

Article

Clinically Relevant *Acinetobacter* spp. Proliferate but Contribute Little to Phosphate Accumulation Under Enhanced Biological Phosphorus Removal-Simulating Conditions

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Featured Application

Understanding the persistence of opportunistic pathogens belonging to the *Acinetobacter baumannii*–*calcoaceticus* complex in enhanced biological phosphorus removal systems.

Abstract

Wastewater treatment plants (WWTPs) receive clinically relevant *Acinetobacter* species originating from hospitals and municipal sewage, yet their role in enhanced biological phosphorus removal (EBPR) remains unclear. This study evaluated the capacity of *Acinetobacter* spp. to accumulate orthophosphate (P) under conditions simulating the aerobic phase of EBPR and investigated the physiological and molecular determinants of P accumulation. Clinically relevant *A. pittii* and *A. baumannii* exhibited comparable growth to the model P-accumulating species *A. junii* and successfully proliferated in synthetic wastewater. However, *A. junii* maintained significantly higher P uptake rates, accumulated more intracellular polyP granules, and continued to remove P after entering the stationary phase, whereas P removal by *A. pittii* and *A. baumannii* reached a plateau. While *A. junii* and *A. pittii* contained a single *ppk1* gene, two distinct *ppk1* homologues and the corresponding PPK1 isoforms were identified in *A. baumannii*. Nevertheless, neither *ppk1* copy number, *ppk1* expression, nor the abundance of key enzymes involved in canonical polyP metabolism explained the enhanced P accumulation capacity of *A. junii*. Proteomic analyses suggested that continued P uptake in *A. junii* was associated with physiological adaptations that support the maintenance of cellular fitness during the stationary phase. Consequently, EBPR systems may favour the persistence and proliferation of clinically relevant *Acinetobacter* spp. without substantially benefiting from their contribution to P removal.

Keywords: *Acinetobacter baumannii*; *Acinetobacter junii*; *Acinetobacter pittii*; polyphosphate kinase (PPK); polyphosphate; wastewater



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1. Introduction

Polyphosphate (polyP) granules are widespread intracellular inclusions found in numerous bacterial taxa. Historically, this phenomenon was first recognized in the field of clinical microbiology, the earliest developed branch of microbiological research. PolyP granules were described in *Corynebacterium diphtheriae* by Babeş at the end of the 19th century and later became a characteristic cytological feature used in the microscopic identification of diphtheria bacilli through the classical Neisser staining method [1,2].

The environmental significance of bacterial polyP accumulation became evident in the second half of the 20th century, when increasing orthophosphate (P) loads from municipal and industrial wastewaters were recognized as a major cause of eutrophication in aquatic ecosystems. The need to limit P discharge stimulated the development of advanced wastewater treatment technologies capable of removing dissolved P before effluent release into the environment. In the 1970s, activated sludge microorganisms were found to remove P from wastewater through intracellular polyP storage [3,4]. This discovery led to the development of enhanced biological phosphorus removal (EBPR), a tertiary wastewater treatment process that exploits the ability of microorganisms to remove dissolved P from wastewater and retain it within the biomass.

In the EBPR technology, biologically treated wastewater is subjected to alternating anaerobic and aerobic conditions. During the anaerobic phase, polyP-accumulating microorganisms take up readily available carbon sources and store them intracellularly as polyhydroxyalkanoates. The energy required for this process is obtained through partial hydrolysis of intracellular polyP, resulting in the release of P into the surrounding medium. During the subsequent aerobic phase, the stored polyhydroxyalkanoates are utilized for bacterial energy generation and multiplication, enabling the uptake of P in amounts exceeding those previously released. The excess P is then stored intracellularly as polyP granules, resulting in net P removal from the treated wastewater [5,6].

Among the microorganisms associated with EBPR, members of the genus *Acinetobacter* attracted particular attention because of their exceptional capacity for P accumulation and their ability to be readily cultivated under laboratory conditions. Early studies identified *Acinetobacter* spp. among the most efficient P-accumulating bacteria isolated from activated sludge, with intracellular P contents exceeding 10% of cell dry mass [4,7]. Although the central role of *Acinetobacter* in EBPR became less certain following the introduction of culture-independent methods that revealed the complexity of P-accumulating populations in activated sludge [6], *Acinetobacter* remains one of the most extensively studied model organisms for investigating bacterial P accumulation. Its robust growth in pure culture and pronounced polyP accumulation capacity continue to make it a valuable model for studying the physiology and molecular basis of bacterial P storage.

Recent studies have renewed interest in polyP metabolism in clinically important *Acinetobacter* species. In *A. baumannii*, polyP has been implicated not only in P storage but also in bacterial persistence and pathogenicity [8]. Deficiency of *ppk1* gene resulted in reduced tolerance to antibiotic, oxidative, heat, and starvation stress, as well as impaired motility, biofilm formation, and host inflammatory responses. These findings indicate that polyP reserves represent multifunctional intracellular structures that contribute to bacterial adaptation under both environmental and host-associated conditions.

The synthesis and turnover of polyP are primarily mediated by polyphosphate kinase (PPK1), polyphosphate phosphotransferase (PPK2), and exopolyphosphatase (PPX). PPK1 catalyses the synthesis of polyP from ATP and is considered the principal enzyme responsible for polyP accumulation in bacteria. In contrast, PPK2 utilizes polyP as a P donor for nucleotide phosphorylation, whereas PPX hydrolyses polyP chains and releases P. The importance of this enzymatic machinery has been demonstrated in both EBPR [9,10] and clinical pathogens [8,11,12].

A. baumannii and *A. pittii* belong to the clinically important *Acinetobacter calcoaceticus*–*baumannii* complex. *A. baumannii* has attracted considerable attention because of its rapid acquisition of antimicrobial resistance, whereas *A. pittii* has only recently emerged as a recognized opportunistic pathogen and remains comparatively understudied despite its increasing clinical importance [13]. Both species can disseminate from infected or colonized patients through hospital and urban sewage systems to wastewater treatment plants

(WWTP) [14,15]. *A. baumannii* survives conventional wastewater treatment processes, while its partial removal from wastewater occurs primarily through incorporation into activated sludge flocs [15,16]. Even chlorination of effluent does not eliminate this opportunistic pathogen [17]. Consequently, dissemination of *A. baumannii* from hospital patients to natural environments through urban wastewater systems has been documented [18].

This raises the question of whether the human pathogens *A. baumannii* and *A. pittii*, upon entering WWTPs, can contribute to biological P removal during tertiary wastewater treatment. The aim of this study was to investigate the capacity of different *Acinetobacter* strains to remove P under conditions simulating the aerobic phase of EBPR. Multiplication, P uptake, intracellular polyP granule formation, *ppk1* expression, and the abundance of proteins involved in polyP metabolism were compared among representatives of *A. junii*, *A. pittii*, and *A. baumannii*. The study further aimed to identify physiological and molecular characteristics associated with efficient biological P removal within the genus *Acinetobacter*.

2. Materials and Methods

2.1. Bacterial Strains

For the P-accumulation assay, three *Acinetobacter* species were chosen. *A. junii* DSM1532 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. This strain corresponds to NRRL B-8058, originally described in U.S. Patent No. 3,980,557 [19] as *A. phosphatovorans*, associated with P removal from wastewater. It was subsequently included within the broadly defined *A. calcoaceticus* group and later reassigned to *A. junii* following taxonomic revision of the genus.

