Phylotype Dynamics of Bacterial P Utilization Genes in Microbialites and Bacterioplankton of a Monomictic Endorheic Lake

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spaceAbstract Microbes can modulate ecosystem function since they harbor a vast genetic potential for biogeochemical cy- cling. The spatial and temporal dynamics of this genetic di- versity should be acknowledged to establish a link between ecosystem function and community structure. In this study, we analyzed the genetic diversity of bacterial phosphorus utiliza- tion genes in two microbial assemblages, microbialites and bacterioplankton of Lake Alchichica, a semiclosed (i.e., endorheic) system with marked seasonality that varies in nu- trient conditions, temperature, dissolved oxygen, and water column stability. We focused on dissolved organic phosphorus (DOP) utilization gene dynamics during contrasting mixing and stratification periods. Bacterial alkaline phosphatases (*phoX* and *phoD*) and alkaline beta-propeller phytases (*bpp*) were surveyed. DOP utilization genes showed different dy- namics evidenced by a marked change within an intra-annual period and a differential circadian pattern of expres-

spacesion. Although Lake Alchichica is a semiclosed system, this dynamic turnover of phylotypes (from lake circulation to strat- ification) points to a different potential of DOP utilization by the microbial communities within periods. DOP utilization gene dynamics was different among genetic markers and among assemblages (microbialite vs. bacterioplankton). As estimated by the system's P mass balance, P inputs and out- puts were

similar in magnitude (difference was <10 %). A theoretical estimation of water column P monoesters was used to calculate the potential P fraction that can be remineralized on an annual basis. Overall, bacterial groups including Proteobacteria (Alpha and Gamma) and Bacteroidetes seem to be key participants in DOP utilization responses.

Keywords Extracellular enzymes · DOP utilization · Phytase · P turnover · Phylotype seasonality · Microbial functional diversity

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Introduction

Dissolved organic phosphorus (DOP) is an abundant phos-phorus (P)

able 1Phylotype assignation of DOP utilization genes *phoD*, *phoX*, and *bpp*; richness and diversity estimators; as well as coverage for each group (microbialites and bacterioplankton) and period (circulation and stratification) are shown

	phoX (sequences *opp* (sequences obtained = 58)					
SCoverage (%)	OTUs	Chao	Shannon (*H'*)	Coverage (%)		
2 88	15	33	2.3527	ɜ 74		

fraction in aquatic systems, representing up to 80% of the total dissolved phosphorus in the water column of

oligotrophic systems [1, 2]. Yet DOP is the less known P fraction [3]. Organic phosphorus transformations occ

urefficiently in natural systems, as revealed by surface water rapid P-turnover rates [2, 4]. We know so far

rthat microbes are key components involved in reutilizing dissolved organic nutrient fractions [5, 6]; theref

ore, DOP utilization by microbes has significant implications for carbon uptake and export [7]. A recogni

zed DOP transformation process is the synthesis of extracellular phosphatases that become active to face Pst

rvation in the water column with P monoesters and diesters as the main substrates [8–11]. Another interesting

example for organic phosphorus utilization consists of phytate (myo-inositol hexakisphosphate or IP6) degrada

tion. Phytate represents approximately 75 to 80 % of the total phosphorus found in nature [12]. This molecul

e, abundant in vegetal tissues, accumulates in the soil but is a minor component of marine sediments, indicati

ng its efficient degradation/utilization in the water column [13]. Recently, a bacterial alkaline phytase (bee-

tap-propeller phytase, *bpp*) was identified in a variety of biological systems, revealing its vast potential

for phytate degradation [14, 15]. Phytases hydrolyze phytate into less phosphorylated myo-inositol residue

sand inorganic phosphates [16], and their activity as organic phosphate releases metals chelated by phytate (e.g., Fe^{+3}, Zn^{+2}

