Appl Microbiol Biotechnol (1998) 49: 202–209

ORIGINAL PAPER

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The effect of growth conditions on the biodegradation of tributyl phosphate and potential for the remediation of acid mine drainage waters by a naturally-occurring mixed microbial culture

Received: 2 June 1997 / Received revision: 15 September 1997 / Accepted: 19 September 1997

Abstract The biodegradation of tributyl phosphate (Bu_3-*P*, TBP), releasing phosphate at a high enough concentration locally to precipitate uranium from solution, was demonstrated by a mixed culture consisting primarily of pseudomonads. The effect of various parameters on Bu_3-*P* biodegradation by growing cells is described. Growth at the expense of Bu_3-*P* as the carbon and phosphorus source occurred over a pH range from 6.5 to 8, and optimally at pH 7. Bu_3-*P* biodegradation was optimal at 30 °C, reduced at 20 °C and negligible at 4 °C and 37 °C. Incorporation of Cu or Cd inhibited, and Ni, Co and Mn reduced its degradation. Inorganic phosphate (above 10 mM) and kerosene (up to 1 g/l) reduced Bu_3-*P* biodegradation significantly, but nitrate had no effect. Sulphate (10–100 mM) was inhibitory. When pregrown biomass was used the fastest rates of tributyl and dibutyl phosphate biodegradation were 25 μmol h^{-1} mg $protein^{-1}$ and 37 μmol h^{-1} mg $protein^{-1}$ respectively. Microcarrier-immobilised biomass decontaminated uranium-bearing acid mine waste water by uranium phosphate precipitation at the expense of Bu_3-*P* hydrolysis in the presence of 35 mM SO_4^{2-}. At pH 4.5, 79% of the UO_2^{2+} was removed at a flow rate of 1.4 ml/h on a 7-ml test column.

Introduction

Tributyl phosphate (Bu_3-*P*, TBP) is used in uranium extraction and nuclear fuel reprocessing (McKay 1964), in aircraft hydraulics fluids, herbicide solutions, surface coating compositions and in other applications as a plasticizer or defoamer (Jones and Brown 1987). Bu_3-*P* can be a primary waste contaminant or a co-contaminant with metals, persisting, for example, in low-activity streams from nuclear fuel reprocessing or in fuel rod storage ponds with uranyl ion at an approximate ratio of 2:1 (Macaskie 1991).

Bu_3-*P*, a phosphotriester, is hydrolysed chemically via the intermediates dibutyl and monobutyl phosphate (Bu_2-*P* and Bu-*P*) to butanol (3 mol/mol) and phosphate (P_i:1 mol/mol) (Belskii 1977). The pathway of biodegradation may be similar (Rosenberg and Alexander 1979). A stepwise hydrolytic removal of methyl groups was observed in trimethyl phosphate-degrading *Hyphomicrobium* sp. (Ghisalba et al. 1987). Biodegradation of Bu_3-*P* as the sole phosphorus source was reported for two strains of *Pseudomonas* (Rosenberg and Alexander 1979) and an *Acinetobacter* sp. (Stoner and Tien 1995). The degradation rate was slow (loss of 150 μg/ml Bu_3-*P* over 28 days), and the degradation pathway was not investigated (Stoner and Tien 1995). The enzymology of Bu_3-*P* degradation is obscure although the participation of phosphotriesterases, diesterases and monoesterases is implicated (Ghisalba et al. 1987).

Three aspects justify this study. First, Bu_3-*P* is an organophosphorus compound requiring treatment before disposal and, secondly, it is a potential phosphate (P_i) donor for heavy-metal accumulation, where liberated P_i can precipitate with metals (Thomas and Macaskie 1996). In addition, this phosphotriester could serve as a model compound for more toxic organophosphates, e.g. pesticides and some nerve gas agents.

The biodegradation of Bu_3-*P* by a mixed culture, predominantly of *Pseudomonas* spp., was reported previously (Thomas and Macaskie 1996). Uranium was simultaneously deposited as hydrogen uranyl phosphate (Thomas and Macaskie 1996), in a similar way to that observed previously with a heavy-metal-accumulating *Citrobacter* sp. hydrolysing glycerol 2-phosphate (Macaskie and Dean 1985; Macaskie et al. 1992). In both cases the release of P_i from the phosphoester substrates was high enough to exceed, locally, the solubility product of HUO_2PO_4.

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Wastes from both mining and nuclear fuel reprocessing are hostile for most metabolic functions. They are usually acidic, containing substantial levels of nitrate (nuclear fuel reprocessing wastes) or sulphate (acid mine drainage waters), metal ions and often organic components (e.g. kerosene in nuclear fuel reprocessing wastes).

A versatile system is required for the degradation of Bu_3-*P* under a wide range of conditions. This study investigates the effect of some environmental and physiological constraints on the biodegradation of Bu_3-*P* by the mixed culture. The potential for the decontamination of uranium-bearing acid mine drainage water (AMD) is investigated, using immobilised cells challenged with AMD in a continuous flow-through system.

Materials and methods

Acid mine drainage water (AMD)

Samples of naturally leached, uranium-bearing acid mine wastewater were as previously described (Roig et al. 1995; Macaskie et al. 1997) and were provided from the ENUSA mine by Dr. M.G. Roig (University of Salamanca, Spain). The physicochemical parameters of the waters were as described previously (Roig et al. 1995; Macaskie et al. 1997: Table 1). Wastewater (pH 3.5) was brought to pH 4.5, or as stated, by addition of NaOH, and ferric hydroxide flocs removed by sedimentation under gravity prior to use. The wastewater was stored at 4 °C.

