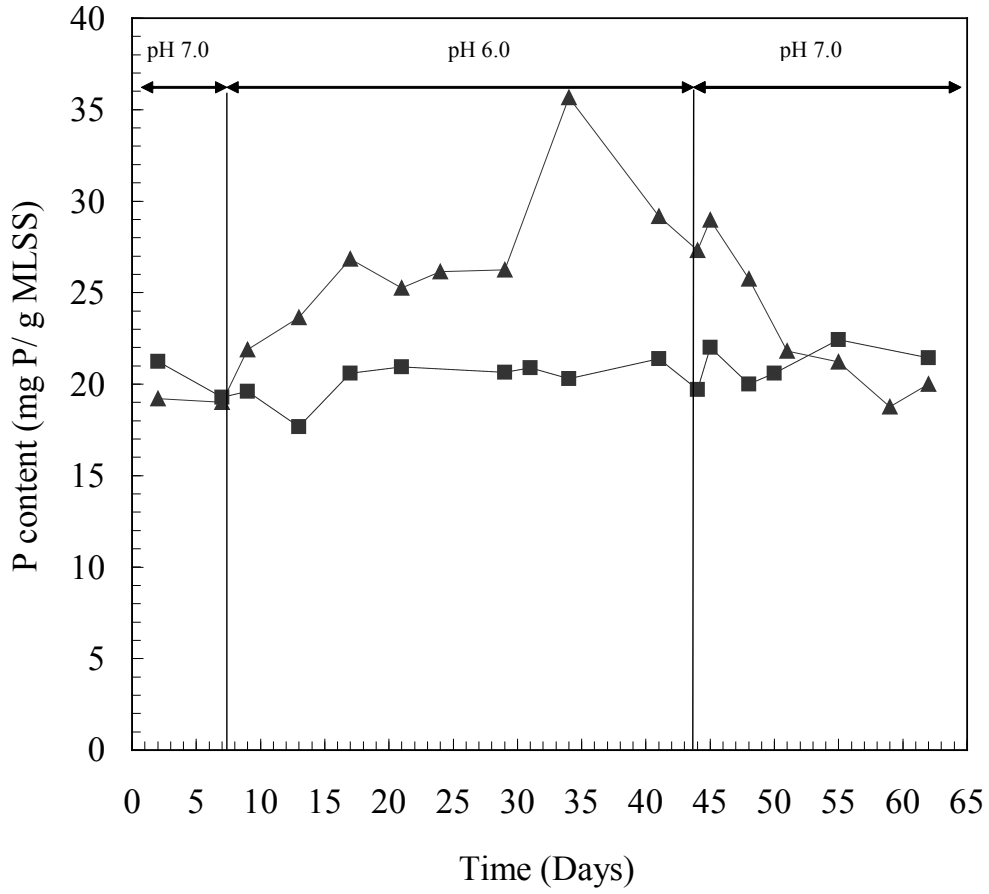

Figure 3.6. Phosphorus content of activated sludge during the extended pH 6.0 trial in the pilot plant (■) and during the same period in the main works (▲).

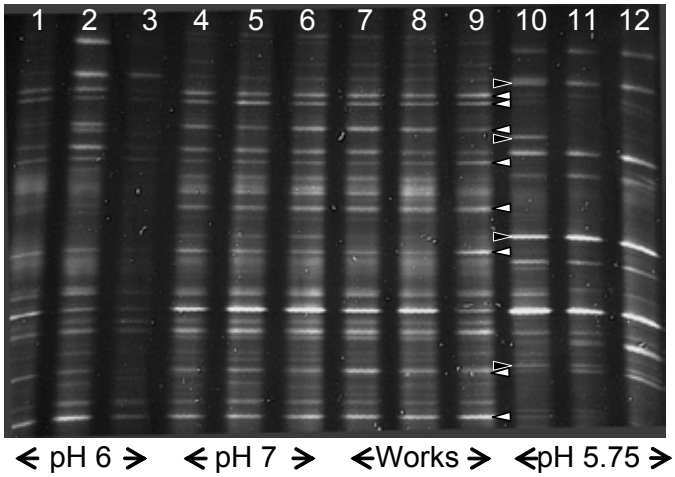

Figure 3.7. Denaturing gradient gel electrophoresis-banding profiles of bacterial 16S rRNA genes PCR-amplified from pilot-plant during pH 6.0 phase – (1) day 15, (2) day 23, (3) day 30 – pH 7.0 phase, (4) day 2, (5) day 3, (6) day 4 – main works, (7) day 2, (8) day 4, (9) day 16 – pH 5.75 phase, (10) day 2, (11) day 7, (12) day 10. Arrows indicate changes in banding profile of pH 5.75 samples relative to the main works. Right-facing black arrows indicate appearance/increase of band, left-facing white arrows indicate band decrease/ disappearance.

Figure 3.7 shows typical DGGE banding patterns. A similar banding pattern was observed in all cases and showed no obvious relationship to pH change, except for those samples taken during the pH 5.75 run. Significant changes in the microbial community structure of the pilot-plant occur during this 5.75 phase. A similar pattern emerged upon DGGE analysis of the test and control full-scale lanes, with pH overdosing causing significant changes in the plant microbial ecology (data not shown). This therefore is the one possible reason for treatment failure. Owing to acid pulses in the full-scale trial, pH values of <3.5 were recorded (Fig. 3.3). Given the molecular ecology results and the changes observed in community structure at pH 5.75, this demonstrates clearly the sensitivity of the microbial biomass to pH values less than 6.0 and confirms the need for more controlled acid dosing in any subsequent ASBPR trial.

3.3 Further Studies

NI Water Ltd are in the process of identifying a further, more appropriate, site for a second trial which would not suffer from the same washout problems encountered at Mullaghglass. Additionally, this aspect of the study has highlighted that pH control is critical for process performance, particularly to prevent overdosing which leads to a serious decline in effluent quality through failure of the treatment process. Future trials will therefore focus on (i) assessing the reliability and robustness of ASBPR and (ii) addressing the possible practicable measures to prevent overdosing. Laboratory studies are continuing to identify if any other parameter (e.g. sludge age, hydraulic retention time, nutrient load and/or dissolved oxygen levels) are involved in the enhanced P removal observed at acid pH.

4 Understanding how Phosphorus is Removed during Wastewater Treatment

Understanding the biochemistry of P uptake and the polymerisation of this accumulated P into polyP is central to furthering an exploitation of ASBPR at the process level. Optimum P removal from wastewater will be achieved only if the rates of P acquisition and polyP formation are functioning at their maximum velocities; a decrease in either will affect P uptake and storage negatively. Using two 'process-relevant' isolates (*Acinetobacter baumannii* 5B2 and *B. cepacia* AM19), obtained from the project team's activated sludge studies and displaying ASBPR (Mullan *et al.*, 2002), the team began dissecting the underlying biochemistry and genetics of the acid-stimulated phenomenon.

4.1 Assay Development

Central to the study of both microbial P transport and polyP turnover is the availability of robust assays to quantify P uptake and its accumulation as polyP. Traditionally, such studies have used ^{32}P and assessed the uptake and assimilation of P over time by measuring the increase in radioactivity levels in the biomass. However, the use of radioactivity is increasingly becoming prohibitive because of containment and disposal issues. Additionally, the methods available for polyP quantification are either: (i) inexact, varying in product recovery yields obtained; (ii) cumbersome, requiring extensive extraction and enrichment steps; or