A. pittii IN3 was isolated in 2014 from urban sewage entering the WWTP in Zagreb, Croatia. Although this species is a potential human pathogen, this strain exhibits a susceptible phenotype to all clinically relevant antibiotics [14].

A. baumannii Sava4 was recovered in 2015 from the Sava River water in Croatia, downstream of the Zagreb WWTP discharge. The strain was characterized as extensively drug-resistant and belongs to sequence type ST-195 within international clonal lineage 2 (IC2). It was closely related to clinical strains [18].

A. baumannii RUH134 was recovered in 1982 from human urine during the nosocomial outbreak at Rotterdam University Hospital, the Netherlands. This multidrug-resistant strain belongs to ST-348 and has been widely used as a reference strain for IC2 [20].

2.2. Orthophosphate Uptake Assay

Bacterial strains were pregrown on nutrient agar plates at 37 °C/24 h. One 10 µL loop of each culture was separately suspended by vortexing in 9 mL of sterile physiological solution. One mL of each suspension was added into bottles containing the 200 mL of synthetic wastewater.

Synthetic wastewater was used to provide standardized and reproducible cultivation conditions, as the composition of wastewater effluents varies substantially over time and among WWTPs. The composition of the synthetic wastewater, designed to simulate the wastewater entering an EBPR system, was as follows (in mg/L of distilled water): Na-propionate 300; peptone 100; MgSO₄ 10; CaCl₂ 6; KCl 30; yeast extract 20; KH₂PO₄ 88 [21]. The pH of the synthetic wastewater was adjusted to 7.00 ± 0.02 with 1M NaOH or 1M HCl before autoclaving (121 °C/20 min). The bottles were sealed and continuously aerated with filtered air through a central opening during incubation at 24 °C for 48 h. The experimental setup was designed to simulate selected aerobic aspects of EBPR and did not include the anaerobic-aerobic cycling characteristic of full-scale EBPR systems. A bottle without bacterial inoculum served as an abiotic control.

For preliminary cultivation experiments, bacteria were grown in nutrient broth (Biolife, Milan, Italy), containing beef extract (3 g/L) and peptone (5 g/L), under the same incubation conditions as described for synthetic wastewater.

Concentration of P and viable bacteria were followed during 48 h of incubation. The P concentration was determined using a DR/2500 Hach spectrophotometer (Hach Company, Loveland, CO, USA) and the molybdovanadate method, with a measurement error of ± 0.1 mg P-PO₄/L. Bacterial concentration was determined as colony forming units (CFU/mL) after incubation of serially diluted samples on nutrient agar plates (Biolife, Milan, Italy) at 37 °C/24 h. The P uptake assay was performed in biological triplicate, with each sample analysed in technical triplicate for both P concentration and CFU counts.

Intracellular polyP granules were visualized by Neisser staining under oil immersion microscopy, appearing dark blue against a yellow-stained cytoplasm. Additionally, polyP granules were detected by DAPI fluorescence microscopy as green fluorescent signals, while DNA appeared blue.

2.3. Whole-Genome Sequencing of *Acinetobacter* spp.

Bacterial strains were incubated at 37 °C for 16 h in nutrient broth, after which bacterial biomass was harvested by centrifugation. High-molecular-weight genomic DNA was extracted using the MagBio HighPrep HMW DNA Kit (MagBio Genomics, Gaithersburg, MD, USA) according to the manufacturer's instructions. DNA concentration was measured using a Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA), and DNA integrity and purity were assessed by agarose gel electrophoresis. DNA libraries were prepared using the EXP-NBD114.24 kit and SQK-LSK114 Ligation Sequencing Kit (Oxford Nanopore Technologies, Oxford, UK) and sequenced on the Oxford Nanopore PromethION platform by Novogene GmbH.

Basecalling of raw sequencing data was performed using Guppy v6.4.2 (Oxford Nanopore Technologies), followed by demultiplexing according to sample-specific barcodes. Sequencing statistics and read quality were assessed using NanoPlot v1.29.1 [22] and reads with quality scores above Q7 were retained for further analysis. High-quality reads were assembled de novo using Unicycler v0.4.8 [23]. Chromosomal and plasmid sequences were identified, and chromosomal assemblies were circularized. Protein-coding sequences were predicted using GeneMarkS v4.17 [24]. Quality-filtered reads were analysed using the PubMLST database (<https://pubmlst.org/>, accessed on 11 March 2026) to determine sequence types (STs). Acquired antimicrobial resistance genes were identified using ResFinder (<https://genepi.food.dtu.dk/resfinder/>, accessed on 12 March 2026).

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession numbers JBXOUS000000000, JBXOUT000000000, JBXOUV000000000, and JBXOUU000000000. Corresponding raw sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1436526.

2.4. RNA Extraction and RT-qPCR

The expression of the *ppk1* gene, responsible for polyP synthesis, was determined in cells from the logarithmic and stationary phases of growth in synthetic wastewater. In addition, the effect of cultivation medium on *ppk1* expression was assessed by comparing stationary-phase cultures grown in nutrient broth and synthetic wastewater.

After 8 h and 28 h of incubation at 24 °C in synthetic wastewater, bacterial biomass was harvested by centrifugation. Total RNA was extracted from bacterial cell pellets using the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). Cell pellets were lysed in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and mechanically disrupted by

bead beating (Zymo Research) using a high-speed homogenizer for 10 min. Following lysis, ethanol was added, and the lysate was processed according to the manufacturer's instructions. Genomic DNA was removed by on-column DNase I treatment, and RNA was eluted in RNase-free water. RNA concentration and purity were determined on a NanoDrop spectrophotometer (Thermo Scientific) and checked by agarose gel electrophoresis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed in a one-step amplification reaction using the Luna[®] Universal One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, USA), with 2 µL of diluted total RNA (1 ng/µL) used as the template for each sample. RT-qPCR cycling conditions consisted of reverse transcription at 55 °C for 10 min, initial denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 60 s. To confirm amplification specificity, melting-curve data were collected. PCR reactions were performed on a Mic Real-Time PCR Cycler (Bio Molecular Systems, Upper Coomera, QLD, Australia) and analysed using Workbench v2.0.3 software (Bio Molecular Systems).

Species-specific primers targeting the *ppk1* gene (Supplementary Table S1) were designed using the PrimerQuest tool (Integrated DNA Technologies, Coralville, IA, USA). In *A. baumannii* strains containing two *ppk1* genes, primers were designed to specifically amplify the *ppk1A* gene (GM002657 in RUH134 and GM002362 in Sava4, respectively). Primer specificity was confirmed using the NCBI Nucleotide BLAST tool (<https://blast.ncbi.nlm.nih.gov/> accessed on 12 May 2026). The high variability of the *ppk1* gene across *Acinetobacter* species precluded interspecies comparisons, as primer design had to be tailored to each species individually. Based on previous similar studies, the *clpX* gene, encoding the ATP-dependent ClpX protease subunit involved in cellular protein quality control, was used as the reference housekeeping gene for normalization of relative gene expression because of its stable constitutive expression [12].

All PCR reactions were carried out in three biological replicates, each with two technical replicates. No-template control reactions were included to monitor contamination. Statistical analysis of Pfaffl-corrected gene fold changes [25] was performed in R v4.3.3 [26] using Welch's *t*-test, with amplification efficiencies determined from individual amplification curves in Workbench v2.0.3 software (Bio Molecular Systems).