Organisms and culture conditions

A mixed culture growing at the expense of Bu_3-*P* as the sole carbon and phosphorus source was isolated as described previously (Thomas and Macaskie 1996). The mixed culture, predominantly of pseudomonads (Thomas and Macaskie 1996), was grown for 2 days in minimal medium (MM: as below), and inoculated (10 ml) into 90 ml MM comprising (g/l) $CaCl_2$ 0.025, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 0.1, $(NH_4)_2SO_4$ 0.5, Na_2 EDTA 0.015, $ZnSO_4 \cdot 7H_2O$ 0.0066, $MnCl_2 \cdot 4H_2O$ 0.00171, $FeSO_4 \cdot 7H_2O$ 0.0015, $CoCl_2 \cdot 6H_2O$ 0.000483, $CuSO_4 \cdot 5H_2O$ 0.000471, $NaMoO_4 \cdot 2H_2O$ 0.000453, 3-(*N*-morpholino)propanesulphonic acid (MOPS) 5.22. The pH was adjusted to 7 with 1 M NaOH, and 0.53 g/l of Bu_3-*P* (final concentration: 2 mM) was added as the sole carbon and phosphorus source. The stock Bu_3-*P* (BDH Chemicals, UK) contained no other phosphate species detectable by ^{31}P nuclear magnetic resonance spectroscopy. The MM was autoclaved (121 °C, 15 min). Bu_3-*P* was self-sterile and was added directly after autoclaving; uninoculated controls gave no colonies on plating onto Bu_3-*P*-unsupplemented nutrient agar plates and no growth in liquid culture in Bu_3-*P*-supplemented minimal medium. Cultures were maintained statically at 30 °C or (as stated) on a rotary shaker (160 rpm). Growth was monitored turbidimetrically (A_{600}) and samples (1 ml) taken periodically were stored at −20 °C for later analysis.

Effect of aeration, temperature, kerosene and pH on Bu_3-*P* degradation

Aliquots (10 ml) of the mixed culture (A_{600} of 0.35; stationary phase, 72 h) were harvested by centrifugation (7000 rpm, MSE high-speed 18, ambient temperature), washed twice in sterile isotonic saline ("saline", 8.5 g/l NaCl), resuspended in 10 ml MM and inoculated into 90 ml MM (250-ml conical flask). Controls were Bu_3-*P*-unsupplemented cultures, and Bu_3-*P*-supplemented cell-free media as appropriate. To determine initially the effect of temperature, the cultures were incubated statically at 4 °C, 20 °C, 30 °C and 37 °C. The effect of aeration was investigated by comparison of growth (as bacterial protein) in shaken and unshaken cultures at 30 °C. The effect of pH was investigated by pH adjustment before inoculation by the addition of either concentrated HCl (Fisher Scientific, UK) to pH 5–6.5, or 10 M NaOH to pH 7.5–9.0. Controls were as above, at the appropriate pH. The aerobic cultures were maintained at 30 °C on a rotary shaker (160 rpm), with growth monitored turbidimetrically (A_{600}). The effect of kerosene (aerobic cultures) was investigated at pH 7 and 30 °C, with kerosene to 0.25 g/l, 0.5 g/l and 1 g/l, with and without Bu_3-*P*, with appropriate controls.

Effect of anions on Bu_3-*P* biodegradation

Phosphate (KH_2PO_4) was added to MM to a final concentration of 0–50 mM, and the pH re-confirmed. Aliquots (10 ml) of the mixed culture (A_{600} of 0.35) in stationary phase (72 h) were harvested, washed twice in saline and resuspended in 10 ml MM with appropriate phosphate. The suspension was inoculated into 90 ml MM containing the corresponding concentration of P_i, and incubated aerobically with Bu_3-*P*. The effect of sodium nitrate and sodium sulphate was investigated in a similar way, at concentrations of 0–100 mM, with appropriate controls (above). The cultures were maintained aerobically at 30 °C with, growth monitored turbidimetrically (A_{600}) and samples stored as before.

Effect of metals on Bu_3-*P* degradation

Metal salts (100 μM or 1 mM final concentrations) were Cu $(NO_3)_2 \cdot 3H_2O$ (BDH, UK), $MnCl_2 \cdot 4H_2O$ (BDH, UK), Co $(NO_3)_2 \cdot 6H_2O$ (Fisons, UK), $Cd(NO_3)_2 \cdot 4H_2O$ (BDH, UK) or $Ni(NO_3)_2 \cdot 6H_2O$ (BDH, UK). Metals were added to MM (100 ml), filter-sterilised and transferred to 250-ml conical flasks with checks for chemical precipitation. Aliquots (10 ml) of the mixed culture (metal unsupplemented: A_{600} of 0.35, 72 h) were harvested, washed as before and resuspended in 100 ml metal-supplemented MM. Growth was aerobic (30 °C) with controls and sampling as above.

Biodegradation of Bu_3-*P* and Bu_2-*P* by the mixed culture

A 100-ml inoculum of the mixed culture (A_{600} of 0.35) was transferred to an airlift fermenter (volume 2.3 l), constructed in the laboratory. MM (1.9 l) with Bu_3-*P* was added, and further supplemented with an additional 0.53 g/l Bu_3-*P* after 48 h to give a final A_{600} of 0.535. Biomass was harvested by centrifugation (7000 rpm, MSE high-speed 18), washed twice in saline and stored as a pellet (4 °C) until required. Cells were resuspended in 1.8 l MM (pH 7), to give a protein concentration of 203 μg/ml. Samples (100 ml) were transferred into 250-ml conical flasks. Bu_3-*P* or Bu_2-*P* was added to 0.25–2 mM. Controls and conditions (30 °C, aerobic) were as before.