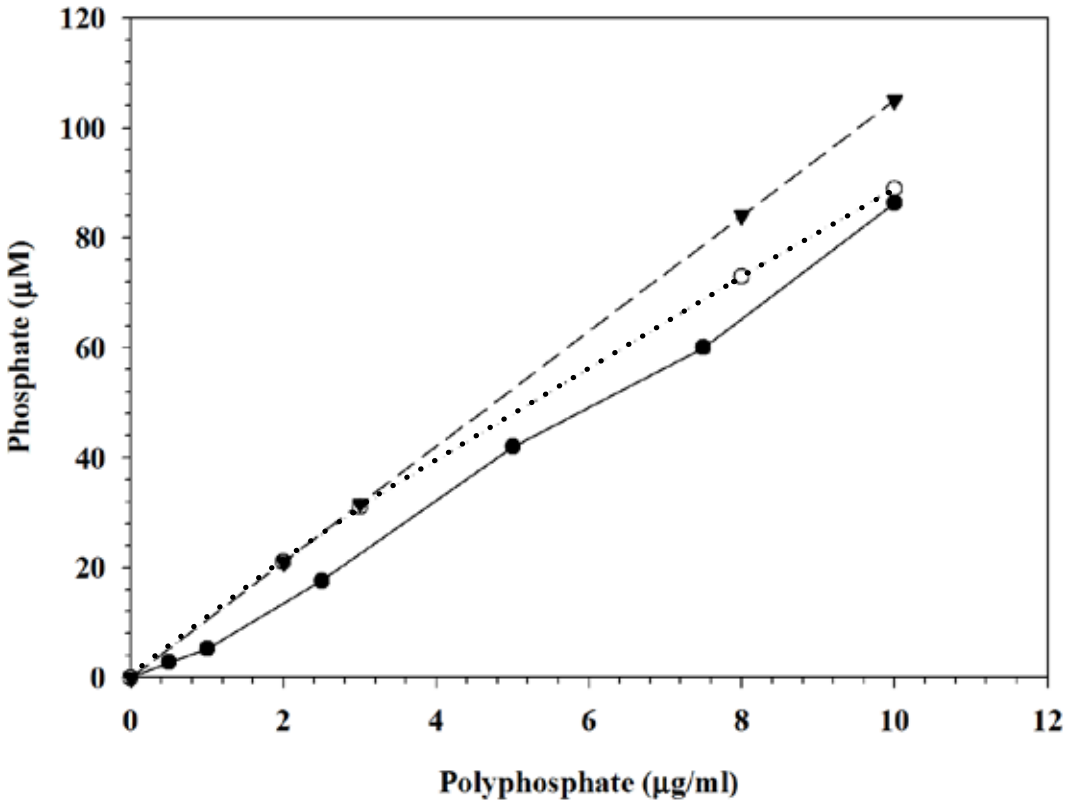

Figure 4.1. Comparison of polyphosphate (polyP) quantification using the ScPpx assay (○) and total acid hydrolysis (●) with the theoretical phosphate content (▼) of the Type 65 polyP standard.

(iii) indirect, therefore subject to interference by other cellular enzymes if cell extracts are used.

Two new methods of polyP quantification have recently been published. The first is based on the fluorometric reaction of DAPI with polyP, whereby excitation of the DAPI–polyP complex at 415 nm and detection at 550 nm allows for its quantitative determination (Aschar-Sobbi *et al.*, 2008; Günther *et al.*, 2009). The second approach, developed to analyse polyP in yeast cells, enzymatically assays extracted polyP using a purified yeast exopolyphosphatase (ScPpx) (Werner *et al.*, 2005). Extraction and purification of the cellular polyP is achieved using silica-gel columns. These novel methods therefore hold considerable promise for the detection and quantification of polyP in microbial cells: the ScPpx based assay has however been used only for polyP detection in yeast, algal and plant cells whilst the DAPI approach has not been evaluated critically with respect to polyP detection in cell extracts, although it has been used for polyP quantification in both *Escherichia coli* (Schurig-Briccio *et al.*, 2009) and *Streptomyces lividans* (Negoda *et al.*, 2009). By modifying and combining these methods an optimised protocol for polyP extraction, purification and quantification from bacterial cells was developed. A publication on this is in preparation.

To ascertain the effectiveness of the DAPI- and ScPpx-based protocols, with a view to their application in quantifying polyP, these methods were compared using known concentrations of polyP from standard Type 65 phosphate glass. The values obtained were compared with those produced after complete hydrolysis of the standard polyP samples in 2N HCl (used as a positive control) and the theoretical concentration of phosphate in the Type 65 polyP standard (Figs 4.1 and 4.2). Both assay systems showed a good correlation with the acid hydrolysis and the calculated theoretical phosphate content of the Type 65 standard.

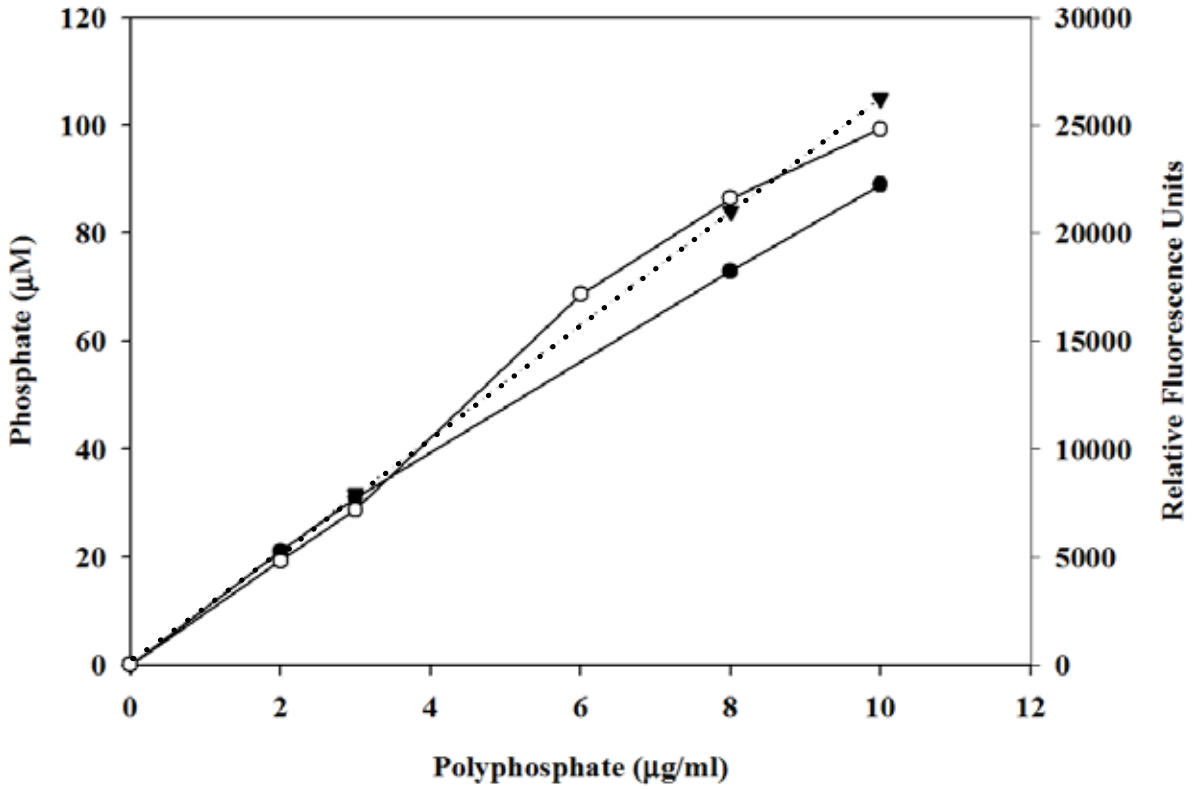

Figure 4.2. Comparison of polyphosphate (polyP) quantification using the DAPI assay (○) and total acid hydrolysis (●) with the theoretical phosphate content (▼) of the Type 65 polyP standard.

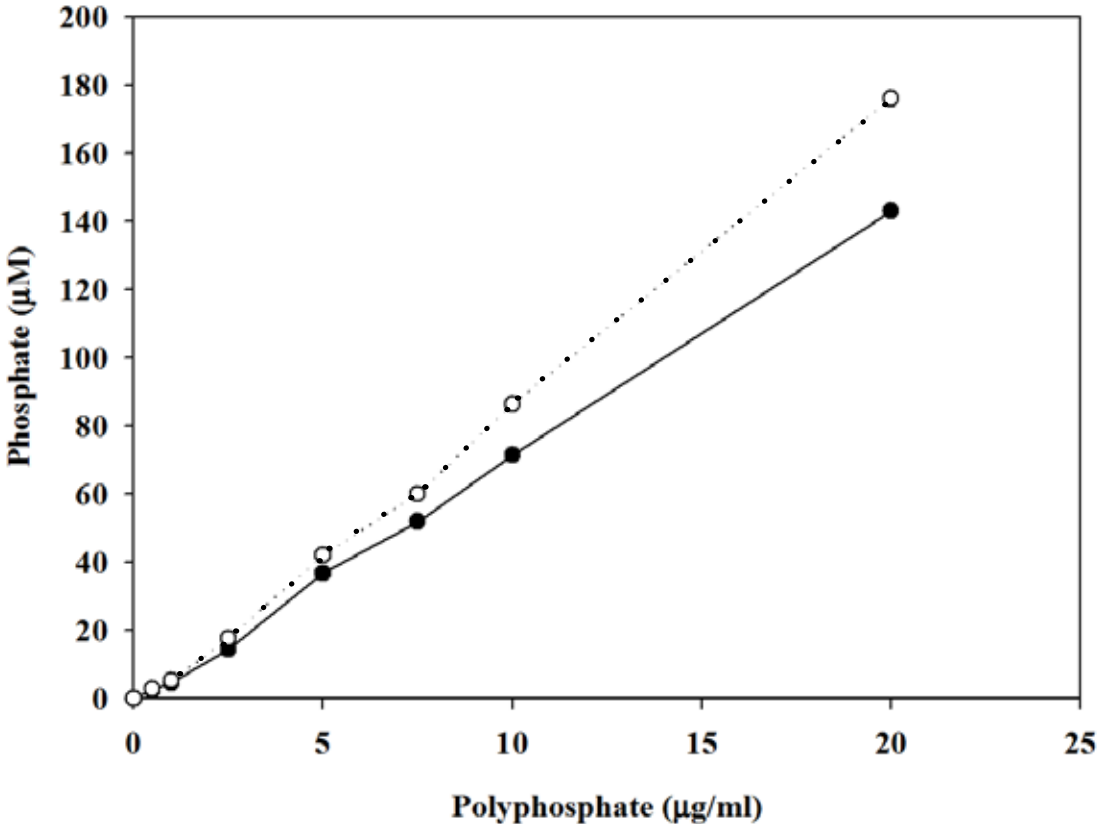

Figure 4.3. Effect of polyP chain length on the ScPpx assay. (○) Type 65 polyP; (●) Type 25 polyP.