2.5. Proteomic Analysis and Identification of PPK1 Protein

In addition to transcriptional analysis of the *ppk1* gene, proteomic analysis was performed to confirm the presence of the corresponding PPK1 protein. Proteomic analysis was conducted on samples collected during the logarithmic and stationary phases of bacterial growth in synthetic wastewater, and the relative abundance of the PPK1 protein between these phases was determined.

Protein extraction was performed using an SDS-based lysis buffer (4% SDS, 100 mM Tris-HCl pH 8.0–8.5, 10 mM EDTA). Frozen bacterial pellets were resuspended in lysis buffer and lysed by sonication. Lysates were clarified by centrifugation at 16,000× *g* for 20 min at room temperature to prevent SDS precipitation, and the supernatant containing solubilized proteins was collected.

To ensure compatibility with LC-MS/MS analysis, proteins were precipitated using a chloroform/methanol precipitation method [27] to remove SDS. Protein pellets were subsequently resuspended in denaturing buffer (6M urea, 2M thiourea in 10 mM Tris buffer, pH 8.0), reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAA), and digested overnight with sequencing-grade trypsin (Promega, Madison, WI, USA) at 37 °C according to manufacturer instructions.

Resulting peptides were desalted and analysed by nanoUHPLC-MS/MS using a nanoUHPLC system (Easy-nLC 1200) coupled to a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operated in data-dependent acquisition (DDA) mode. Peptides were separated on a C18 analytical column (EASY-Spray™, 75 µm × 250 mm, 2 µm particle size, Thermo Scientific) using a linear gradient of increasing acetonitrile concentration in 0.1% formic acid. MS1 survey scans were acquired at high resolution, followed by MS/MS fragmentation of the most intense precursor ions.

Raw mass spectrometry data were processed in Proteome Discoverer 2.4 (Thermo Scientific), using Sequest HT as the search engine. Minimal peptide length was set to 6, maximal number of missed cleavage sites was set to 2. Carbamidomethylation was defined as static modification and methionine oxidation and methionine loss from protein N-terminus as dynamic modifications. Protein sequence databases were generated from WGS data of *Acinetobacter* spp. following gene prediction and functional annotation using the NCBI Prokaryotic Genome Annotation Pipeline (release 2025-05-06.build7983), resulting in predicted protein-coding sequences used for peptide and protein identification. Reversed protein sequences were used as decoy database, peptides were filtered at FDR = 0.05 and proteins were also identified at FDR level 0.05. For protein quantification label-free quantification approach was employed and values were normalized according to total peptide amount. *p*-values adjusted for multiple comparisons according to Benjamini–Hochberg procedure (*q*-values) were used to judge statistical significance. Change in protein expression between logarithmic and stationary phase was considered significant if corresponding *q*-value was lower than 0.05. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE [28] partner repository with the dataset identifier PXD080552.

2.6. Comparative Phylogenetic Analysis of *ppk1* Gene and Protein Sequences

Comparison of *ppk1* gene and protein sequences among the four studied *Acinetobacter* strains was conducted to assess their similarities. Phylogenetic analyses were performed separately using aligned *ppk1* nucleotide sequences and corresponding PPK1 amino acid sequences generated in our study to compare relationships at the gene and protein levels. Sequences were aligned using the MAFFT v7.505 software [29] with default parameters. Aligned *ppk1* nucleotide sequences were used to generate an initial Neighbor-Joining (NJ) phylogenetic tree from a maximum-likelihood distance matrix. The tree was subsequently optimised under the Jukes-Cantor substitution model using the ape v5.8.1 [30] and phangorn v2.12.1 [31] R packages. Branch support was assessed using 1000 bootstrap replicates, and the final tree was visualised as a phylogram with branch lengths proportional to substitutions per site. Aligned PPK1 amino acid sequences were analysed similarly, with phylogenetic tree optimisation performed using the LG amino acid substitution model.

3. Results

3.1. Differences in Orthophosphate Uptake Among *Acinetobacter* spp.

Four *Acinetobacter* strains successfully multiplied under conditions simulating the aerobic phase of EBPR, reaching the stationary growth phase after 24 h of incubation (Figure 1A). Bacterial concentrations increased by 1.5–1.8 log CFU/mL during the incubation period, indicating successful growth of all strains under EBPR conditions. Similar increases in viable bacterial concentrations were observed for *A. junii*, *A. pittii*, and both *A. baumannii* strains, indicating that the clinically relevant species multiplied as efficiently as the environmental strain *A. junii*.