Analysis of biomass, residual alkyl phosphates and phosphate

Stored samples were thawed and centrifuged (13 000 rpm, 4 min 20 °C: Heraeus Sepatech Biofuge A) and the supernatant analysed for residual Bu_3-*P* or Bu_2-*P* by gas chromatography (610 series gas chromatograph; ATI Unicam, UK), by the method of Kuno et al. (1991), adapted using a PEG 20 M macrobore column (Pye Unicam). The supernatant P_i was measured by an adaptation of the method of Pierpoint (Pierpoint 1957; Yong and Macaskie 1995). Protein was measured using the copper sulphate/bicinchoninic acid kit according to the manufacturers' instructions (Sigma).

The bioremediation of uranium AMD by immobilised Bu_3-*P*-biodegrading culture

Microcarriers (0.3 g Cytopore 2: Pharmacia AB, Sweden; from Pharmacia Biotechnology, St. Albans, Hertfordshire, England)

were suspended in 30 ml phosphate-buffered saline (29 mM KH_2PO_4, 29 mM K_2HPO_4, 145 mM NaCl; 1 h), autoclaved (20 min), harvested by centrifugation and resuspended in MM (300 ml) with 2 mM (0.53 g/l) Bu_3-*P*, inoculated with cells (30 ml inoculum; A_{600} of 0.35) and further supplemented with Bu_3-*P* (0.53 g/l) after 48 h and 64 h. The microcarriers were transferred to a glass column (7 ml, Pharmacia) and washed with saline (100 ml). AMD (2 l; Table 1) was brought to pH 4, 4.5 or 5 with 10 M NaOH, supplemented with Bu_3-*P* to 2 mM and pumped upwards through the column (flow rate of 1.4 ml/h). Samples of inflow and outflow solutions were collected and stored at −20 °C. Microcarrier-bound biomass was estimated as protein extracted into 0.5 M NaOH at 100 °C for 10 min, and measured using the copper sulphate/bicinchoninic acid kit (Sigma, UK). The total protein in the column was 0.10 g (0.0145 g protein/ml or 0.33 g protein/g dry weight microcarrier). Controls used mine water unsupplemented with TBP.

Treatment of results

All experiments were done in triplicate on three separate occasions and the data for each experiment were pooled and calculated as the mean of three experiments ± standard deviation (SD; $n = 3$).

Results

Effect of aeration, temperature, pH and kerosene on growth and Bu_3-*P* biodegradation by the culture

Preliminary tests used statically incubated flasks, where the cell doubling time at 30 °C and 20 °C was 72 ± 5 h and 152 ± 4.9 h respectively. No growth or Bu_3-*P* biodegradation was noted in corresponding cultures incubated at 4 °C or 37 °C. All subsequent tests were done at 30 °C. The predominant culturable organisms under these conditions were identified as pseudomonads using the API20 NE system (Thomas and Macaskie 1996). Since *Pseudomonas* spp. are obligately aerobic and no nitrate was added as an alternative electron acceptor, subsequent tests were done aerobically. The doubling time at 30 °C was reduced to 28.4 ± 1.4 h (c.f. above) and, accordingly, aerobic cultures were used in all subsequent experiments. Previous tests showed less than stoichiometric phosphate release into the medium from Bu_3-*P* biodegradation, attributed to polyphosphate-accumulating organisms within the culture (Thomas and Macaskie 1996). Aerated cultures at 30 °C degraded 2 ± 0.03 mM Bu_3-*P* and released 1.4 ± 0.02 mM P_i.

No growth was observed in any Bu_3-*P*-unsupplemented cultures in this study, and no Bu_3-*P* biodegradation was noted in uninoculated cultures. The optimal temperature for growth and Bu_3-*P* biodegradation was 30 °C (above) and the optimal pH was 7 (e.g. 0.48 ± 0.03 mM residual Bu_3-*P* after 50 h). Reduced growth at pH 7.5 and reduced Bu_3-*P* biodegradation were noted at pH 6.5 and 7.5 (0.73 ± 0.02 and 0.73 ± 0.03 mM respectively after 50 h), with some residual activity at pH 6, 8 and 9 and none at pH 5 (Table 2). This narrow pH range may limit industrial application, since the "target" wastes are acidic and would require pH adjustment before this method could be used (c.f. later).

Kerosene is a Bu_3-*P* diluent used in nuclear fuel reprocessing at an equivalent amount (w/w) to Bu_3-*P* (Ashley et al. 1987). As a potential carbon source, kerosene could be used as a growth substrate in preference to Bu_3-*P*. The presence of kerosene reduced the rate of Bu_3-*P* biodegradation by the culture, e.g. from 29 ± 1 μmol/h in unsupplemented cultures to 21 ± 1 μmol/h (0.25 g/l and 0.5 g/l kerosene), with an initial lag (30 h) in the latter. The addition of 1 g/l kerosene gave no apparent lag and the initial rate of loss of Bu_3-*P* was similar to that of the unsupplemented control, with growth similar to that of unshaken controls. The kerosene probably formed a surface layer restricting access of air to the culture and may also have trapped some Bu_3-*P* within it.