The effect of polyP chain length on the effectiveness of the assay system was determined using both Type 25 and Type 65 polyP. Whereas the DAPI assay was not dependent on polyP chain length, the effectiveness of ScPpx protocol was reduced significantly (Figs 4.3 and 4.4). DAPI fluorescence was linear within the 0–10 µg/ml range (Fig. 4.3). The application of the DAPI-based method for samples containing high polyP concentrations requires dilution prior to quantification.

Another advantage of the DAPI-based assay over the ScPpx system is that direct polyP quantification might be achievable using either whole cells or cell-free extracts without the need for prior biopolymer extraction (which can lead to loss of polyP and an underestimation of cellular polyP content). Indeed, there are no suitable approaches for the extraction of short chain, but potentially physiologically important, polyP (Ault-Riche *et al.*, 1998). (This can mainly be attributed to the dependence of extraction efficiency on polyP chain length. It has been proposed that polyP of at least 60 residues is required for effective extraction [Ault-Riche *et al.*, 1998]). The ability to quantify polyP directly using the DAPI-based approach would therefore offer a significant advantage over other polyP quantification systems. This was therefore tested using whole cells and cell-free extracts prepared from *Pseudomonas putida* KT2440. Direct polyP quantification using DAPI was compared with the ScPpx protocol, which requires the prior extraction and purification of the polyP as a precursor to its enzymatic hydrolysis. No change in DAPI fluorescence could be detected in either whole cells or cell-extracts prepared from *P. putida* KT2440 during growth, although the development of polyP granules could be followed using fluorescence microscopy (data not shown) and was detected using the ScPpx assay. From these results, it appears that the DAPI-based assay is not suitable for the direct detection of polyP. This might be because of interference and/or quenching of the fluorescence signal due to polyP forming intracellular complexes with polyhydroxybutyrate, calcium or protein (e.g. Negoda *et al.*, 2009).

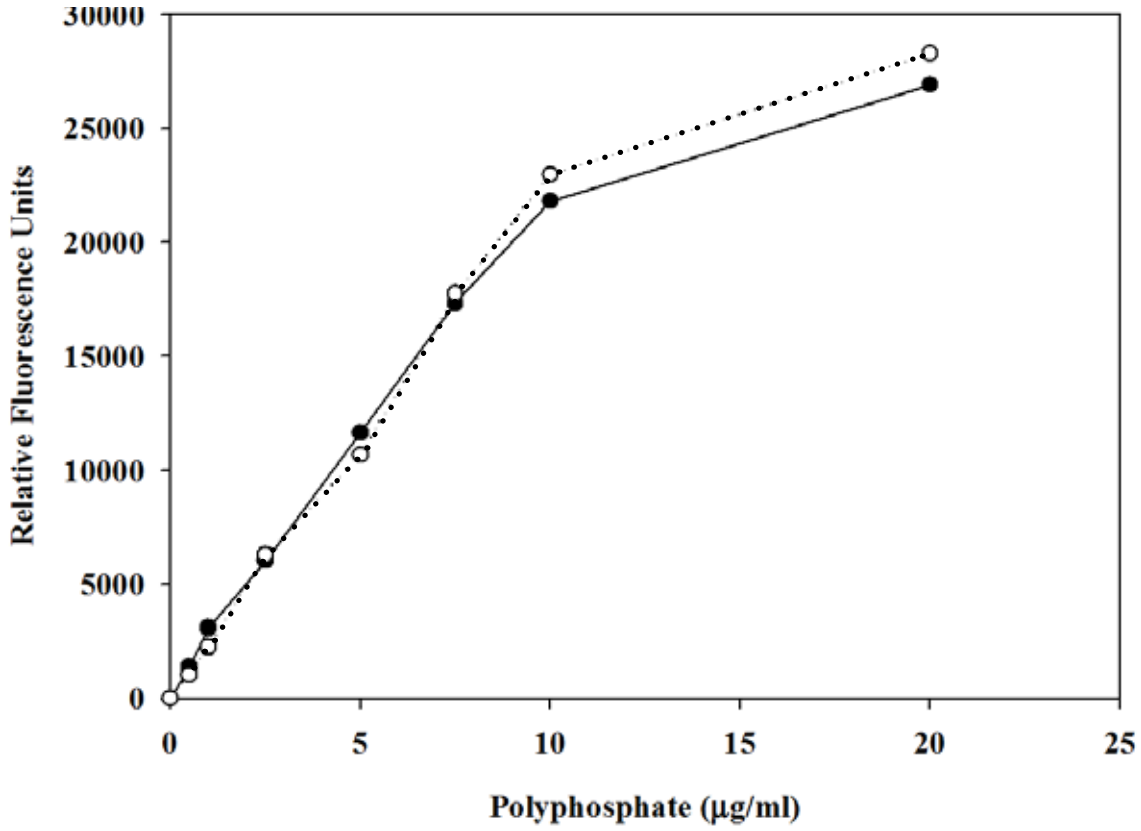

Figure 4.4. Effect of polyphospate (polyP) chain length on the DAPI assay. (○) Type 65 polyP; (●) Type 25 polyphosphate.

To further evaluate the DAPI-based methodology, a polyP extraction and purification step was introduced into the procedure: this has not previously been included in any studies employing the DAPI-based approach. Accordingly, the method developed by Werner *et al.* (2005) for polyP extraction and purification using PCR-product purification columns was employed. When combined with the DAPI assay, and using a calibration graph based on the reaction of DAPI with a Type 65 polyP standard, polyP accumulation in cells of *P. putida* KT2440 could be quantified. Similar polyP levels were obtained using the three assay methods with 15 µg polyP/mg of cellular protein, 22 µg polyP/mg of cellular protein and 21.2 µg polyP/mg of cellular protein accumulated by *P. putida* KT2440 cells upon completion of growth, using either acid hydrolysis, ScPpx or the DAPI assay systems respectively.

The development of these protocols was an important milestone in the life of the project as it allowed the examination of both P transport and polyP accumulation in cells of the chosen isolates, *Acinetobacter baumannii* 5B2 and *B. cepacia* AM19. Of particular significance is the technical simplicity and non-radioactive nature of the assays. As such, these protocols provide a significant and powerful tool for the study of polyP metabolism and will allow for the further elucidation of the physiological role played by this ubiquitous phosphate biopolymer.

4.2 Characterisation of Phosphate Transport Systems in Cells of *Acinetobacter baumannii* 5B2 and *Burkholderia cepacia* AM19

Two major P transport systems have been identified in bacteria. The inorganic phosphate transport (Pit) system is a constitutive, low-affinity transporter that is energised by the proton motive force and consists of a single membrane component. The system specifically transports neutral metal phosphate ($MeHPO_4$) chelates via a $MeHPO_4/H^+$ symport. The phosphate-specific transport (Pst) system is a high-affinity ATP-binding cassette (ABC) transporter which belongs to the group of P starvation (pho-regulated) genes whose expression is triggered by P limitation. This system uses a periplasmic-binding protein to sequester and deliver either $H_2PO_4^-$ or

HPO_4^{2-} (but not $MeHPO_4$) to the membrane-spanning transporter complex; ATP hydrolysis catalyses the translocation process. The periplasmic-binding protein appears to have no preference for either $H_2PO_4^-$ or HPO_4^{2-}. The Pst promoter is expressed at a low, basal level when P is present in excess but is derepressed up to 100-fold when external P concentrations fall below 4 μM.

In a series of P transport studies designed to: (i) determine the number of systems present; (ii) assess their mode of energisation; and (iii) ascertain any requirement for a periplasmic solute binding protein, it was demonstrated that P transport at acid pH is not due to Pit but rather involves a novel system analogous to Pst in that it requires a periplasmic binding protein and is energised by ATP. Kinetically, this acid pH P transporter has a K_M comparable to that of Pit but a V_{max} similar to P starvation-induced Pst. However, this Pst-like transporter operating at acid pH occurs under conditions of P excess known to repress the classical Pst system; for example, enhanced phosphate uptake occurs in *B. cepacia* AM19 cells grown at pH 6.0 even when the phosphate concentration in the medium is greater than 1 mM. At this external P concentration, the classical pho regulon, which controls Pst, is inactive. How the distribution of the phosphate species in aqueous solution varies as a function of pH was also investigated. At pH 7.5 there is an almost equal distribution of $H_2PO_4^-$, HPO_4^{2-}, and $MeHPO_4$. At pH 5.5, 92% of P is in the $H_2PO_4^-$ form. Previous studies have shown that the classical Pst system has no preference for $H_2PO_4^-$ or HPO_4^{2-}.