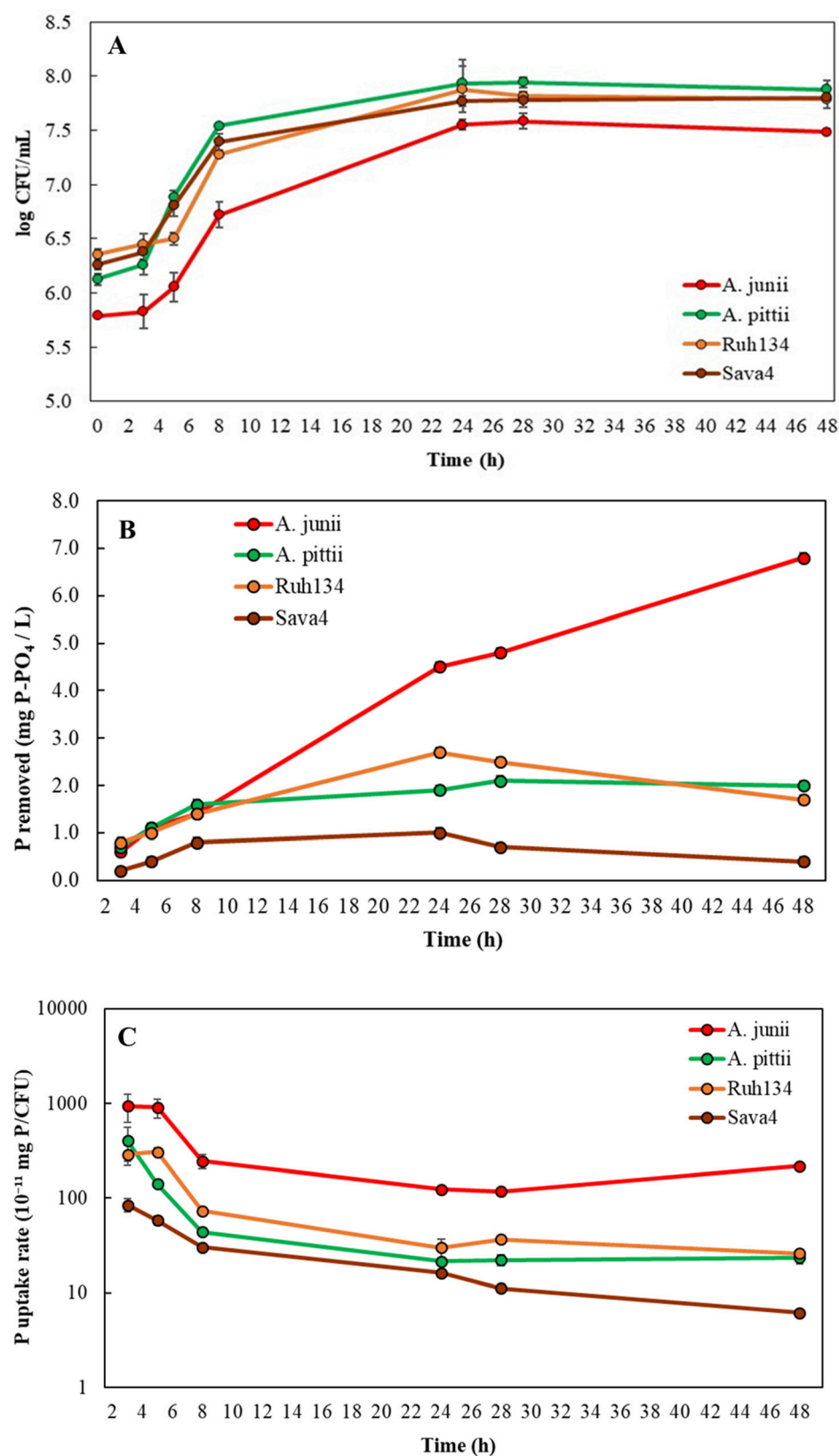


Figure 1. Viable cell counts (A), P removed from synthetic wastewater (B), and P uptake rates (C) of four *Acinetobacter* strains in a representative P uptake assay. $t_0(\text{P-PO}_4 \text{ (mg/L)}) = 22.9 \pm 0.1$. Final pH values varied from 6.54 to 6.65. Values represent means of technical triplicate measurements; standard deviations are shown.

In reactors inoculated with *A. pittii* and the two *A. baumannii* strains (Sava4 and RUH134), the amount of P removed from synthetic wastewater reached a plateau upon

entry into the stationary growth phase. In contrast, soluble P continued to be removed in the reactor inoculated with *A. junii* until the end of monitoring (Figure 1B). Consequently, the final net P removal achieved by *A. junii* was 3.4–17-fold higher than in the other strains. No decrease in soluble P concentration exceeding the measurement error of the method was observed in the abiotic control after 48 h of incubation. Given the comparable growth of all strains (Figure 1A), biomass-associated P assimilation alone is unlikely to account for the observed differences in P removal.

The P uptake rates were highest during the early stages of incubation and subsequently decreased in all cultures (Figure 1C). The decline was particularly pronounced in *A. baumannii* Sava4 strain, which exhibited the lowest P uptake rates throughout the experiment. Despite these temporal changes, *A. junii* maintained markedly higher P uptake rates than *A. pittii* and both *A. baumannii* strains. The P uptake rates of *A. junii* were significantly higher ($p < 0.05$) than those of the other strains during both the logarithmic (8 h) and stationary (28 h) growth phases, exceeding them by 3.4–8.2-fold and 3.2–10.6-fold, respectively (Figure 1C). The same strain-specific pattern was confirmed in three independent biological P uptake assays, and CFU-normalized P uptake rates from all assays are provided in Supplementary Figure S1.

Neisser staining confirmed a higher abundance of intracellular polyP granules visible as dark blue in cells of *A. junii* compared to *A. pittii* and *A. baumannii* (Supplementary Figure S2A), and this pattern remained consistent throughout the experiment. A similar trend was observed using DAPI fluorescence microscopy (Supplementary Figure S2B). These observations are consistent with the superior P removal performance of *A. junii*.

3.2. Genome Organisation of *Acinetobacter* spp.

To investigate the genetic basis underlying differences in the behaviour of *Acinetobacter* strains under EBPR-mimicking conditions, WGS was performed. The acquired antimicrobial resistance determinants were identified in *A. baumannii* and *A. pittii*, but not in *A. junii* (Table 1).

Table 1. Distribution of acquired antimicrobial and disinfectant resistance genes identified by whole-genome sequencing in four *Acinetobacter* strains. The *bla*_{OXA-66} gene is an intrinsic OXA-51-like β -lactamase in *A. baumannii*.

Antibiotic/ Disinfectant Class	<i>A. junii</i> DSM1532	<i>A. pittii</i> IN3	<i>A. baumannii</i> Sava4	<i>A. baumannii</i> RUH134
β -lactam	-	<i>bla</i> _{OXA-421} , <i>bla</i> _{ADC-25}	<i>bla</i> _{OXA-23} , <i>bla</i> _{ADC-25} , <i>bla</i> _{OXA-66}	<i>bla</i> _{ADC-25} , <i>bla</i> _{OXA-66}
Aminoglycosides	-	-	<i>aac</i> (3)-Ia, <i>aph</i> (3')-Via, <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib, <i>aadA1</i>	<i>aac</i> (3)-Ia, <i>aac</i> (6')-Im, <i>aph</i> (6)-Id, <i>aph</i> (3'')-Ib
Tetracyclines	-	-	<i>tet</i> (B)	<i>tet</i> (B)
Sulfamethoxazole	-	-	<i>sul1</i>	-
Chloramphenicol	-	-	<i>catA1</i>	-
Spectinomycin	-	-	<i>aadA1</i>	-
Quaternary ammonium compounds	-	-	<i>qacE</i>	-

The environmental strain *A. baumannii* Sava4 exhibited a markedly higher level of resistance compared to the reference strain *A. baumannii* RUH134. The detected antimicrobial resistance genes were consistent with previously reported phenotypic resistance of this

strain [18] to clinically relevant antibiotics according to EUCAST criteria [32], including carbapenems, aminoglycosides, tetracyclines, and trimethoprim/sulfamethoxazole. In addition, the presence of chromosomally located *qacE* gene suggests potential resistance to quaternary ammonium compounds commonly used as disinfectants. In *A. pittii*, acquired resistance genes were detected, but these were not associated with clinically relevant carbapenem resistance in the absence of overexpression or accompanying permeability defects.

The WGS revealed that *A. pittii* harbours a single plasmid pML4_1 (101.66 kb), while *A. baumannii* Sava4 contains two plasmids pA0292-2 (26.18 kb) and pII_RAB9 (71.29 kb). Of all detected resistance genes, only the aminoglycoside resistance gene *aph(3')-VIa* was located on the pII_RAB9 plasmid, indicating its potential contribution to the horizontal transfer of aminoglycoside resistance.

The *ppk1* gene was present in all strains; however, sequence variability among strains indicates genetic heterogeneity within the studied *Acinetobacter* spp. (Figure 2A,B).