Microbial assemblage	Period	Cutoff	*phoD* (sequences obtained = 180)			
			OTUs	Chao	Shannon (*H'*)	
Microbialites	Circulation	0.05	23	0 58	2.51	80
	Stratification					
Bacterioplankton	Circulation					
	Stratification					

arting to understand the identen-tity of the microorganis ms that harbor P transformation genes [3, 18], the effect

ors that drive the presence, diversity, or expression of P transformation genes/proteins [10, 11, 18, 19], as

well as their spatial and temporal replacement or turnover [4, 20]. Remineralization patterns and rates may vary

ry due to shifts in bacterial assemblages [4, 21, 22], but the patterns and mechanisms of functional gene turn

overstill remain unclear. Here, we studied the DOP transformation potential in microbial lites and bacterioplan

kton of Lake Alchichica. Recent surveys in natural waters have shown that alkaline phosphatases (AP) *phoD* and

phoX are more abundant than *phoA*, the classical model to study alkaline phosphatases [10, 11]. Here, these two

bacterial alkaline phosphatases, *phoX* and *phoD*, and alkaline beta-propeller phytases, *bpp*, were surveyed in

environmental DNA and RNA (Table 1). Since shifts in the bacterial assemblages structure could affect remineral

ization patterns [4, 21], the dynamics of these calcium-dependent metallo enzymes was followed during lake circ

ulation and stratification periods, which offer contrasting physicochemical conditions within the system. A ph

osphorus mass balance was performed to frame P status a nd utilization in Lake Alchichica.

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Study Area

Alchichica is a maar lake located in the easternmost region of the Trans-Volcanic Belt, Puebla, Mexico (19° 24′ N, 97° 24′ W; 2,340 m a.s.l.). It is a saline and alkaline lake (salinity 8.5 g l^{-1}, pH 9.5) that exhibits a particular chemical signature where sodium is abundant [23–25], calcium concentration is particularly low (~0.3 mM), and Mg/Ca ratio is high. Alchichica's water column production seems to be limited mainly by nitrogen, although phosphorus and silica may alter- natively limit phytoplankton productivity [26, 27]. Lake Alchichica is warm-monomictic [28, 29]. Stratification period occurs from April to December and circulation, in the dry winter, from January to March; the water column (Z_{mean} = 40.9 m and Z_{max} = 62 m) mixes completely by the end of January or early February. Stratification and circulation pe- riods offer contrasting (intra-annual) physicochemical and nutrimental conditions [19] within the system (Fig. 1).

Two different microbial assemblages characterize Alchichica's microbiota: bacterioplankton and a littoral ring of microbialites. Macek et al. [30] found that picoplankton dynamics follow a regular pattern, linked to the hydrological cycle of this system. Nutrient availability relates to the tempo- ral pattern and the vertical zonation of the bacterioplankton components [31]. Two microbialite morphotypes can be found in Lake Alchichica (columnar and spongy morpholo- gy); their microbial composition shows high prokaryote diver- sity [32]. Microbialites have shown relevant activity related to the N cycle, such as high N_2 fixation rates [33, 34] as well as potential for DOP utilization through bacterial alkaline phos- phatases [19].

Environmental Characterization

Water samples from the littoral zone, central station of the lake with depths of 5 m (surface mixed layer), 25 m (metalimnion), and 61 m (1 m above the bottom)—detailed description of the thermal structure of Alchichica can be consulted in Macek et al. [30]—and from nearby wells were taken in 2011: during lake circulation (February) and the well-established stratifica- tion (August). Physicochemical characterization of the water column and the littoral zone (water surrounding the microbialites) included temperature, dissolved oxygen (DO), pH, and conductivity of the water column and of the water surrounding the microbialites, recorded with a YSI 6600 mul- tiparameter probe. Water samples for nutrient analysis includ- ed determinations of soluble reactive phosphorus (SRP), NO^-, NO^-, dissolved inorganic nitrogen (DIN), soluble re-

spacein polypropylene containers. Samples were analyzed (within 24 to 48 h) with a segmented flow Autoanalyzer (Skalar San-Plus) following the standard methods adapted by Grasshoff et al. [35] and the circuits indicated by Kirkwood [36]. Total P and N were analyzed following the methods proposed by Valderrama [37], and total particulate P (TPP) was calculated as the difference between total P − SRP (this represents the minimum benchmark of organic phosphorus since SRP con- tains some organic species).