4.3 PolyP Accumulation in Cells of *Acinetobacter baumannii* 5B2 and *Burkholderia cepacia* AM19

Cells of *Acinetobacter baumannii* 5B2 grown in defined mineral salts medium showed maximal P removal and accumulation of polyP at pH 6.0. This was accompanied by an increase in the activity of the polyP synthesising enzyme PPK. Using universal bacterial PPK primers, a fragment of 1.3 Kbp whose translated amino acid sequence was compared with known bacterial PPK sequences was obtained. Significant similarities were only observed with PPKs from other *Acinetobacter* sp.: *Acinetobacter* sp. ADP1 and *Acinetobacter baumanii* PPK showed the highest levels of amino acid similarity, 74% and 73% respectively.

Maximal P removal and accumulation of polyP in cells of *B. cepacia* AM19 occurred at pH 5.5 with levels, respectively, 2.5-fold and 3.3-fold those of cells grown at pH 7.5. PPK activity was detected only in cells grown at pH 5.5 (manuscript in preparation).

4.4 Further Studies

Despite the progress made during this study, many fundamental questions remain unanswered with respect to the ASBPR phenomenon:

- Why is the rate of phosphate transport into the cell increased at acid pH?
- Has it a protective role against cytoplasmic acidification?
- What triggers polyP biosynthesis? Is it increased intracellular phosphate concentration due to increased rates of phosphate transport? Is it elevated intracellular ATP levels, caused by increased rates of oxidative phosphorylation and/or proton motive force increases resulting from reduced external pH?
- Or is polyP produced as a direct response to acid pH – perhaps because elevated intracellular polyP levels have a protective role?

To begin answering these questions, the creation of a bank of *B. cepacia* AM19 mutants carrying deletions in those genes responsible for phosphate transport and its assimilation into polyP is planned. Mutagenesis will be carried out using either the commercially available TargeTron Gene Knockout System, which specifically disrupts bacterial genes by the insertion of Group 11 introns via a site-specific retrohoming mechanism, or a reporter system based on *pyrF* – developed in the laboratory of Victor de Lorenzo (Galvão and de Lorenzo, 2005). Analysing P transport and polyP metabolism in these mutant cells will allow the team to unravel further the biochemistry and genetics involved in ASBPR.

5 Improving Biotechnological Approaches to Phosphorus Removal from Wastewater

The results of both Objectives 1 and 3 indicate clearly that a key requirement for successful ASBPR is the maintenance of operational pH at 6.0. Herein lies the difficulty with the process. Those isolates obtained in Objective 2 and further analysed in Objective 3 displayed, for the most part, maximal P removal at pH 5.5. However, this pH, both in the pilot plant and full-scale trial, had a detrimental affect on final effluent quality with respect to sludge-settling and nitrification. Additionally, within the full-scale trial there was difficulty in maintaining pH within the desired acidic range, encountering frequent overdosing problems leading to sludge disintegration and loss of nitrification (Fig. 3.3). As a consequence, the ASBPR process has not yet been shown capable of meeting current compliance limits. A further trial site is currently being investigated and attempts are under way to address these dosing problems with the project's industrial partners – using, for example, a multipoint pH dosing system.

One further issue became apparent in the laboratory scale studies: the stability of the ASBPR process itself. This was manifested in an inconsistency in P removal results using activated sludge inocula where ASBPR could be demonstrated in some samples while not in others (even for the same sewage treatment works sampled on consecutive days). This might imply that some further underlying stress was responsible for inducing the ASBPR phenomenon or at least was involved in an interplay with acid stress, and thus contributed to the enhanced P removal that was observed. In the latter few months of the project, the team therefore began looking at other stresses that could be used either alone or in conjunction with the acid process.

The accumulation of polyP is central to the success of any bio-P removal process. PolyP is ubiquitous in the biological world with a wide range of cellular functions (Kornberg *et al.*, 1999). An understanding of the conditions under which microbial cells accumulate polyP in nature is therefore important to stimulate the development of alternative biological systems for P removal. As an example, extensive accumulation of polyP has been detected in *E. coli* in response to osmotic stress and to defects in DNA metabolism or to nutritional stress imposed by either nitrogen, amino acid or phosphate limitation (Ault-Riche *et al.*, 1998; Rao *et al.*, 1998; Amado and Kuzminov, 2009). Similarly, *Pseudomonas aeruginosa* mucoid strain 8830 accumulates intracellular polyP, particularly during the stationary phase and in response to phosphate and amino acid limitations (Ault-Riche *et al.*, 1998; Kim *et al.*, 1998) while both the unicellular alga *Dunaliella salina* and the yeast *Saccharomyces cerevisiae* utilise their intracellular polyP reserves to provide a pH-stat mechanism to counterbalance alkaline stress (Pick *et al.*, 1990; Bental *et al.*, 1991; Pick and Weiss, 1991; Castro *et al.*, 1995). PolyP accumulation has also been observed upon exposure of the freshwater sponge *Ephydatia muelleri* to various organic pollutants (Imsiecke *et al.*, 1996). PolyP may also play an important role in the physiological adaptation of microbial cells during growth and development, and in their response to nutritional and environmental stresses: it is this adaptation to stress which could be particularly applicable to the development of novel treatment technologies (Ault-Riche *et al.*, 1998). Additionally, a recent report by Schulz and Schulz (2005) describes the presence of large polyP inclusions in the giant sulfur oxidising marine bacterium *Thiomargarita namibiensis*. Accumulation of polyP occurred in an aerobic environment when *T. namibiensis* was supplied with reduced sulfur as an energy and electron source.

Attempts were made to assess whether the observations made by Schulz and Schulz (2005) in the marine environment might translate to the terrestrial and wastewater spheres, thus finding biotechnological application in the development of alternative or supplementary treatment strategies for P removal. Would the addition of reduced sulfur compounds to activated sludge under aerobic conditions similarly stimulate microbial polyP accumulation and thereby increase the P removal capacity of the indigenous sludge microflora? The question is relevant since sulfur-oxidising bacteria are widespread in the terrestrial

environment and are present in wastewater treatment systems (Shooner *et al.*, 1996; Cha *et al.*, 1999; Kohno, 1998; Ito *et al.*, 2004).

The effect of reduced sulfur compound addition on the aerobic uptake of phosphate by an activated sludge inoculum from Hilden Sewage Treatment Works grown on mineral salts medium containing glucose (0.5 g/l) at pH 7.2 is shown in Table 5.1. Glucose was added to encourage growth of the organoheterotrophic sludge population (Lai *et al.*, in press). It was chosen because of its widespread use by sludge microorganisms (of 46 environmental bacteria isolated in the current project, all used glucose as a carbon source; unpublished results). However, glucose is rarely found in the activated sludge system being fermented in either in the sewer or during primary settlement. Had reduced sulfur addition increased P removal significantly, further studies using a synthetic sewage based on skim milk would have been necessary (McGrath *et al.*, 2001).

Only addition of the S^{iv} species, sodium sulfite (0.5 mM), gave rise to an increase in P removal, with 0.284±0.041 mmol/l phosphate removed across the 5 replicates upon sodium sulfite supplementation compared to 0.243±0.012 in the unamended controls: this represents an average increase of 17% in phosphate removal (Lai *et al.*, in press). The addition of sodium thiosulfate (S^{ii}), sodium hydrosulfite (S^{iii}) and potassium tetrathionate (S^{v}: 0.5 mM) did not increase P removal: decreased growth yield was however observed with these compounds (results not shown). Reducing agents can act as inhibitors of microbial growth (Riondet *et al.*, 2000). For example, sodium hydrosulfite can easily cross the cell membrane and has been shown to dramatically affect ∆pH, thereby reducing viability (Waché *et al.*, 2002).

The observed increase in P removal upon addition of sodium sulfite was unrelated to increased medium sulfur or sodium counter ion concentration as addition of sodium sulfate (0.5 mM) to the sludge inoculum produced no effect (Table 5.1). The maximum phosphate removal was observed in the presence of 0.5–1 mM sodium sulfite at pH 7.2: sulfite concentrations up to 5 mM showed no inhibitory affect on microbial growth yield (results not shown; Lai *et al.*, in press). Similarly, Babich and Stotzky (1978a) reported no decrease in the growth yields of *Bacillus cereus*, *Serratia marcescens*, *E. coli and Pseudomonas aeruginosa* at pH 7.0 in nutrient broth supplemented with up to 10 mM sulfite: at 50 mM sulfite the growth yield of all bacteria was however reduced. Salts of sulfite exist as quadrivalent-sulfur substances (sulfur dioxide [SO_2], sulfurous acid [H_2SO_3], sulfite [SO_3^{2-}] and bisulfite [HSO_3^-]) in a pH-sensitive equilibrium. The predominant sulfur species depends upon pH and the acid dissociation constant, which in turn is dependent on temperature and medium ionic strength. Under the physiological conditions used in this study SO_3^{2-} however predominates (Babich and Stotzky, 1978a; Schimz, 1980). As pH decreases, HSO_3^-, H_2SO_3 and SO_2 concentrations increase. The inhibitory effect of sulfite on yeast and lactic acid bacteria at acid pH has been attributed to increased SO_2 concentrations leading to ATP depletion and DNA damage (Schimz, 1980). Indeed, P removal in the presence of sulfite (0.5 mM) decreased at acidic pH: 0.201 mmol/L phosphate was removed under otherwise identical conditions at pH 5.0 compared with 0.284 mmol/L at pH 7.2 (results not shown: Lai *et al.*, in press).