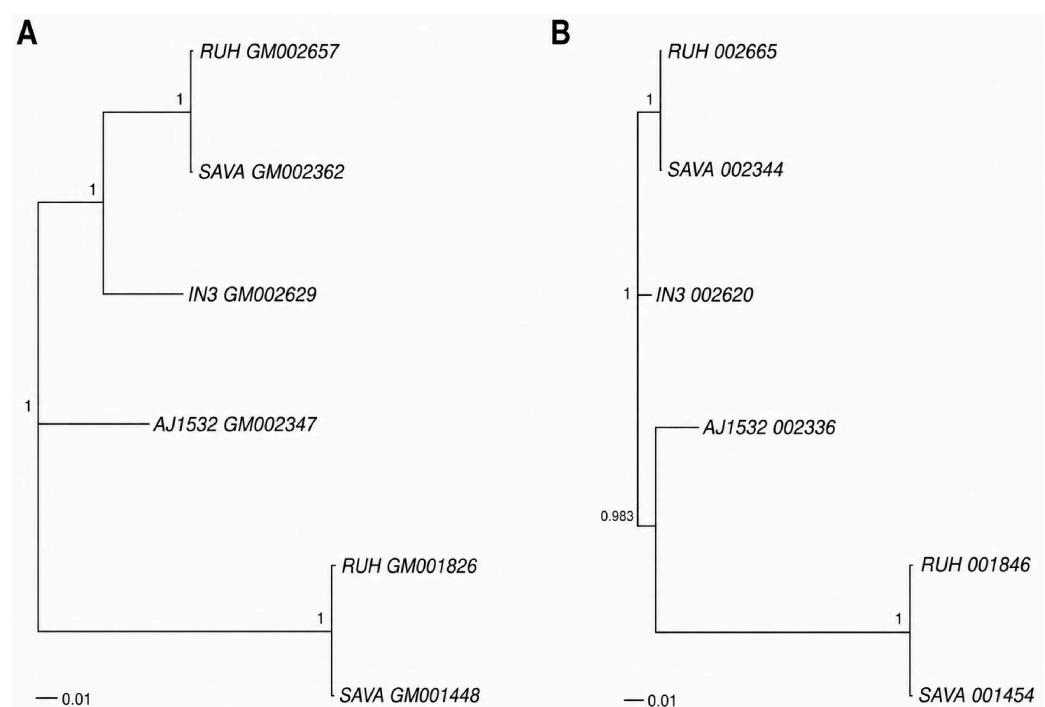


Figure 2. Comparative phylogenetic analysis of *ppk1* genes (A) and PPK1 amino acid sequences (B) among the studied *Acinetobacter* strains. Trees are presented as phylograms with branch lengths proportional to substitutions per site. *A. baumannii* strains Sava4 (SAVA) and RUH134 (RUH) each contained two *ppk1* genes, whereas *A. junii* (AJ1532) and *A. pittii* (IN3) contained a single *ppk1* gene. Bootstrap support values are shown at internal nodes. Gene and protein identifiers are indicated next to strain labels.

No mobile genetic elements were identified in the genomic regions flanking the *ppk1* genes. *A. junii* and *A. pittii* harboured a single *ppk1* gene, whereas two distinct *ppk1* sequences were identified in both *A. baumannii* strains. These two *ppk1* homologues shared 66.5% sequence identity.

The *ppk1* genes GM002657 and GM002362 from *A. baumannii* RUH134 and *A. baumannii* Sava4, respectively, were more similar to *ppk1* from *A. junii* and *A. pittii* than to their corresponding homologues. Pairwise sequence comparison using EMBOSS Needle showed that two *ppk1* sequences (GM002657 and GM002362) identified in this study shared 98.8% similarity with the *ppk1A* gene described by Roberge et al. [12]. The remaining two sequences (GM001826 and GM001448) exhibited 98.2% similarity to *ppk1B* reported in the

same study. Based on these results, the corresponding genes and proteins were designated as *ppk1A*/PPK1A and *ppk1B*/PPK1B, respectively.

3.3. Expression of *ppk1* Gene

The P uptake assay demonstrated that *A. junii* continued to remove P from synthetic wastewater during the stationary growth phase, whereas P removal by *A. pittii* and both *A. baumannii* strains reached a plateau upon entry into the stationary growth phase. Because *ppk1* encodes the principal enzyme responsible for bacterial polyP synthesis and is therefore considered a key gene in biological P removal, relative *ppk1* expression was examined in logarithmic and stationary growth phases. Owing to the high sequence variability of *ppk1* among *Acinetobacter* species and the use of species-specific primers (Supplementary Table S1), expression levels could only be compared between growth phases within individual strains and not among species. No significant differences in *ppk1* expression were observed between the logarithmic and stationary growth phases in any of the investigated strains during incubation in synthetic wastewater (Figure 3).

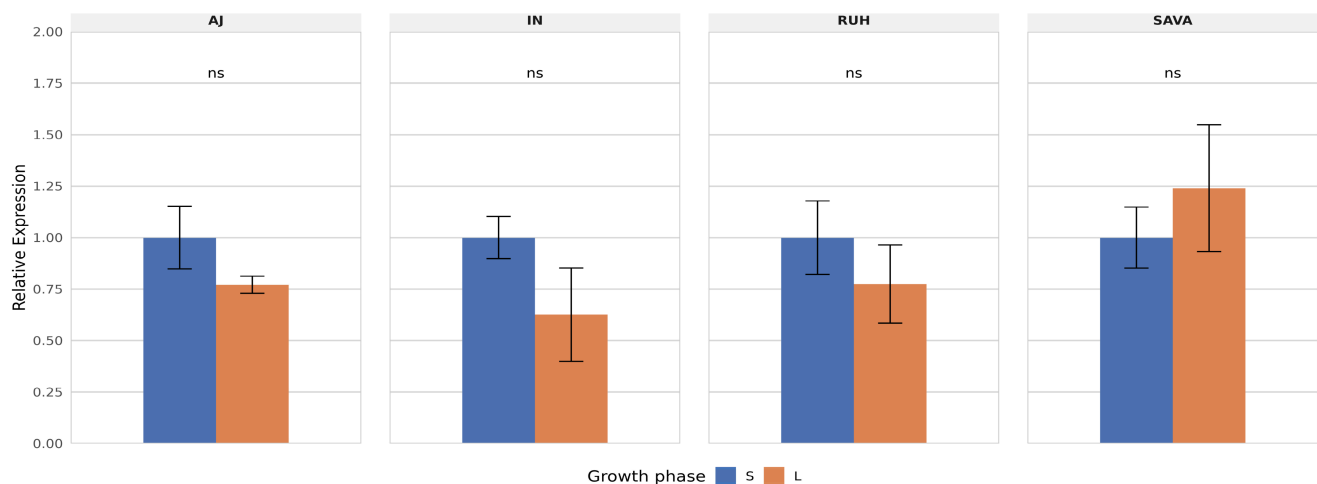


Figure 3. Relative *ppk1* gene expression in logarithmic (L) and stationary (S) growth phases of four *Acinetobacter* strains. Expression levels were determined by RT-qPCR in *A. junii* DSM1532 (AJ), *A. pittii* IN3 (IN), *A. baumannii* Sava4 (SAVA) and *A. baumannii* RUH134 (RUH). Relative expression values were normalized to the stationary phase (S = 1). Error bars represent the standard error of the mean. Statistical significance between growth phases was assessed using Welch's *t*-test. ns, not significant.

However, comparison of stationary-phase cultures grown in synthetic wastewater and nutrient broth revealed significantly higher *ppk1* expression in *A. pittii* and both *A. baumannii* strains cultivated in nutrient broth (Supplementary Figure S3). In contrast, no significant differences in *ppk1* expression between the two cultivation media were observed in *A. junii*.

3.4. Proteomic Analysis and Identification of PPK1 Protein

LC-MS/MS analysis identified more than 2000 distinct proteins in all four examined *Acinetobacter* strains during both logarithmic and stationary growth phases in synthetic wastewater. Proteomic analysis confirmed the presence of two distinct PPK1 protein variants in both *A. baumannii* strains (Table 2), consistent with the identification of two putative *ppk1* genes by WGS analysis (Figure 2A,B).