Table 1 The chemical composition of ENUSA water. Na does not precipitate as the phosphate *per se*, but Na (and NH_4^+) can be incorporated into the uranyl phosphate precipitate to form $NaUO_2PO_4$ and $NH_4UO_2PO_4$ respectively (Yong and Macaskie 1995). Mg and Ca are not included because they are unlikely to form insoluble phosphate extensively at low pH. The metal ions included in the calculation of total available metals are in bold. The analyses were as in Roig et al. (1995) and Macaskie et al. (1997). ENUSA wastewater was sampled in October 1993. (*I*) Native wastewater, (*II*) the pH was increased to 4.5 with NaOH; this precipitated-out the iron. Data are shown for filtered wastewater in each case. *NT* not tested

Analyte	Concentration (ppm)		Concentration (mM)	
	pH 3.5	pH 4.5	pH 3.5	pH 4.5
SO_4^{2-}	3339(I)	3375(II)	34.8(I)	35.16(II)
NH_4^+	10.6(I)	NT	0.59(I)	NT
Mg	639(I)	623(II)	26.3(I)	25.64(II)
Ca	499(I)	495(II)	12.4(I)	12.34(II)
Mn	79.3(I)	79.1(II)	1.44(I)	**1.44(II)**
Na	78.7(I)	106(II)	3.42(I)	4.61(II)
UO_2^{2+}	38.6(I)	35.0(II)	0.14(I)	**0.13(II)**
Al	29.9(I)	24.4(II)	1.11(I)	**0.91(II)**
Fe	12.9(I)	0.66(II)	0.23(I)	**0.012(II)**
Zn	4.37(I)	4.38(II)	0.066(I)	**0.067(II)**
Ni	2.90(I)	2.94(II)	0.049(I)	**0.050(II)**
Cu	0.17(I)	0.18(II)	0.0027(I)	**0.0028(II)**
Total available metals			3.0 mM(I)	2.6 mM(II)

Table 2 The effect of pH on the growth, biodegradation of tributyl phosphate (Bu_3-*P*) and phosphate release by the mixed culture. *NG* no growth

pH	Doubling time (h)	Inorganic phosphate release (mmol l^{-1})		Bu_3-*P* degradation (mmol l^{-1})	
		30 h	70 h	30 h	70 h
5	NG	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
6	NG	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
6.5	28.5 ± 1.6	0.81 ± 0.02	1.15 ± 0.04	0.82 ± 0.05	1.63 ± 0.03
7	28.4 ± 1.7	0.71 ± 0.03	1.11 ± 0.04	1.22 ± 0.08	1.92 ± 0.09
7.5	145 ± 3.1	0.22 ± 0.01	0.89 ± 0.03	0.71 ± 0.06	1.61 ± 0.10
8	150 ± 3.0	0.00 ± 0.00	0.58 ± 0.05	0.00 ± 0.00	0.71 ± 0.05
9	240 ± 2.9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.02

Effect of anions on Bu_3-*P* degradation

Inorganic phosphate inhibited growth and Bu_3-*P* biodegradation at concentrations of 10 mM and above (Fig. 1). Phosphate-unsupplemented controls gave growth as in Table 2. The residual Bu_3-*P* concentration after 24 h was 1.45 ± 0.02 mM with 0.5, 1, 2 or 5 mM inorganic phosphate and all cultures showed a lag before onset of Bu_3-*P* degradation (20 h, Fig. 1B). The inhibition of Bu_3-*P* biodegradation at 10–50 mM P_i suggests possible phosphate-mediated regulation (see Discussion). However, high concentrations of P_i *per se* are unlikely in nuclear fuel cycle wastes (Macaskie 1991) or uranium mining wastes but, in contrast, the effects of nitrate and sulphate are pertinent to their role as the major counterions in nuclear fuel cycle wastes and AMD waters respectively (see Introduction and Table 1). Accordingly, the effect of these on Bu_3-*P* biodegradation was investigated as shown in Fig. 2. Addition of nitrate (up to 100 mM) had little effect on Bu_3-*P* biodegradation or phosphate release (Fig. 2), e.g. 1.68 ± 0.06 mM and 1.78 ± 0.04 mM Bu_3-*P* degraded with 10 mM and 100 mM NO_3^- respectively, compared to 1.54 ± 0.05 mM for the control. This compares with nitrate levels in wastes of, for example, 1.2 mM in some radium-loaded waste waters and 140 mM in nuclear fuel storage pond water (Macaskie 1991). The addition of 10 mM and 100 mM sulphate reduced the amount of Bu_3-*P* biodegraded significantly (1.39 ± 0.07 mM and 0.57 ± 0.07 mM respectively), with a pronounced delay (more than 36 h) with the latter. This inhibition could be problematic for the treatment of AMD waters (c.f. Table 1), but nuclear fuel reprocessing wastes do not contain substantial SO_4^{2-} , e.g. 5.2 mM SO_4^{2-} for waste streams of low activity and 13 mM for radium-loaded waste waters (Macaskie 1991).