Table 5.1. Effect of various reduced sulfur compounds (0.5 mmol/l) on the phosphate removal capabilities of activated sludge microorganisms after 24 hours growth on basal mineral salts medium supplemented with 0.5g/l glucose and 1 mmol/l phosphate at pH 7.2.

Sulfur compound (0.5 mmol./l)	Phosphate removed (mmol./l) †	% phosphate removed
None (control)	0.243±0.012	100
Sodium sulfite	0.284±0.041	117
Sodium hydrosulfite	0.229±0.057	94
Sodium thiosulfate	0.221±0.047	91
Potassium tetrathionite	0.241±0.031	99
Sodium sulfate‡	0.231±0.027	95

† Results are a mean of 5 replicates. ‡Sodium sulfate was added at a concentration of 0.5 mmol/l as a positive control-giving medium with a similar sulfur concentration to that used in the test cultures.

In parallel with enhanced P uptake, increased intracellular polyP accumulation was observed in the presence of sodium sulfite (Fig. 5.1) with polyP inclusions clearly visible in the treated sludge biomass (results not shown).

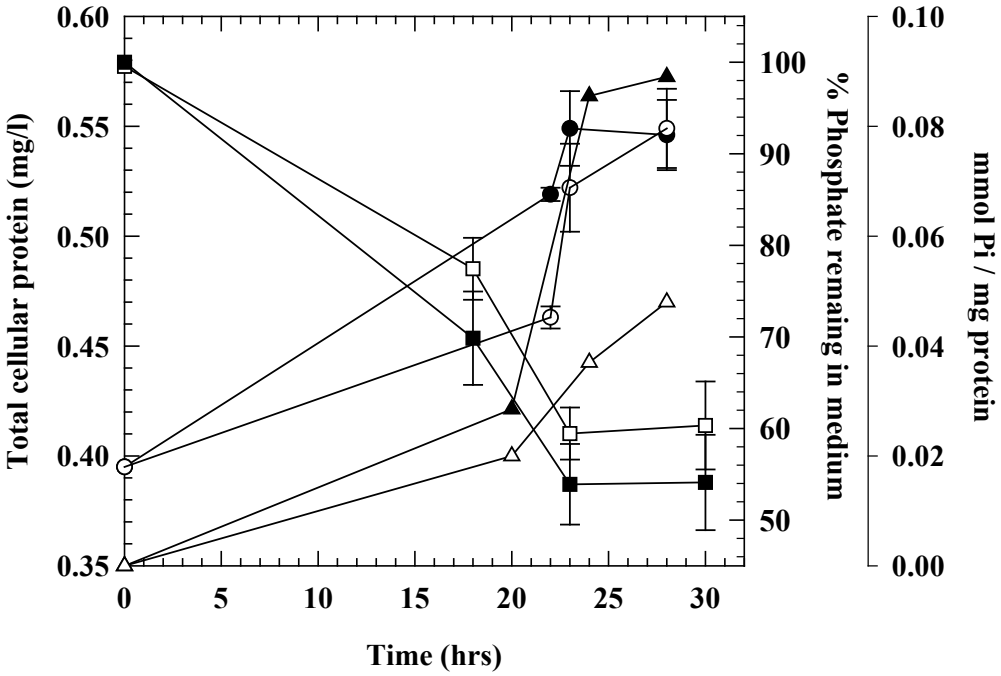

Figure 5.1. Growth (measured by the increase in total cellular protein [●,○], % phosphate removal [■,□] and intracellular polyphosphate accumulation [mmol Pi/mg protein: ▲,△]) by an activated sludge inoculum grown on 0.5g/l glucose mineral salts medium in the presence (closed symbols) and absence of sodium sulfite (0.5 mM) (open symbols).

Intracellular polyphosphate levels in cultures incubated with sulfite reached a maximum of 0.089 mmol phosphate/mg of total cellular protein compared to 0.048 mmol phosphate/mg of total cellular protein in control cultures (Fig. 5.1). Furthermore, enhanced phosphate removal and polyP accumulation occured whilst sulfite concentrations in the growth medium remained high: 85% of the sulfite remained in the media upon completion of microbial growth (Lai *et al.*, in press).

This represents the first report of polyP accumulation by activated sludge microorganisms in response to the addition of sulfur compounds. In contrast, Harold and Sylvan (1963) describe the suppression of polyP formation in *Aerobacter aerogenes* in the presence of sulfate, sulfite, cysteine and oxidised glutathione. However, the authors do report polyP accumulation in response to sulfur starvation. To avoid the potential for sulfur starvation in the current project's cultures Mg $SO_4.7H_2O$ (0.2g/l) was routinely added to the mineral salts medium. The exact relationship between sulfite addition, increased phosphate removal, and polyphosphate accumulation is at present unclear but may be caused by either an increased abundance of chemolithotrophic sulfur oxidisers in the sludge biomass or a stress response to the addition of sulfite. Owing to its antioxidant and antimicrobial properties, sulfite is used widely in the food and beverage industry as a preservative. Studies have shown that sulfite and its anionic solubility products can exert a range of stresses on the microbial cell – from the modification of nucleic acids and proteins to the alteration of cellular ATP levels. PolyP accumulation in response to environmental stress is a well-documented phenomenon with polyP itself having a wide range of biological functions (reviewed by Kornberg *et al.*, 1999). For example, polyP has been implicated in bacterial RNA and protein turnover, with accumulation reported in response to nutritional limitation, osmotic stress, pH change or even exposure to various organic pollutants. Given therefore that enhanced phosphate removal and polyP accumulation occur while sulfite concentrations in the growth medium remain high, this may well point to a sulfite-induced stress response. However, despite the fact that sulfite was shown to enhance both P removal and polyP accumulation, the levels of enhancement achieved were not sufficient to meet current European P discharge consents, even in combination with acid stimulation.

5.1 Further Studies

The last few months of the project have been marked by a potentially significant breakthrough in attempts to develop a new bio-P removal process. Although the underlying basis of the phenomenon has yet to be resolved fully, it seems to revolve around how long microbial cells are maintained in the stationary phase and may go some way towards explaining the variable performance of the ASBPR system. Whether this is due to the production of signalling compounds or is a cellular response to the stresses imposed by stationary-phase-nutrient limitation has yet to be elucidated (nor indeed the influence of acid on this). Nevertheless, if the stationary phase conditions are altered, the P removal response is altered significantly. This result has been confirmed using individual microorganisms (including those sequenced isolates and the process relevant strains discussed in Section 3.1), in inocula from activated sludge plants, and by employing the surrogate activated sludge culture 'Polytox'. The significance of these results is that enhanced P removal can be seen consistently and in cultures not previously shown to display the ASBPR phenomenon. Using this system, increases in P removal of 33% for activated sludge, 109% for Polytox and 22% for the environmental isolates *Acinetobacter calcoaceticus* and *Aeromonas hydrophilia* have been observed. Future studies will focus on assessing the potential of incorporating this new finding into the P removal process using a 2 l laboratory-scale bioreactor.

6 Relevance to Policy/Decision Makers and Other Stakeholders

Freshwater systems are considered eutrophic if the P concentration exceeds 25 µg/l (McGrath and Quinn, 2003). Of 496 Irish lakes monitored under Article 4(4) of the Local Government of Ireland's Water Pollution 1977 (Water Quality Standards for Phosphorus Regulations, 1998) (S.I. 258 of 1998), 95 currently do not comply with these P targets, while 36.6% of river monitoring stations surveyed are also non-compliant (EPA, 2006). In Northern Ireland, both Loughs Neagh and Erne are highly eutrophic (The Environment and Heritage Service, 1999). These elevated P concentrations are a direct result of anthropogenic discharge from agricultural, industrial or municipal sewage sources.