As PPK1, PPK2, and PPX represent key enzymes involved in polyP accumulation and turnover, their relative abundance was further examined. The abundance of PPK1 remained unchanged between logarithmic and stationary growth phases in all examined strains, consistent with the stable *ppk1* transcription levels determined by RT-qPCR. Likewise, no

significant differences were observed in the abundance of PPK2 or PPX between growth phases (Table 2).

Table 2. Relative abundance of proteins involved in polyphosphate metabolism (PPK1, PPK2 and PPX) during stationary and logarithmic growth phases of *Acinetobacter* spp. Abundance is expressed as the ratio between stationary and logarithmic phases. Adjusted *p*-value (*q*-value) < 0.05 was considered significant. Both species of *A. baumannii* are endowed with two genes encoding the PPK1.

Species, Isolate	Protein	Abundance Ratio (stac/log)	<i>q</i> -Value
<i>A. junii</i> DSM1532	PPK1	1.297	0.714
	PPK2	1.011	0.997
	PPX	1.097	0.997
<i>A. pittii</i> IN3	PPK1	1.361	0.812
	PPK2	1.187	0.950
	PPX	0.966	0.997
<i>A. baumannii</i> Sava4	PPK1A	1.175	0.940
	PPK1B	1.406	0.930
	PPK2	1.530	0.810
	PPX	0.724	0.961
<i>A. baumannii</i> RUH134	PPK1A	0.997	0.998
	PPK1B	0.795	0.584
	PPK2	1.022	0.998
	PPX	0.917	0.946

Because no significant differences were detected in the abundance of proteins directly associated with polyP metabolism, proteins showing differential abundance between logarithmic and stationary growth phases were further examined. In *A. junii*, the stationary growth phase was associated with increased abundance of proteins involved in oxidative stress response, protein quality control, respiratory metabolism, glutamate metabolism, and DNA repair, including ATP-dependent chaperone ClpB, catalase/peroxidase HPI, NADH-quinone oxidoreductase subunits NuoG and NuoF, glutamate synthase, and A/G-specific adenine glycosylase ($q < 0.05$).

In *A. pittii*, proteins involved in redox metabolism and regulatory processes, including ferredoxin FdxA, NAD(P)/FAD-dependent oxidoreductases, LysR-family transcriptional regulators, and sensor histidine kinases, were more abundant during the stationary phase. In contrast, proteins associated with siderophore biosynthesis, phenylacetate degradation, and nutrient uptake were less abundant ($q < 0.05$).

In *A. baumannii* strain Sava4, the stationary phase was characterized by increased abundance of proteins involved in nutrient acquisition, membrane transport, and transcriptional regulation, including TonB/Exb-dependent transport systems, ferric acinetobactin transporter BauC, and regulators belonging to the MarR, TetR, MerR, and AraC families. Proteins involved in amino acid transport, ammonium uptake, phenylacetate degradation, and several catabolic pathways were less abundant during the stationary phase ($q < 0.05$).

Similarly, *A. baumannii* RUH134 exhibited increased abundance of proteins associated with nutrient acquisition, membrane transport, and stress adaptation, including TonB-dependent receptors, TonB/Exb energy-transduction proteins, dicarboxylate transport systems, and TetR- and GntR-family regulators. In contrast, proteins involved in

siderophore biosynthesis, phenylacetate degradation, chemotaxis, and several transport systems were less abundant during the stationary phase ($q < 0.05$).

4. Discussion

Emerging nosocomial pathogens, particularly *A. baumannii*, have been repeatedly detected throughout wastewater treatment systems worldwide, including raw sewage, activated sludge, secondary effluents, and biosolids [15,16]. The occurrence of *A. pittii* in municipal wastewater has also been documented [14]. Although historically overshadowed by *A. baumannii*, *A. pittii* has recently attracted increased attention following its recognition as an emerging cause of non-*baumannii* *Acinetobacter* infections and hospital outbreaks worldwide [13]. The ability of these opportunistic pathogens to survive wastewater treatment processes has raised concerns regarding their environmental dissemination and the spread of antimicrobial resistance determinants.

While numerous studies have investigated the occurrence, persistence, and antimicrobial resistance profiles of clinically relevant *Acinetobacter* spp. in WWTPs, little attention has been paid to their potential physiological role within the treatment process itself. In particular, no previous study has examined whether clinically relevant *Acinetobacter* strains entering WWTPs can contribute to EBPR, a process driven by environmental P-accumulating organisms. The present study therefore addressed the largely unexplored link between the environmental fate of clinically relevant *Acinetobacter* spp. and their capacity for P accumulation under conditions simulating tertiary wastewater treatment.

A. junii as well as *A. pittii* and *A. baumannii* successfully multiplied under conditions simulating the aerobic phase of EBPR. These findings support previous observations that *A. baumannii* can persist under various conditions encountered throughout wastewater treatment processes [15,16] and even multiply in secondary wastewater effluent [33]. Therefore, the aerobic phase of EBPR is unlikely to limit clinically relevant *A. pittii* and *A. baumannii*, whose populations may not only survive but also proliferate during tertiary wastewater treatment.

WGS revealed that the environmental strain *A. baumannii* Sava4, recovered in 2015, harboured substantially more acquired antimicrobial and disinfectant resistance genes than the reference IC2 strain RUH134 isolated in 1982, illustrating the remarkable genomic plasticity of *A. baumannii*. Two *ppk1* homologues (*ppk1A* and *ppk1B*) and the corresponding isoforms (PPK1A and PPK1B) were detected in both examined *A. baumannii* strains. The identified *ppk1* shared more than 98% sequence similarity with *ppk1A* and *ppk1B* described by Roberge et al. [12]. The occurrence of two *ppk1* homologues and the corresponding PPK1 isoforms in both examined *A. baumannii* strains, together with their previous detection in *A. baumannii* strain AB5075 by Roberge et al. [12], suggests that this dual *ppk1* arrangement may be a common feature of *A. baumannii*. However, no mobile genetic elements were identified in the genomic regions flanking either *ppk1* homologue, providing no evidence that the dual *ppk1* arrangement arose through horizontal gene transfer. The evolutionary origin and functional significance of this genetic arrangement remain unclear. Although both *A. baumannii* strains harboured two *ppk1* homologues, whereas *A. pittii* and *A. junii* contained a single *ppk1* gene, no apparent differences in growth were observed under EBPR-simulating conditions. These findings suggest that the presence of two *ppk1* homologues does not confer a growth advantage under the tested conditions. A similar observation was reported by Lv et al. [8], who found that deletion of *ppk1* did not impair growth of *A. baumannii*.

Despite comparable growth of all investigated strains, marked differences were observed in their capacity to remove P from synthetic wastewater. The superior P uptake capacity of *A. junii* cannot be explained solely by enhanced bacterial growth, as *A. pittii*

and both *A. baumannii* strains exhibited similar multiplication rates but substantially lower P uptake rates and fewer intracellular polyP granules. These observations are consistent with the historical description of *A. junii* as a bacterium capable of exceptionally efficient P accumulation under wastewater treatment conditions. Intracellular polyP granules in *A. junii* DSM1532 were reported to be detectable as early as the exponential growth phase [19], whereas in majority of other bacteria polyP accumulation is enhanced upon entry into the stationary growth phase or under stress conditions [11]. This characteristic may contribute to the superior P uptake phenotype observed in the present study. In contrast, *A. pittii* and both *A. baumannii* strains removed only limited amounts of P despite successful growth, suggesting that multiplication and P accumulation are not directly coupled traits within the genus *Acinetobacter*.