Effect of metals on Bu_3-*P* degradation

The effect of uranium on cultures growing at the expense of Bu_3-*P* was reported previously (Thomas and Macaskie 1996). UO_2^{2+} (1 mM) inhibited growth and Bu_3-*P* degradation, with some inhibition at 100 μM UO_2^{2+}. Other heavy metals inhibited growth; the doubling time (h) in the presence of 100 μM metal was 62 ± 1 (Mn), 112 ± 2 (Ni), 61 ± 2 (Co) and 330 ± 12 (Cd), increasing in the presence of 1 mM metal to 106 ± 3 (Mn), 195 ± 2 (Ni) and 302 ± 10 (Co), as compared to 28 ± 1 h in unsupplemented controls. There was no

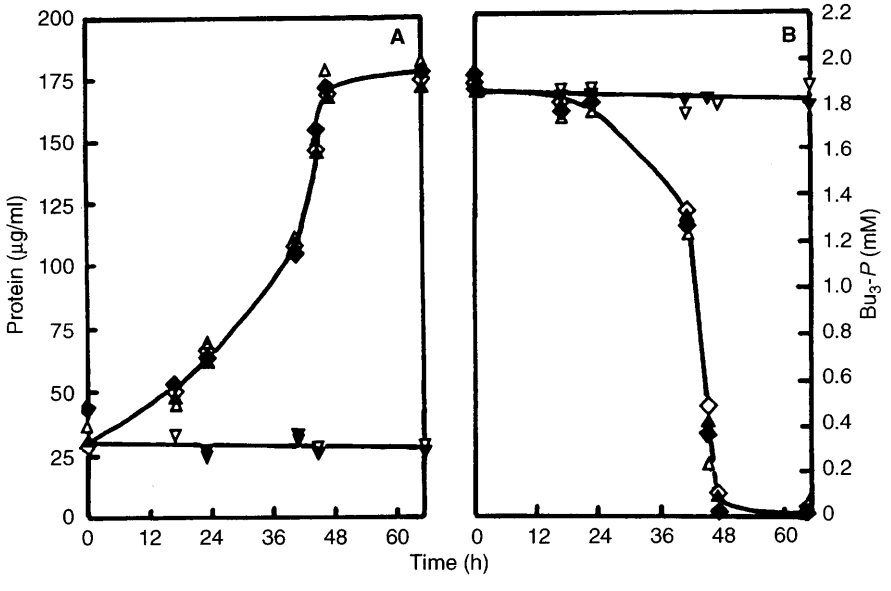

Fig. 1A, B The effect of inorganic phosphate on growth (**A**) and tributyl phosphate (Bu_3-*P*) utilisation (**B**). Cultures were grown (aerobically, 30 °C, pH 7) as described in the text in the presence of P_i at 0 (◆), 1 (◇), 2 (▲), 5 (△), 10 (▼) and 20 (▽) mM

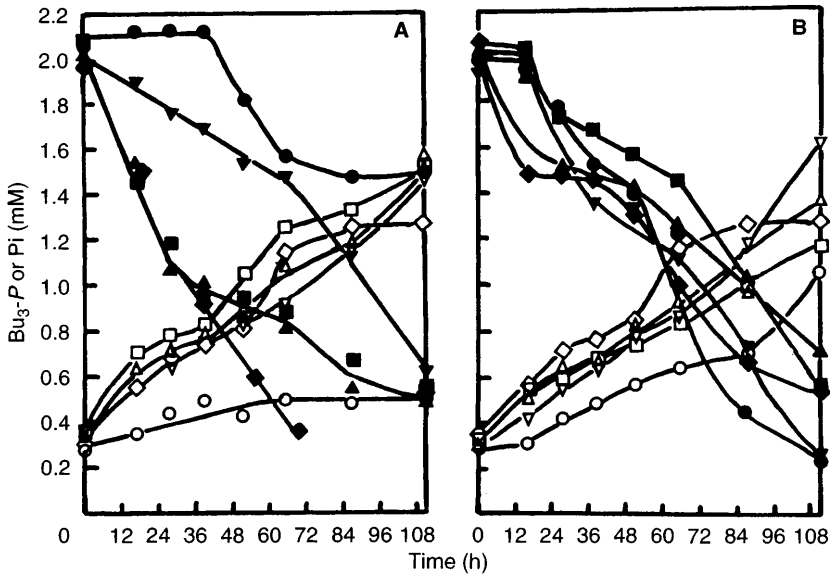

Fig. 2A, B The effect of sulphate (**A**) and nitrate (**B**) on Bu_3-*P* utilisation (*filled symbols*) and P_i release (*open symbols*). Growth was as described in the text, in the presence of sulphate or nitrate at 0 (◆ ◇), 0.1 (■ □), 1 (▲ △), 10 (▼ ▽) or 100 (● ○) mM

growth with Cu at either concentration, or Cd at 1 mM, and no Bu_3-*P* degradation in Cu- or Cd-supplemented cultures.

Mn^{2+} had little effect on Bu_3-*P* biodegradation at 100 μM but at 1 mM this was reduced to 1.27 ± 0.01 mM Bu_3-*P* degraded after 144 h, from 1.99 ± 0.03 mM in the metal-unsupplemented control. The release of P_i with metals was apparently small, but since precipitation of P_i with heavy metals was likely, its release was probably underestimated. Ni^{2+} (100 μM) promoted a biphasic degradation of Bu_3-*P*. Up to 60 h the rate was similar to the control, with 1.4 mM Bu_3-*P* degraded. The residual 0.6 mM Bu_3-*P* was degraded at approximately half of the rate of the control, with 95% removal of Ni^{2+} after 144 h. In contrast, 1 mM Ni^{2+} gave a lag of 60 h, and reduced the total Bu_3-*P* biodegraded to only 0.75 ± 0.03 mM. The addition of 100 μM Co^{2+} also reduced the total Bu_3-*P* degraded, by 25%, from 1.99 ± 0.03 mM to 1.51 ± 0.02 mM, without lag; 1 mM Co^{2+} reduced Bu_3-*P* biodegradation further, to 0.37 ± 0.03 mM degraded.