The Urban Wastewater Treatment Directive and S.I. 258 of 1998, 684 of 2007 and 272 of 2009 now places strict limits on the amount of P released to the aquatic environment. Indeed, the EU Urban Wastewater Treatment Directive requires an effluent standard of no more than 1 mg/l P and/or a minimum percentage P reduction of 80% for large treatment works, particularly from those discharging into 'eutrophication-sensitive' receiving waters (Council of the European Community, 1991 and 1998). As conventional activated sludge processes remove P relatively poorly – with typical removal efficiencies of 20–40% (c.f. the Directive) – P removal technologies must increasingly be incorporated into the activated sludge system. Currently in Ireland, only 17% of wastewater treatment plants (serving above 500 person equivalents discharging to 'sensitive waters') have nutrient-reduction facilities. There is however a strong case for the incorporation of P removal in all treatment plants discharging to inland waters throughout Ireland, with the EPA calling for the construction/upgrade of wastewater facilities to include more advanced treatment options (EPA, 2006; Toner, 2000; Stapleton and Clenaghan, 2000). A further driver for this will be the implementation of the EU Water Framework Directive and the associated Statutory Instruments to reduce external nutrient loading in order to achieve good ecological quality in Ireland's waters by 2015. This will ultimately necessitate the inclusion of a P-removal process at many sewage works. However, the technologies available to achieve these consents are unreliable, unsustainable or uneconomic. For the water industry, the development of alternative Bio-P removal strategies is therefore imperative for the further prevention of eutrophication.

This award has enabled the project team to continue our efforts to develop an alternative biotechnological approach to the EBPR process for bio-P removal and recovery from wastewater. Because of the work reported here, and the ongoing studies this sponsorship has catalysed, it is hoped that more progress in the development of ASBPR or its derivative technologies will be seen. Given that EPBR was first discovered some 60 years ago and its scientific basis is not yet fully resolved, the project team feels well placed to progress in developing a competitive alternative based on these studies. Hence, this research is of benefit to both water companies and legislative organisations. The implication of acid-stimulated P uptake (or any process variant) as a waste-treatment technology is that efficient and reliable P removal may be obtained in a single phase without the need to retro-fit existing sewage plants with anaerobic pre-treatment stages. Such a system might also prove to be more appropriate for the high-volume/low-strength wastewaters typical of temperate regions and would also have advantages with respect to energy consumption and plant footprint. Furthermore, these studies may also have implications in the design of strategies to recover elemental P from sewage, required to replace dwindling global rock-P reserves which are estimated to last only 50–100 years. P-rich sewage sludges, which contain 10–100 times less metals than rock phosphate, could supply a significant proportion of the P used in Europe annually whilst significantly reducing processing costs. Recycling is viable only if a P-rich biomass, such as that potentially obtainable by the project's process, can be produced.

References

Amado L. and Kuzminov, A. (2009). Polyphosphate accumulation in *Escherichia coli* in response to defects in DNA metabolism. *Journal of Bacteriology* **191**, 7410–7416.

Aschar-Sobbi R., Abramov A.Y., Diao C., Kargacin M.E., Kargacin G.J., French R.J. and Pavlov E. (2008). High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. *Journal of Fluorescence* **18**, 859–866.

Ault-Riche, D., Fraley, C.D., Tzeng, C.M. and Kornberg, A. (1998). Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyP in *Escherichia coli*. *Journal of Bacteriology* **180**, 1841–1847.

Babich, H. and Stotzky, G. (1978a). Influence of pH on inhibition of bacteria, fungi, and coliphages by bisulfite and sulfite. *Environmental Research* **15**, 405–417.

Barak, Y. and van Rijn, J. (2000). Atypical polyphosphate accumulation by the denitrifying bacterium *Paracoccus denitrificans*. *Applied and Environmental Microbiology* **66**, 1209–1212.

Barker, P.S. and Dold, P.L. (1996). Denitrification behaviour in biological excess phosphorus removal activated sludge systems. *Water Research* **30**, 769–780.

Barnard, J. and Abraham, K. (2006). Key features of successful BNR operation. *Water Science and Technology* **53**, 1–9.

Bental, M., Pick, U., Avron, M. and Degani, H. (1991). PolyP metabolism in the alga *Dunaliella salina* studied by P^{31}-NMR. *Biochimica Biophysica Acta* **1092**, 21–28.

Brett, S, Guy, J, Morse, G.K. and Lester, J.N. (1997). *Phosphorus Removal and Recovery Technologies*, Selper Publications, London.

Castro, C.D., Meehan, A.J., Koretsky, A.P. and Domach, M.M. (1995). In situ ^{31}P nuclear magnetic resonance for observation of polyP and catabolite responses of chemostat-cultivated *Saccharomyces cerevisiae* after alkalinization. *Applied and Environmental Microbiology* **61**, 4448–4453.

Cha, J.M., Cha, W.S. and Lee, J.H. (1999). Removal of organo-sulfur odour compounds by *Thiobacillus novellus* SRM, sulfur-oxidizing microorganisms. *Process Biochemistry* **34**, 659–665.

Council of the European Community (1991). Council Directive concerning urban waste water treatment (91/271/EC). *Official Journal of the European Communities.* **L135/40**.

Council of the European Community (1998). Council Directive concerning urban waste water treatment (***98/15/EC***) *Official Journal of the European Communities.* **L67/29.**

Dabert, P., Sialve, B., Delgenes, J.P., Moletta, R. and Godon, J.J. (2001). Characterisation of the microbial 16S rDNA diversity of an aerobic phosphorus-removal ecosystem and monitoring of its transition to nitrate respiration. *Applied Microbiology and Biotechnology* **55**, 500–509.

Dawes, E.A. and Senior, P.J. (1973). Energy reserve polymers in microorganisms. *Advances in Microbial Physioogy* **10**, 178–203.

Egli, T. and Zehnder, A.J.B. (2002). Phosphate and nitrate removal. *Current Opinion in Biotechnology* **5**, 275–284.

Eckenfelder, W.W. and Grau, P. (1992). In *Activated Sludge Process Design and Control: theory and practice*, Eckenfelder, W.W., Malina, J.F., Patterson, J.W. (eds) Technomic Publishing: Lancaster, PA.

Environment and Heritage Service NI. (1999). *Eutrophication in Northern Ireland's Waters – Proposals for a Strategy to Control Nutrient Enrichment.* Department of Environment for Northern Ireland.

EPA (Environmental Protection Agency) (2006). *P Regulations – National Implementation Report*, *2005*. Environmental Protection Agency, Ireland.

Forbes, C.M., O'Leary, N.D., Dobson, A.D. and Marchesi, J.R. (2010). The contribution of 'omic'-based approaches to the study of enhanced biological phosphorus removal microbiology *FEMS Microbiology Ecology* 69, 1–15.

Galvão, T.C. and de Lorenzo, V. (2005) Adaptation of the yeast *URA3* selection system to Gram-negative bacteria and generation of a *betCDE Pseudomonas putida* strain. *Applied and Environmental Microbiology* **71** (2), 883–892.

Gieseke, A.T, Tarre, S., Green, M. and de Beer, D. (2006). Nitrification in a Biofilm at Low pH Values: Role of *In Situ* Microenvironments and Acid Tolerance. *Applied and Environmental Microbiology*, **72**, 4283–4292.

Greenberg, A.E., Levin, G. and Kauffman, W.J. (1955). The effect of phosphorus removal on the activated sludge process. *Sewage and Industrial Waters* **27**, 227.

Günther, S., Trutnau, M., Kleinsteuber, S., Hause, G., Bley, T., Röske, I., Harms, H. and Müller, S. (2009). Dynamics of polyphosphate-accumulating bacteria in wastewater treatment plant microbial communities detected via DAPI (4',6'-diamidino-2-phenylindole) and tetracycline labeling. *Applied and Environmental Microbiology* **75**, 2111–2121.

Harold, F.M. (1966). Inorganic polyphosphate in biology: structure, metabolism, and function. *Bacteriological Reviews* **30**, 772–794.

Harold, F.M. and Sylvan, S. (1963) Accumulation of inorganic polyphosphate in *Aerobacter aerogenes:* Environmental control and the role of sulfur compounds. *Journal of Bacteriology* **86**, 222–231.

Hirota, R., Kuroda, A., Kato, J. and Ohtake H. (2010) Bacterial phosphate metabolism and its application to phosphorus recovery and industrial bioprocesses. *Journal of Bioscience And Bioengineering* **109**, **423–432**.

Imsiecke, G., Munkner, J., Lorenz, B., Bachinski, N., Muller, W.E.G. and Schroder, H.C. (1996). Inorganic polyPs in the developing freshwater sponge *Ephydatia muelleri*: effect of stress by polluted waters. *Environmental Toxicological Chemistry* **15**, 1329–1334.

Ito, T., Sugita, K. and Okabe, S. (2004). Isolation, characterization and *in situ* detection of a novel chemolithoautotrophic sulfur-oxidizing bacterium in wastewater biofilms growing under microaerophilic conditions. *Applied and Environmental Microbiology* **70**, 3122–3129.

Jorgensen, K.S. and Pauli, A.S.I. (1995). Polyphosphate accumulation among denitrifying bacteria in activated-sludge. *Anaerobe* **1**, 161–168.

Kim, H.Y., Schlictman, D., Shankar, S., Xie, Z., Chakrabarty, A.M. and Kornberg, A. (1998). Alginate, inorganic polyP, GTP and ppGpp synthesis co-regulated in *Pseudomonas aeruginosa:* implications for stationary-phase survival and synthesis of RNA/DNA precursors. *Molecular Microbiology* **27**, 717–725.