Sampling

Microbialites and bacterioplankton for total DNA and RNA surveys were collected in both periods (circulation and strati- fication). The two morphologically different microbialites (spongy and columnar) were sampled in six stations (n =6 for each morphotype, at a depth of ∼0.30 m) along the lakes littoral zone. Subsamples of ∼10 g were excised and kept frozen (−20 °C) until DNA extraction. After extraction, DNA from all six stations was mixed to obtain a single pooled DNA sample for each microbialite morphotype per period.

At the central station of the lake, water column samples of 0.5 l (triplicates) were filtered through polycarbonate membranes (pore size 0.22 μm, Osmonics; Poretics Corp.) taken at the following water depths: 5 m (surface), 25 m (metalimnion), and 61 m (bottom). Filters were then collected and kept frozen (−20 °C) in DNA-free 2.0 ml tubes, until analysis.

Collection of samples for total RNA extraction was performed in a diel sampling scheme. Microbialite sections from all sampling stations (six microbialite samples) were incubat- ed at on-site conditions (light and temperature). Subsamples were taken (in RNAse-free surfaces) at 0600, 1200, 1800, and 2400 hours; placed in liquid nitrogen; and kept frozen at
−80 °C until analysis.

Nucleic Acid Extraction

DNA extraction was performed using the method of Zhou et al. [38] modified by Centeno et al. [32] in which samples of approximately 5 g of each microbialite were pulverized in liquid nitrogen with a buffer solution (100 mM Tris-HCl, 20 mM NaCl, 100 mM EDTA, pH 8) and 0.06 V of cetyl trimethylammonium bromide (CTAB). A total volume of
0.5 l of water was filtered in 0.22-μm membranes (Durapore, Millipore) to obtain total DNA from bacterioplankton samples. A volume of 3 ml of the extraction buffer was added and the mixtures were then incubated with lysozyme (30 mg ml^{-1}) (Sigma Aldrich, USA) at 37 °C for
30 min. A second incubation followed, adding proteinase K
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spaceactive Si (SRSi), total P (TP), and total N (TN). The samples were collected and filtered through 0.45- and 0.22-μm (HA Millipore™) mixed cellulose membranes, collected and held
space(10 mg ml^{-1}, Sigma Aldrich, USA) and 0.1 V of sodium dodecyl sulfate (SDS) at 55 °C, overnight. Incubations were then centrifuged (20 min, 1800*g*). The aqueous phase of each
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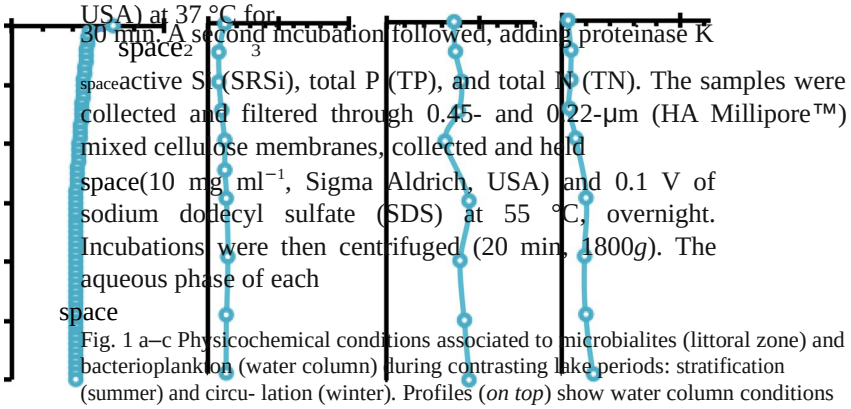
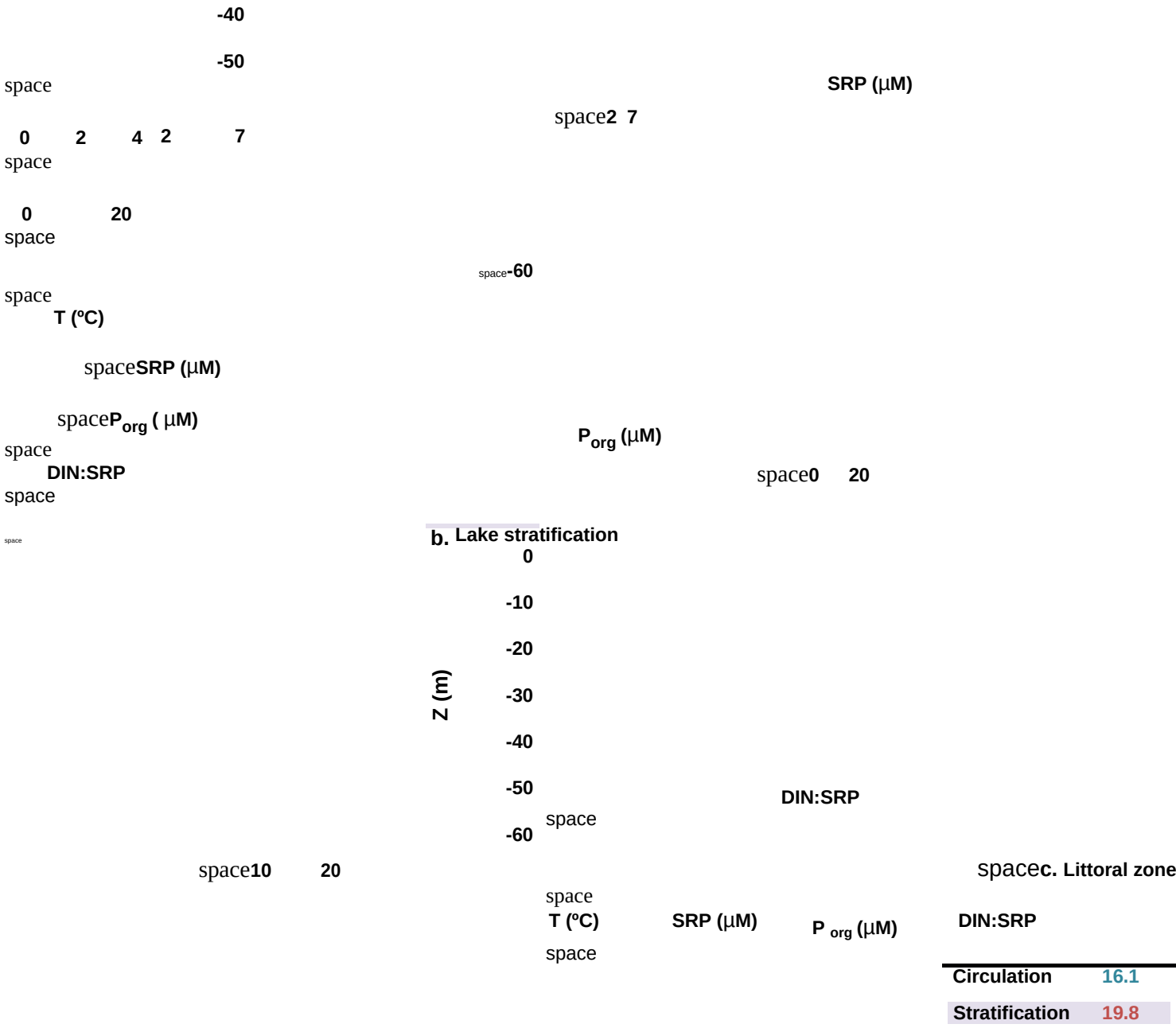