Under the continuously aerated conditions used in this study, substantial PPX activity was not expected, while PPK2 appears to play only a minor role in polyP granule accumulation [12]. Consequently, differences in P uptake among the investigated strains were expected to be associated primarily with *ppk1*, which encodes the principal enzyme responsible for bacterial polyP synthesis. However, neither *ppk1* transcription nor the abundance of the corresponding PPK1 protein differed significantly between logarithmic and stationary growth phases in any of the investigated strains. These findings indicate that during the aerobic phase of EBPR, the enhanced P accumulation phenotype of *Acinetobacter* spp. cannot be explained by the presence, transcription, or abundance of the canonical polyP enzymes involved in polyP synthesis. Similar observations have been reported in laboratory-scale EBPR systems, where deletion of *ppk* did not affect P removal efficiency [10].

The only measurable higher expression of *ppk1* was observed for *A. pittii* and both *A. baumannii* strains in nutrient broth compared to synthetic wastewater, while *A. junii* maintained stable expression in both media. Lv et al. [8] also found that *ppk1* is required for polyP accumulation that enhances persistence and virulence of *A. baumannii* in nutrient-rich LB medium. Nutrient broth is rich in nutrients containing primary organic P compounds, whereas synthetic wastewater contains mainly inorganic P. This may reflect the ecological association of pathogens from *A. calcoaceticus*–*baumannii* complex to nutrient-rich environment, while environmental bacterium *A. junii* is adapted to the diverse P sources present in wastewater.

Proteomic analysis revealed clear differences in stationary-phase physiology among the investigated *Acinetobacter* spp. In *A. junii*, proteins associated with oxidative stress protection, protein quality control, respiratory metabolism, glutamate metabolism, and DNA repair increased in abundance during the stationary phase. In contrast, *A. pittii* and both *A. baumannii* strains exhibited proteomic signatures more consistent with adaptation to nutrient limitation and resource conservation. These observations suggest that continued P accumulation depends on the overall physiological state of the cells rather than on changes in the abundance of enzymes directly involved in polyP metabolism.

The stationary phase is recognized as a dynamic physiological state characterized by active metabolic adaptation rather than cellular inactivity [34]. The continued P removal observed in *A. junii* during the stationary phase indicates that physiologically active cells continued to accumulate polyP despite the dynamic balance between cell division and cell death characteristic of this growth phase. In contrast, the stationary-phase proteomes of *A. pittii* and *A. baumannii* were more consistent with a transition towards resource conservation, which may limit further P accumulation. These findings indicate that sustained P uptake under conditions simulating the aerobic phase of EBPR is linked to the ability of cells to maintain physiological fitness during the stationary phase rather than to differential expression of polyP metabolism enzymes alone. The ability of *A. junii* to continue accumu-

lating polyP during the stationary phase may help explain its exceptional performance in full-scale EBPR systems.

The markedly lower P uptake capacity of *A. baumannii* and *A. pittii* compared with *A. junii* suggests that clinically relevant *Acinetobacter* spp. are unlikely to represent major contributors to biological P removal in wastewater treatment systems. Nevertheless, their ability to multiply under EBPR-simulating conditions indicates that WWTPs may support the persistence of clinically relevant *Acinetobacter* populations and serve as reservoirs of antimicrobial resistance determinants. Particularly concerning is the efficient growth of the extensively drug-resistant *A. baumannii* strain Sava4, demonstrating that even highly resistant lineages can proliferate under conditions encountered during tertiary wastewater treatment. However, clinically relevant *Acinetobacter* spp. are more likely to persist within activated sludge flocs than to become dominant members of the P-accumulating microbial community.

5. Conclusions

This study demonstrates that the tested *A. pittii* and *A. baumannii* strains can successfully multiply under EBPR-simulating conditions. However, despite comparable growth, they exhibited substantially lower P accumulation than *A. junii*, suggesting a limited contribution to P removal in WWTPs.

The superior P uptake capacity of *A. junii* could not be explained by differences in the number of *ppk1* homologues, *ppk1* transcription, or the abundance of the major enzymes involved in polyP metabolism. Instead, the results suggest that sustained P accumulation is associated with the ability of cells to maintain physiological fitness during the stationary phase.

The efficient growth of the extensively drug-resistant *A. baumannii* under EBPR-simulating conditions is of particular concern, as it indicates that tertiary wastewater treatment may support the persistence and proliferation of clinically relevant *Acinetobacter* spp. and their associated antimicrobial resistance determinants.

Consequently, WWTPs should be regarded not only as systems for nutrient removal but also as potential reservoirs for the environmental dissemination of clinically relevant opportunistic pathogens.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app16146852/s1>, Table S1: Primers used in this study; Figure S1: CFU-normalized P uptake rates of four *Acinetobacter* strains in three independent P uptake assays. Values represent means of biological triplicate measurements; standard deviations are shown; Figure S2. Intracellular polyP granules observed after 24 h of incubation using Neisser staining (A) and DAPI fluorescence microscopy (B) in *A. junii*, *A. pittii*, *A. baumannii* Sava4, and *A. baumannii* RUH134. Neisser staining revealed dark blue polyP granules, while DAPI staining showed green fluorescent signals corresponding to intracellular polyP. All micrographs were acquired at 1000× magnification using a 100× oil immersion objective; Figure S3. Relative *ppk1* gene expression in four *Acinetobacter* strains grown in synthetic wastewater (SW) and nutrient broth (NB). Expression levels were determined by RT-qPCR in *A. junii* DSM1532 (AJ), *A. pittii* IN3 (IN), *A. baumannii* Sava4 (SAVA), and *A. baumannii* RUH134 (RUH). Relative expression values were normalized to cells grown in synthetic wastewater (SW = 1). Error bars represent the standard error of the mean. Statistical significance between growth conditions was assessed using Welch's *t*-test. *, *p* < 0.05; ns, not significant.

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Abbreviations

The following abbreviations are used in this manuscript:

CFU	Colony Forming Units
DAPI	4',6-Diamidino-2-Phenylindole
EBPR	Enhanced Biological Phosphorus Removal
LC-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
polyP	Polyphosphate
PPK1	Polyphosphate Kinase 1
PPK2	Polyphosphate Kinase 2
PPX	Exopolyphosphatase
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
WGS	Whole-Genome Sequencing
WWTP	Wastewater Treatment Plant