Biodegradation of Bu_3-*P* and Bu_2-*P* by resuspended cells of the mixed culture

Since the biodegradation of Bu_3-*P* was slow, the breakdown of Bu_2-*P* was also evaluated. Bu_2-*P* can be produced by alkaline hydrolysis of Bu_3-*P* (Belskii 1977) and chemical pretreatment of Bu_3-*P* with NaOH may be feasible, particularly if subsequent neutralisation of the wastewater is required. The rate of Bu_3-*P* biodegrada-

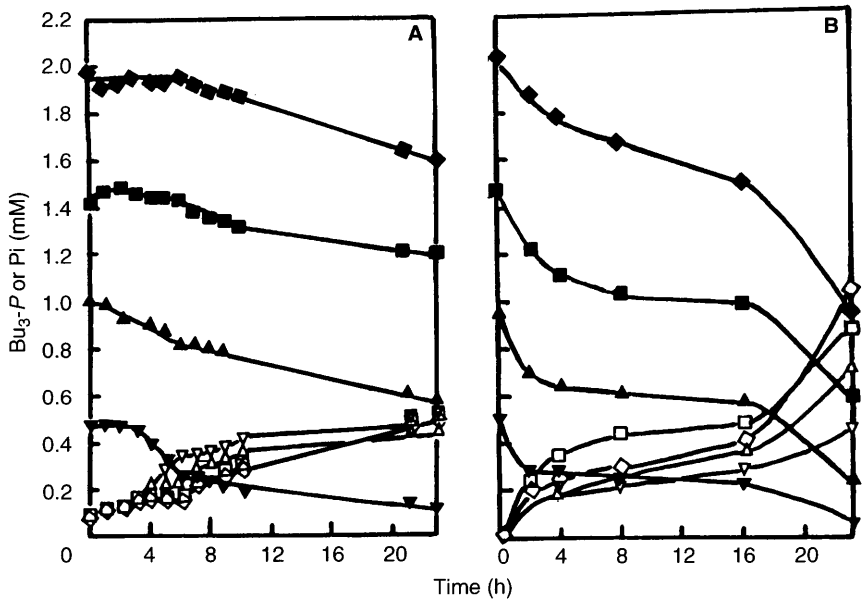

Fig. 3A, B Degradation of Bu_3-*P* (**A**) and Bu_2-*P* (**B**) (*filled symbols*) and P_i release (*open symbols*) by cells challenged with alkyl phosphate at 0.5 (▼ ▽), 1 (▲ △), 1.5 (■ □) or 2 (◆ ◇) mM

tion (Fig. 3A) by resuspended cells of the mixed culture was comparable between 0.25–2 mM Bu_3-*P*, and maximal at 25 ± 0.2 μmol h^{-1} mg $protein^{-1}$. The biodegradation of Bu_2-*P* (Fig. 3B) was biphasic and maximal at 37 ± 1.1 μmol h^{-1} mg $protein^{-1}$.

The use of immobilised biomass for the continuous treatment of waste water

Individually, the metals other than U in the ENUSA water (except Mn^{2+}) would not be sufficiently concentrated to inhibit Bu_3-*P* biodegradation substantially (Table 1), but a combination of these, in the presence of SO_4^{2-} (Table 1) and the low pH (c.f. Table 2) could be problematic for waste remediation. For testing against ENUSA water the biomass was self-immobilised as biofilm on microcarriers and challenged with ENUSA water in a flow-through column at constant flow rate for over 20 h. The columns were at steady state, with constant activity over that time. No significant UO_2^{2+} removal (< 2%) occurred in the Bu_3-*P*-free controls and no significant Bu_3-*P* loss (< 3%) was noted in cell-free columns, in accordance with preliminary tests using a uranium solution (Thomas and Macaskie 1996). UO_2^{2+} was removed from the ENUSA water by precipitation with enzymatically liberated phosphate from Bu_3-*P* biodegradation. At pH 4.5, removal of UO_2^{2+} (124 ± 3 μM or 79.0%) and 100% removal of Bu_3-*P* were observed (Fig. 4A). At pH 4 there was only a slight reduction in the UO_2^{2+} (28 ± 3 μM or 16.7%) and Bu_3-*P* (0.12 ± 0.005 mM or 5.9%) concentration (Fig. 4B), probably attributable to reduced enzyme activity at the lower pH (c.f. Table 1). At pH 5, with 100% hydrolysis of Bu_3-*P*, only 77 ± 3 μM (60%) of the uranium was removed. The reason for the poorer activity at pH 5 than at pH 4.5 is not clear, but, given that the wastewater was not buffered and no organic complexing ligands were present, the formation of hydroxylated uranyl species may have reduced the availability of free UO_2^{2+} for precipitation with phosphate.