Kohno, T. (1988). Morphology, physiology, and nutrition of a sulfur-oxidizing filamentous organism isolated from activated-sludge. *Water Science and Technology* **20**, 241–247.

Kornberg, A. and Fraley, C.D. (2000). Inorganic polyphosphate: a molecular fossil come to life. *ASM News* **66**, 275–280.

Kornberg, A., Rao, N.N. and Ault-Riche, D. (1999). Inorganic polyP: a molecule of many functions. *Annual Review of Biochemistry* **68**, 89–121.

Kortstee, G.J.J., Appeldoorn, K.J., Bonting, C.F.C., Vanniel, E.W.J. and van Veen, H.W. (1994). Biology of polyphosphate-accumulating bacteria involved in enhanced biological phosphorus removal. *FEMS Microbiology Reviews* **15**, 137–153.

Kortstee, G.J.J., Appeldoorn, K.J., Bonting, C.F.C., van Niel, E.W.J. and van Veen, H.W. (2000). Recent developments in the biochemistry and ecology of enhanced biological phosphorus removal. *Biochemistry (Moscow)* **65**, 332–340.

Kuba, T., Smolders, G., van Loosdrecht, M.C.M. and Heijnen, J.J. (1993). Biological phosphorus removal from waste-water by anaerobic- anoxic sequencing batch reactor. *Water Science and Technology* **27**, 241–252.

Kulaev, I.S. (1979). *The Biochemistry of Inorganic polyPs*. J. Wiley and Sons Inc., New York.

Kulaev, I.S. and Vagabov, V.M. (1983). Polyphosphate metabolism in microorganisms. *Advances in Microbial Physiology*. **15**, 731–738.

Kulaev, I.S., Vagabov, V.M. and Kulakovskaya, T.V. (1999). New aspects of inorganic polyphosphate metabolism and function. *Journal of Bioscience and Bioengineering* **88**, 111–129.

Lai, M.W-Y., Kulakova, A.N., Quinn, J.P. and McGrath, J.W. (In press). Stimulation of phosphate uptake and polyphosphate accumulation by activated sludge microorganisms in response to sulfite addition. *Water Science and Technology*.

Levin, G.V. and Shapiro, J. (1965). Metabolic uptake of phosphorus by wastewater organisms. *Journal of the Water Pollution Control Federation* **37**, 800–821.

McGrath, J.W. and Quinn, J.P. (2000). Intracellular accumulation of polyphosphate by the yeast *Candida humicola* G-1 in response to acid pH. *Applied and Environmental Microbiology* **66**, 4068–4073.

McGrath, J.W., Cleary, S., Mullan, A. and Quinn, J.P. (2001). Acid-stimulated enhanced phosphate uptake by activated sludge microorganisms under aerobic laboratory conditions. *Water Research* **35**, 4317–4322.

McGrath, J.W. and Quinn, J.P. (2003). Microbial phosphate removal and polyP production from wastewaters. *Advances in Applied Microbiology* **52**, 75–100.

McGrath, J.W. and Quinn, J.P. (2004). Biological P Removal. In: Valsami-Jones, E. (ed.). *P in Environmental Technology*. E. IWA Publishing, London, pp. 272–286.

Manfredo, S.J., Alvarez, H.M. and Farias, M.E. (2008). Role of polyphosphoates in microbial adaption to extreme environments. *Applied and Environmental Microbiology* **74**, 5867–5874.

Mino, T., van Loosdrecht, M.C.M. and Heijnen, J.J. (1998). Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Research* **32**, 3193–3207.

Mullan, A., McGrath, J.W., Adamson, T., Irwin, S. and Quinn, J.P. (2006). Pilot scale evaluation of the application of low pH-inducible polyphosphate accumulation to the biological removal of phosphate from wastewaters. *Environmental Science and Technology* **40**, 296–301.

Mullan, A., McGrath, J.W. and Quinn, J.P. (2002). Enhanced phosphate uptake and polyphosphate accumulation by *Burkholderia cepacia* grown under low-pH conditions. *Microbial Ecology* **44**, 69–77.

Negoda, A., Negoda, E., Xian, M. and Reusch, R.N. (2009). Role of polyphosphate in regulation of the Streptomyces lividans KcsA channel *Biochimica et Biophysica Acta-Biomembranes* **1788**, 608–614.

Oehmen, A., Lemos, G. and Carvalho, Z. (2007). Advances in enhanced biological phosphorus removal: from micro to macro scale. *Water Research* **41**, 2271–2230.

Pick, U., Bental, M., Chitlaru, E. and Weiss, M. (1990). PolyP-hydrolysis – a protective mechanism against alkaline stress. *FEBS Biochemistry Letters* **274**, 15–18.

Pick, U. and Weiss, M. (1991). PolyP hydrolysis within acidic vacuoles in response to amine- induced alkaline stress in the halotolerant alga *Dunaliella salina*. *Plant Physiology* **97**, 1234–1240.

Rao, N.N., Liu, S. and Kornberg, A. (1998). Inorganic polyP in *Escherichia coli*: the phosphate regulon and the stringent response. *Journal of Bacteriology* **180**, 2186–2193.

Rao, N.N., Gomez-Garcia, M. and Kornberg, A. (2009). Inorganic polyphosphate: essential for growth and survival. *Annual Review of Biochemistry* **78**, 605–647.

Schimz, K.L. (1980). The effect of sulfite on the yeast *Saccharomyces cerevisiae*. *Archives of Microbiology* **125**, 89–95.

Schulz, H.N. and Schulz, H.D. (2005) Large sulfur bacteria and the formation of phosphorite. *Science* **307**, 416–418.

Schurig-Briccio, L.A., Farias, R.N., Rintoul, M.R. and Rapisarda, V.A. (2009) Phosphate-enhanced stationary-phase fitness of *Escherichia coli* is related to inorganic polyphosphate level. *Journal of Bacteriology* **191**, 4478–4481.

Seviour, R.J. and McIlroy, S. (2008). The microbiology of phosphorus removal in activated sludge processes – the current state of play. *Journal of Microbiology* **46**, 115–124.

Shooner, F, Bousquet, J. and Tyagi, R.D. (1996). Isolation, phenotypic characterization, and phylogenetic position of a novel, facultatively autotrophic, moderately thermophilic bacterium, *Thiobacillus thermosulfatus* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **46**, 409–415.

Srinath, E.G., Sastry, C.A. and Pillai, S.C. (1959). Rapid removal of phosphorus from sewage by activated sludge. *Experientia* **15**, 339–340.

Stapleton, L. and Clenaghan, C. (2000). Future outlook. In: L. Stapleton, M. Lehane & P. Toner (eds). *Ireland's Environment. A. Millennium Report*. Environmental Protection Agency, Dublin.

Streichan, M., Golecki, J. R. and Schon, G. (1990). Polyphosphate-accumulating bacteria from sewage plants with different processes for biological phosphorus removal. *FEMS. Microbial Ecology* **73**, 113–124.

Tarre, S. and Green, M. (2004). High rate nitrification at low pH using suspended and attached biomass type reactors. *Applied and Environmental Microbiology* **70**, 6481–6487.

Toner, P.F. (2000). *Ireland's Environment: The Main Issues.* Environmental Protection Agency, Dublin.

Upton, J., Hayes, E. and Churchley, J. (1996). Biological phosphorus removal at Stratford Upon Avon, UK: The effect of influent wastewater characteristics on effluent phosphate. *Water Science and Technology* **33**, 73–80.

van Loosdrecht, M.C.M., Hooijmans, C.M., Brdjanovic, D. and Heijnen, J.J. (1997). Biological phosphate removal processes. *Applied Microbiology and Biotechnology* **48**, 289–296.

van Niel, E.W., Appeldoorn, K.J., Zehnder, A.J. and Kortstee, G.J. (1998). Inhibition of anaerobic phosphate release by nitric oxide in activated sludge. *Applied and Environmental Microbiology* **64**, 2925–2930.

Victorio, L., Gilbride, K.A., Allen, D.G. and Liss, S.N. (1996). Phenotypic fingerprinting of microbial communities in wastewater treatment systems. *Water Research* **30**, 1077–1087.

Water Environment Federation (1998). *Biological and Chemical Systems for Nutrient Removal: A special publication*. Water Environment Federation, Alexandria, Virginia.

Werner, T.P., Amrhein, N., Freimoser, F.M. (2005). Novel method for the quantification of inorganic polyphosphate (iPoP) in *Saccharomyces cerevisiae* shows dependence of iPoP content on the growth phase. *Archives of Microbiology* **184**, 129–136.

Wiame, J.M. (1947). The metachromatic reaction of hexametaphosphate. *Journal of the American Chemical Society* **69**, 3146–3147.

Wiame, J.M. (1948). The occurrence and physiological behavior of two metaphosphate fractions in yeast. *Journal of Biological Chemistry* **178**, 919–929.

Yeoman, S., Stephenson, T., Lester, J.N. and Perry, R. (1988). The removal of P during wastewater treatment: a review. *Environmental Pollution* **49**, 183–233.