References

1. Babes, V. Beobachtungen über die metachromatischen Körperchen, Sporenbildung, Verzweigung, Kolben- und Kapselbildung pathogener Bakterien. *Z. Hyg. Infekt.* **1895**, *20*, 412–437. [\[CrossRef\]](#)
2. Neisser, A. Eine neue Methode des Diphtherie-Bacillen Nachweises. *Cent. Bakteriolog.* **1897**, *22*, 697–700.
3. Barnard, J.L. Nutrient removal in biological systems. *Water Wastes Eng.* **1974**, *11*, 33–36.
4. Fuhs, G.W.; Chen, M. Microbiological basis of phosphate removal in activated sludge. *Appl. Microbiol.* **1975**, *29*, 424–434.
5. Mino, T.; van Loosdrecht, M.C.M.; Heijnen, J.J. Microbiology and biochemistry of the enhanced biological phosphate removal process. *Water Res.* **1998**, *32*, 3193–3207. [\[CrossRef\]](#)
6. Roy, S.; Guanglei, Q.; Zuniga-Montanez, R.; Williams, R.B.H.; Wuertz, S. Recent advances in understanding the ecophysiology of enhanced biological phosphorus removal. *Curr. Opin. Biotechnol.* **2021**, *67*, 166–174. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Sidat, M.; Bux, F.; Kusan, H.C. Phosphate accumulation by bacteria isolated from activated sludge. *Water SA* **1999**, *25*, 175–179.
8. Lv, H.; Zhou, Y.; Liu, B.; Guan, J.; Zhang, P.; Deng, X.; Li, D.; Wang, J. Polyphosphate kinase is required for the processes of virulence and persistence in *Acinetobacter baumannii*. *Microbiol. Spectr.* **2022**, *10*, e01230-22. [\[CrossRef\]](#) [\[PubMed\]](#)
9. McMahon, K.D.; Dojka, M.A.; Pace, N.R.; Jenkins, D.; Keasling, J.D. Polyphosphate kinase from activated sludge performing enhanced biological phosphorus removal. *Appl. Environ. Microbiol.* **2002**, *68*, 4971–4978. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Nan, Y.P.; Zhou, G.B.; Yuan, L.J. Function of polyphosphate kinase gene in biological phosphate removal during the wastewater treatment process. *Huan Jing Ke Xue* **2017**, *38*, 1529–1535. [\[PubMed\]](#)
11. Rao, N.N.; Gómez-García, M.R.; Kornberg, A. Inorganic polyphosphate: Essential for growth and survival. *Annu. Rev. Biochem.* **2009**, *78*, 605–647. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Roberge, N.; Neville, N.; Douchant, K.; Noordhof, C.; Boev, N.; Sjaarda, C.; Sheth, P.M.; Jia, Z. Broad-spectrum inhibitor of bacterial polyphosphate homeostasis attenuates virulence factors and helps reveal novel physiology of *Klebsiella pneumoniae* and *Acinetobacter baumannii*. *Front. Microbiol.* **2021**, *12*, 764733. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Bello-López, E.; Escobedo-Muñoz, A.S.; Guerrero, G.; Cruz-Córdova, A.; Garza-González, E.; Hernández-Castro, R.; Zarain, P.L.; Morfín-Otero, R.; Volkow, P.; Xicohtencatl-Cortes, J.; et al. *Acinetobacter pittii*: The emergence of a hospital-acquired pathogen analyzed from the genomic perspective. *Front. Microbiol.* **2024**, *15*, 1412775. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Hrenović, J.; Goić-Barišić, I.; Ganjto, M.; Kazazić, S.; Kovačić, A.; Skorić, D.; Šeruga Musić, M. Isolates of *Acinetobacter pittii* from municipal wastewater of the City of Zagreb, Croatia. In Proceedings of the 10th International Symposium on the Biology of *Acinetobacter*, Athens, Greece, 3–6 June 2015; p. 56.

15. Higgins, P.G.; Hrenović, J.; Seifert, H.; Dekic, S. Characterization of *Acinetobacter baumannii* from water and sludge line of secondary wastewater treatment plant. *Water Res.* **2018**, *140*, 261–267. [CrossRef] [PubMed]
16. Pulami, D.; Kämpfer, P.; Glaeser, S.P. High diversity of the emerging pathogen *Acinetobacter baumannii* and other *Acinetobacter* spp. in raw manure, biogas plants digestates, and rural and urban wastewater treatment plants with system specific antimicrobial resistance profiles. *Sci. Total Environ.* **2023**, *859*, 160182. [PubMed]
17. Karumathil, D.P.; Yin, H.B.; Kollanoor-Johny, A.; Venkitanarayanan, K. Effect of chlorine exposure on the survival and antibiotic resistance of *Acinetobacter baumannii* in water. *Int. J. Environ. Res. Public Health* **2014**, *11*, 1844–1854. [PubMed]
18. Šeruga Musić, M.; Hrenović, J.; Goić-Barišić, I.; Hunjak, B.; Škorić, D.; Ivanković, T. Emission of extensively-drug resistant *Acinetobacter baumannii* from hospital settings to the natural environment. *J. Hosp. Infect.* **2017**, *96*, 323–327. [PubMed]
19. University Patents Inc. Phosphorous Removal from Waste Water. U.S. Patent 3,980,557, 14 September 1976.
20. Nigro, S.J.; Hall, R.M. Antibiotic resistance islands in A320 (RUH134), the reference strain for *Acinetobacter baumannii* global clone 2. *J. Antimicrob. Chemother.* **2012**, *67*, 335–338. [CrossRef] [PubMed]
21. Hrenović, J.; Rožić, M.; Sekovanić, L.; Anić-Vučinić, A. Interaction of surfactant-modified zeolites and phosphate accumulating bacteria. *J. Hazard. Mater.* **2008**, *156*, 576–582. [CrossRef] [PubMed]
22. De Coster, W.; Rademakers, R. NanoPack2: Population-scale evaluation of long-read sequencing data. *Bioinformatics* **2023**, *39*, btad311. [PubMed]
23. Wick, R.R.; Judd, L.M.; Gorrie, C.L.; Holt, K.E. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* **2017**, *13*, e1005595. [CrossRef] [PubMed]
24. Besemer, J.; Lomsadze, A.; Borodovsky, M. GeneMarkS: A self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. *Nucleic Acids Res.* **2001**, *29*, 2607–2618. [PubMed]
25. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. [CrossRef] [PubMed]
26. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2021; Available online: <https://www.R-project.org/> (accessed on 12 March 2026).
27. Wessel, D.; Flüggé, U.I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **1984**, *138*, 141–143. [CrossRef] [PubMed]
28. Perez-Riverol, Y.; Bandla, C.; Kundu, D.J.; Kamatchinathan, S.; Bai, J.; Hewapathirana, S.; John, N.S.; Prakash, A.; Walzer, M.; Wang, S.; et al. The PRIDE database at 20 years: 2025 update. *Nucleic Acids Res.* **2025**, *53*, D543–D553. [PubMed]
29. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [CrossRef] [PubMed]
30. Paradis, E.; Schliep, K. ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* **2019**, *35*, 526–528. [PubMed]
31. Schliep, K.; Potts, A.J.; Morrison, D.A.; Grimm, G.W. Intertwining phylogenetic trees and networks. *Methods Ecol. Evol.* **2017**, *8*, 1212–1220. [CrossRef]
32. The European Committee on Antimicrobial Susceptibility Testing. *Breakpoint Tables for Interpretation of MICs and Zone Diameters*, Version 16.0; EUCAST: Växjö, Sweden, 2026.
33. Hrenović, J.; Goić-Barišić, I.; Kazazić, S.; Kovačić, A.; Ganjto, M.; Tonkić, M. Carbapenem-resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, 2014. *Euro Surveill.* **2016**, *21*, 30195. [CrossRef] [PubMed]
34. Navarro Llorens, J.M.; Tormo, A.; Martínez-García, E. Stationary phase in gram-negative bacteria. *FEMS Microbiol. Rev.* **2010**, *34*, 476–495. [CrossRef] [PubMed]

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