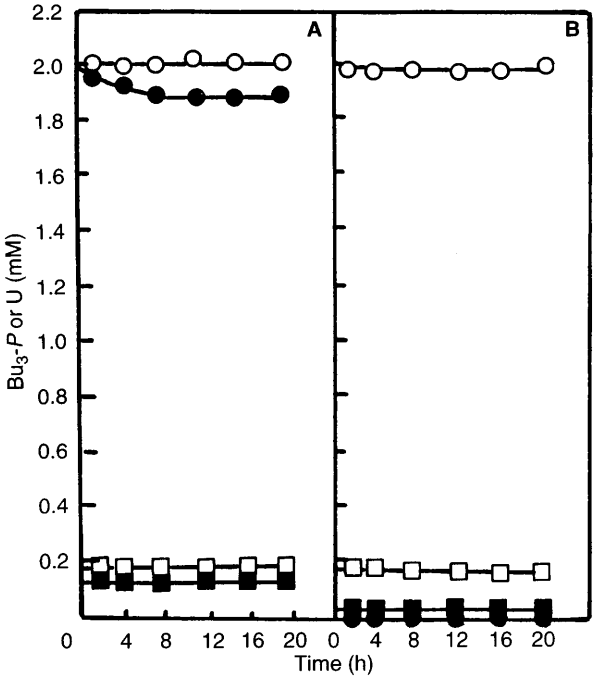

Fig. 4A, B The bioremediation of ENUSA acid mine drainage water. The culture was immobilised as a biofilm and challenged with a flow of ENUSA water of the composition shown in Table 1 and as described in Materials and methods. The pH of the flow was adjusted with NaOH to 4.0 (**A**) or 4.5 (**B**). ○ Bu_3-*P* in column input solution, ● Bu_3-*P* in outflow solution, ❒ UO_2^{2+} in input solution, ■ UO_2^{2+} in outflow solution

Discussion

This study demonstrates utilisation of Bu_3-*P* as the sole source of carbon and phosphorus, in contrast to previous studies where it was provided as the sole utilisable phosphorus source (Rosenberg and Alexander 1979; Stoner and Tien 1995). It can be difficult to use an organophosphorus compound to fulfil both requirements. This is because the cellular demand for carbon is greater than that for phosphorus; cleavage of substrate and consumption of carbon lead to an excess of phosphate within the cell, which can inhibit further uptake. This potential paradox is characteristic of tight regulation of phosphate transport systems via the *pho* regulon (Torriani 1990). In the example of the "Ugp paradox" (Brzoska et al. 1994), uptake of glycerol phosphate via the Ugp system in *Escherichia coli* is repressed by liberated P_i via the *pho* regulon, even though the cellular requirement for carbon is still high; In the absence of an alternative transport pathway (GlpT) the cell can starve (Brzoska et al. 1994). Utilisation of the phosphonate herbicide glyphosate by a *Pseudomonas* was suggested to be limited by a similar paradox (Dick 1991; R.E. Dick, personal communication).

In the present case some inhibition by phosphate was seen, but this occurred above 10 mM P_i. In the case of *E. coli* the threshold for *pho*-mediated control is much lower (below 1 mM) (Torriani 1990) and end-product inhibition by phosphate is more likely in the present case. Although the nature of the Bu_3-*P* transporter is unknown, it probably operates outside the *pho* regulon. Degradation of Bu_2-*P* was more rapid than that of Bu_3-*P*, suggesting that uptake of Bu_3-*P* could be the rate-limiting step. Other studies have shown that strains of *Pseudomonas* isolated from the mixed culture are ampicillin-resistant (Thomas et al. 1997), and that growth in the presence of ampicillin stabilises the ability of the pure isolates to grow at the expense of Bu_3-*P*; this property is otherwise very unstable (Thomas et al. 1997a). Stable growth was associated with the presence of an approximately 22- to 24-kb fragment of DNA isolated by two methods but the function was not assigned (Thomas et al. 1997a,b).

The biphasic degradation of Bu_2-*P* (Fig. 3) could suggest that time is required for a cellular adaptation. The data could also indicate the initial biosorption of Bu_2-*P* (0.2–0.3 mM) onto the cells, but the release of an equivalent amount of phosphate suggests otherwise. It is possible that an antiport system is operating, whereby the uptake of Bu_2-*P* occurs concomitantly with a phosphate efflux (antiport) from the cells; such an exchange has been reported for other organophosphorus compounds (Cook 1989), while the GlpT system for glycerol phosphate uptake is known to be a P_i antiporter (Elvin et al. 1985; Ambudkar et al. 1986).

In addition to providing fundamental information on the physiology of Bu_3-*P* utilisation, this study also aimed to show a possible application to the treatment of metal-loaded wastewaters via generation of P_i as a precipitant ligand. The low pH and high metal and SO_4^{2-} content of uranium-bearing AMD waters could pose a problem for biological treatment.

The growing culture was sensitive to pH, with optimal growth at pH 7 (Table 2). However, the immobilised culture effectively remediated the AMD water at acidic pH. Possibly localised phosphate accumulation at the surface of the biofilm provided a buffer against the pH of the bulk solution. ENUSA water contains 35 mM SO_4^{2-}, which significantly reduces Bu_3-*P* biodegradation by free cells (Fig. 2). The water also contains a number of toxic heavy metals (Table 1), the combined effect of which could prevent Bu_3-*P* biodegradation. However, despite these factors, the immobilised biomass decontaminated the AMD water at pH 4.5. A previous study using *Citrobacter* liberating P_i from glycerol 2-phosphate showed the effective remediation of ENUSA waste water at pH values substantially below the optimum of the mediating phosphatase (Macaskie et al. 1997). The use of glycerol 2-phosphate as a phosphate donor would be economically unattractive, and this study shows that the culture of pseudomonads can achieve the same result at the expense of Bu_3-*P* hydrolysis. The process could be further improved using Bu_2-*P* as the phosphate donor, giving a greater rate of phosphate release (37 as opposed to 25 μmol h^{-1} mg $protein^{-1}$), but the extra cost of alkaline hydrolysis of Bu_3-*P* may counteract this. Partial neutralisation of the wastewater (to pH 4.5) is required. Bu_3-*P* could be introduced into more concentrated NaOH for partial hydrolysis and the hydrolysate then used for neutralisation of the water.