Acronyms and Annotations

ABC	ATP binding cassette
ADP	Adenosine-5’-diphosphate
ASBPR	Acid stimulated biological P removal
ATP	Adenosine-5’-triphosphate
COD	Chemical oxygen demand
DAPI	4’,6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
EBPR	Enhanced biological phosphorus removal
GDP	Guanosine-5’-diphosphate
GTP	Guanosine-5’-triphosphate
MLSS	Mixed liquor suspended solids
NDEBPR	Nitrification-denitrification enhanced biological phosphorus removal
P	Phosphorus
PHB	Poly-β-hydroxybutyrate
PHV	Poly-β-hydroxyvalerate
Pit	Inorganic phosphate transport
PolyP	Polyphosphate
Pst	Phosphate specific transport
VFA	Volatile fatty acid

An Ghníomhaireacht um Chaomhnú Comhshaoil

Is í an Gníomhaireacht um Chaomhnú Comhshaoil (EPA) comhlachta reachtúil a chosnaíonn an comhshaol do mhuintir na tíre go léir. Rialaímid agus déanaimid maoirsiú ar ghníomhaíochtaí a d'fhéadfadh truailliú a chruthú murach sin. Cinntímid go bhfuil eolas cruinn ann ar threochtaí comhshaoil ionas go nglactar aon chéim is gá. Is iad na príomh-nithe a bhfuilimid gníomhach leo ná comhshaol na hÉireann a chosaint agus cinntiú go bhfuil forbairt inbhuanaithe.

Is comhlacht poiblí neamhspleách í an Ghníomhaireacht um Chaomhnú Comhshaoil (EPA) a bunaíodh i mí Iúil 1993 faoin Acht fán nGníomhaireacht um Chaomhnú Comhshaoil 1992. Ó thaobh an Rialtais, is í an Roinn Comhshaoil agus Rialtais Áitiúil a dhéanann urraíocht uirthi.

ÁR bhFREAGRACHTAÍ

CEADÚNÚ

Bíonn ceadúnais á n-eisiúint againn i gcomhair na nithe seo a leanas chun a chinntiú nach mbíonn astuithe uathu ag cur sláinte an phobail ná an comhshaol i mbaol:

- áiseanna dramhaíola (m.sh., líonadh talún, loisceoirí, stáisiúin aistrithe dramhaíola);
- gníomhaíochtaí tionsclaíocha ar scála mór (m.sh., déantúsaíocht cógaisíochta, déantúsaíocht stroighne, stáisiúin chumhachta);
- diantalmhaíocht;
- úsáid faoi shrian agus scaoileadh smachtaithe Orgánach Géinathraithe (GMO);
- mór-áiseanna stórais peitreail.
- Scardadh dramhuisce

FEIDHMIÚ COMHSHAOIL NÁISIÚNTA

- Stiúradh os cionn 2,000 iniúchadh agus cigireacht de áiseanna a fuair ceadúnas ón nGníomhaireacht gach bliain.
- Maoirsiú freagrachtaí cosanta comhshaoil údarás áitiúla thar sé earnáil - aer, fuaim, dramhaíl, dramhuisce agus caighdeán uisce.
- Obair le húdaráis áitiúla agus leis na Gardaí chun stop a chur le gníomhaíocht mhídhleathach dramhaíola trí comhordú a dhéanamh ar líonra forfheidhmithe náisiúnta, díriú isteach ar chiontóirí, stiúradh fiosrúcháin agus maoirsiú leigheas na bhfadhbanna.
- An dlí a chur orthu siúd a bhriseann dlí comhshaoil agus a dhéanann dochar don chomhshaol mar thoradh ar a ngníomhaíochtaí.

MONATÓIREACHT, ANAILÍS AGUS TUAIRISCIÚ AR AN GCOMHSHAOL

- Monatóireacht ar chaighdeán aeir agus caighdeáin aibhneacha, locha, uiscí taoide agus uiscí talaimh; leibhéil agus sruth aibhneacha a thomhas.
- Tuairisciú neamhspleách chun cabhrú le rialtais náisiúnta agus áitiúla cinntí a dhéanamh.

RIALÚ ASTUITHE GÁIS CEAPTHA TEASA NA HÉIREANN

- Cainníochtú astuithe gáis ceaptha teasa na hÉireann i gcomhthéacs ár dtiomantas Kyoto.
- Cur i bhfeidhm na Treorach um Thrádáil Astuithe, a bhfuil baint aige le hos cionn 100 cuideachta atá ina mór-ghineadóirí dé-ocsaíd charbóin in Éirinn.

TAIGHDE AGUS FORBAIRT COMHSHAOIL

- Taighde ar shaincheisteanna comhshaoil a chomhordú (cosúil le caighdéan aeir agus uisce, athrú aeráide, bithéagsúlacht, teicneolaíochtaí comhshaoil).

MEASÚNÚ STRAITÉISEACH COMHSHAOIL

- Ag déanamh measúnú ar thionchar phleananna agus chláracha ar chomhshaol na hÉireann (cosúil le pleananna bainistíochta dramhaíola agus forbartha).

PLEANÁIL, OIDEACHAS AGUS TREOIR CHOMHSHAOIL

- Treoir a thabhairt don phobal agus do thionscal ar cheisteanna comhshaoil éagsúla (m.sh., iarratais ar cheadúnais, seachaint dramhaíola agus rialacháin chomhshaoil).
- Eolas níos fearr ar an gcomhshaol a scaipeadh (trí cláracha teilifíse comhshaoil agus pacáistí acmhainne do bhunscoileanna agus do mheánscoileanna).

BAINISTÍOCHT DRAMHAÍOLA FHORGHNÍOMHACH

- Cur chun cinn seachaint agus laghdú dramhaíola trí chomhordú An Chláir Náisiúnta um Chosc Dramhaíola, lena n-áirítear cur i bhfeidhm na dTionscnamh Freagrachta Táirgeoirí.
- Cur i bhfeidhm Rialachán ar nós na treoracha maidir le Trealamh Leictreach agus Leictreonach Caite agus le Srianadh Substaintí Guaiseacha agus substaintí a dhéanann ídiú ar an gcrios ózóin.
- Plean Náisiúnta Bainistíochta um Dramhaíl Ghuaiseach a fhorbairt chun dramhaíl ghuaiseach a sheachaint agus a bhainistiú.

STRUCHTÚR NA GNÍOMHAIREACHTA

Bunaíodh an Ghníomhaireacht i 1993 chun comhshaol na hÉireann a chosaint. Tá an eagraíocht á bhainistiú ag Bord lánaimseartha, ar a bhfuil Príomhstiúrthóir agus ceithre Stiúrthóir.

Tá obair na Gníomhaireachta ar siúl trí ceithre Oifig:

- An Oifig Aeráide, Ceadúnaithe agus Úsáide Acmhainní
- An Oifig um Fhorfheidhmiúchán Comhshaoil
- An Oifig um Measúnacht Comhshaoil
- An Oifig Cumarsáide agus Seirbhísí Corparáide

Tá Coiste Comhairleach ag an nGníomhaireacht le cabhrú léi. Tá dáréag ball air agus tagann siad le chéile cúpla uair in aghaidh na bliana le plé a dhéanamh ar cheisteanna ar ábhar imní iad agus le comhairle a thabhairt don Bhord.

NDP
National Development Plan 2007 - 2013

Science, Technology, Research and Innovation for the Environment (STRIVE) 2007-2013

The Science, Technology, Research and Innovation for the Environment (STRIVE) programme covers the period 2007 to 2013.

The programme comprises three key measures: Sustainable Development, Cleaner Production and Environmental Technologies, and A Healthy Environment; together with two supporting measures: EPA Environmental Research Centre (ERC) and Capacity & Capability Building. The seven principal thematic areas for the programme are Climate Change; Waste, Resource Management and Chemicals; Water Quality and the Aquatic Environment; Air Quality, Atmospheric Deposition and Noise; Impacts on Biodiversity; Soils and Land-use; and Socio-economic Considerations. In addition, other emerging issues will be addressed as the need arises.

The funding for the programme (approximately €100 million) comes from the Environmental Research Sub-Programme of the National Development Plan (NDP), the Inter-Departmental Committee for the Strategy for Science, Technology and Innovation (IDC-SSTI); and EPA core funding and co-funding by economic sectors.

The EPA has a statutory role to co-ordinate environmental research in Ireland and is organising and administering the STRIVE programme on behalf of the Department of the Environment, Heritage and Local Government.

epa
Environmental Protection Agency
An Ghníomhaireacht um Chaomhnú Comhshaoil

ENVIRONMENTAL PROTECTION AGENCY
PO Box 3000, Johnstown Castle Estate, Co. Wexford, Ireland
t 053 916 0600 f 053 916 0699
LoCall 1890 33 55 99
e info@epa.ie w http://www.epa.ie

SSTI
Strategy for Science, Technology and Innovation

Comhshaol, Oidhreacht agus Rialtas Áitiúil
Environment, Heritage and Local Government