In conclusion, microcarrier-immobilised biomass provides an effective method for the removal of uranium from AMD water despite the poor prognosis offered by preliminary batch tests using free cells. The use of microcarriers may allow the localised accumulation of a layer of phosphate to shield the cells from metal toxicity, via their interception exterior to the cell. Despite the higher capital outlay, microcarriers may prove to be a superior immobilisation support for use under aggressive conditions, and future studies will aim to quantify and evaluate these advantages.

Acknowledgements The authors wish to thank Pharmacia AB, Sweden for the gift of microcarriers and ENUSA and Dr. M.G. Roig, Spain (funded by the European Community: contract EV5V-CT 93-0251) for the acid mine waste water. The support of the BBSRC (studentship 93302170 to R.A.P.T.) is acknowledged, with thanks.

References

Ambudkar SV, Larson TH, Maloney PC (1986) Reconstitution of sugar phosphate transport systems of *Escherichia coli*. J Biol Chem 261: 9083–9086

Ashley NV, Pope NR, Roach DJW (1987) Feasibility study of the application of biotechnology to nuclear waste treatment. In: Department of the Environment: Commissioned Research on Radioactive Waste Management, DOE/RW/88.008

Belskii VE (1977) Kinetics of the hydrolysis of phosphate esters. Russian Chem Rev 46: 828–841

Brzoska P, Rimmele M, Brzostek K, Boos W (1994) The Ugp paradox: the phenomenon that glycerol 3-phosphate, exclusively transported by the *Escherichia coli* Ugp system, can serve as a sole source of phosphate but not as a sole source of carbon is due to *trans* inhibition of Ugp-mediated transport by phosphate. In: Torriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms. Cellular and molecular biology. ASM, Washington, pp 30–36

Cook A M (1996) Combined carbon and phosphorus or carbon and sulphur substrates. In: Hamer G, Egli T, Snozzi M (eds) Mixed and Multiple Substrates and Feedstocks. European Federation of Biotechnology, Braunschweig, pp 71–84

Dick RE, (1991) Microbial degradation of the herbicide glyphosphate, PhD thesis, Queens University, Belfast, UK

Elvin CM, Hardy CM and Rosenberg H (1985) Pi exchange mediated by the *glpT*-dependent *sn*-glycerol 3-phosphate transport system in *Escherichia coli*. J Bacteriol 161: 1054–1058

Ghisalba O, Kuenzi M, Ramos Tombo GM, Schar HP (1987) Microbial degradation and utilisation of selected organophosphorus compounds: strategies and applications. Chimia 41: 206–215

Jones CJ, Brown DA (1987) Science and technology of tributyl phosphate. 2. In: Shulz WW, Navratil JD (eds) Miscellaneous industrial uses. CRC, Boca Raton, Fla, pp 35–135

Kuno Y, Hina T, Akiyama T, Matsui M (1991) Simultaneous determination of tributyl phosphate and dibutyl phosphate in spent fuel reprocessing streams by gas chromatography. J Chromatogr 537: 489–493

Macaskie LE (1991) The application of biotechnology to the treatment of wastes produced from the nuclear fuel cycle: biodegradation and bioaccumulation as a means of treating radionuclide-containing streams. Crit Rev Biotechnol 11: 41–112

Macaskie LE, Dean ACR (1985) Uranium accumulation by immobilised cells of a *Citrobacter* sp. Biotechnol Lett 7: 457–462

Macaskie LE, Empson RM, Cheetham AK, Grey CP, Skarnulis AJ (1992) Uranium bioaccumulation by a *Citrobacter* sp. as a result of enzymatically mediated growth of polycrystalline HUO_2PO_4. Science 257: 782–784

Macaskie LE, Yong P, Doyle TC, Roig MG, Diaz M, Manzano T (1997) Bioremediation of uranium-bearing wastewater: biochemical and chemical factors influencing bioprocess application. Biotechnol Bioeng 53: 100–109

McKay HAC (1964) The physical chemistry of tri-*n*-butyl phosphate solutions. Belg Chem Ind 12: 1278–1285

Pierpoint WS (1957) The phosphatase and metaphosphatase activities of pea extracts. Biochem J 65: 67–76

Roig MG, Manzano T, Diaz M, Pascual MJ, Paterson M, Kennedy JF (1995) Enzymatically-enhanced extraction of uranium from biologically leached solutions. Int Biodeterior Biodegrad 35: 93–127

Rosenberg A, Alexander M (1979) Microbial cleavage of various organophosphorus insecticides. Appl Environ Microbiol 37: 886–891

Stoner DL, Tien AJ (1995) Method and compositions for the degradation of tributyl phosphate in chemical waste mixtures. US patent 543375

Thomas RAP, Macaskie LE (1996) Biodegradation of tributyl phosphate by naturally-occurring microbial isolates. Environ Sci Technol 30: 2371–2375

Thomas RAP, Greated A, Lawlor K, Bailey M, Macaskie LE (1997a) Stabilisation of tributyl phosphate-biodegradative ability of naturally-occurring pseudominads using ampicillin. Biotechnol Techniques 11: 781–785

Thomas RAP, Morby AP, Macaskie LE (1997b) The biodegradation of tributyl phosphate by naturally-occuring microbial isolates. FEMS Microbiol Lett 155: 155–159

Torriani A (1990) From cell membrane to nucleotides: the phosphate regulon in *Escherichia coli*. BioEssays 12: 371–376

Yong P, Macaskie LE (1995) Enhancement of uranium bioaccumulation by a *Citrobacter* sp. via enzymatically-mediated growth of polycrystalline $NH_4UO_2PO_4$. J Chem Technol Biotechnol 63: 101–108