Fig. 1 a–c Physicochemical conditions associated to microbialites (littoral zone) and bacterioplankton (water column) during contrasting lake periods: stratification (summer) and circu- lation (winter). Profiles (*on top*) show water column conditions and a table (*below*) shows littoral zone ambient conditions



sample was carefully separated and extracted twice with a 25:24:1 solution of phenol/chloroform/isoamyl alcohol and once more with 24/1 chloroform/isoamyl alcohol. DNA pre- cipitation was conducted at −20 °C, adding 0.1 volume of sodium acetate (3 M), 2 volumes of 2-propanol, and 2 µl of GlycoBlue (Ambion Inc., USA). Precipitated DNA was washed twice with 80 % ethanol and resuspended in molecu- lar grade water. DNA samples were further purified with Mini Spin columns (DNeasy Blood & Tissue kit, Qiagen, Alameda, CA) following the instructions of the manufacturer and pre- cipitated again. Purified DNA was stored at −20 °C until analysis.

Total RNA was extracted from microbialite (spongy- morphotype) samples that were collected and

frozen in liquid nitrogen in a circadian sampling scheme (0600, 1200, 1800, and 2400 hours). Total RNA extraction was performed using the RNA PowerSoil® Total RNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) following the protocol of the manufacturer. RNA was then purified with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) including a step with DNAseI (Qiagen) to avoid DNA contamination. Immediately after RNA purification, primer-conducted com- plementary DNA (cDNA) synthesis was performed using the Reverse Transcription System (Promega, WI, USA) with

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space0.5 μg of each primer (Invitrogen). Reactions of 20 μl were incubated in the following sequence: 10 min at room temper- ature, 15 min at 42 °C, and finally 5 min at 95 °C to inactivate reverse transcriptase. cDNA obtained was kept frozen (at

−20 °C) until analysis. Samples were verified for DNA con-

tamination after DNAse incubation with PCR before reverse transcription.

Amplification of DOP Utilization Genes

Partial sequences of alkaline phosphatases (*phoX* and *phoD*) and beta-propeller phytase (*bpp*) were amplified using reported de- generate primers [10, 15, 39]. PCR-specific conditions, primers used, and partial sequence length are shown in Table 2 (Online Resource 1). In a general scheme, PCRs were performed using

∼10 ng of DNA and cDNA in a final reaction mixture of 25 μl,

containing 2.5 μl of 10× ViBuffer A (Vivantis, Oceanside, USA), a specific $MgCl_2$ concentration (Table 2, Online Resource 1), 0.2 mM of each deoxynucleotide triphosphate,

0.5 μg $μl^{-1}$ bovine serum albumin (BSA, Biolabs, New England), and 0.5 U of Taq DNA Polymerase (Vivantis, Oceanside, USA). PCR products obtained from five reactions were pooled and then gel purified using QIAquick spin columns (Qiagen). DNA and cDNA amplicons were used to build clone libraries for each season and sample. Amplified fragments were ligated to pCR®2.1 vector following the manufacturer's sugges- tions (Original TA Cloning Kit, Invitrogen, Carlsbad, CA, USA); *Escherichia coli* TOP-10 chemically competent cells

were transformed and selected with ampicillin (100 μg ml^{-1}) and X-Gal (80 μg ml^{-1}); M13 primers were used to screen the fragments (∼100 transformant clones were picked for each DNA sample, and transcript libraries had a lower efficiency, with ∼50 clones per sample).

Sequence Analyses

To explore diversity and dynamics of the genetic markers surveyed, operational taxonomic units (OTUs) were defined and considered as P-gene phylotypes. Phylotype assignation was done using cutoff values of 0.25 and 0.05 (see Table 1), following the similitude cutoff of 0.25 has been defined in

spacesilico for *phoD* [40], and cutoff value of 0.05 suggested for the analysis of functional genes [41]. Although both cutoff values (0.25 and 0.05) were used for OTU assignation (Table 1), a theoretical analysis of *phoD* sequences indicates a specific sequence cutoff of 0.25 [40], and the resulting phy- lotypes were used further to compare phylotypes among groups and periods (Fig. 3). In contrast, while no specific cutoff values have been determined for *phoX* and *bpp* (per- haps due to the complexity for the reconstruction of the evo- lution of these proteins), a cutoff of 0.05, suggested for func- tional genes [41], was used to examine phylotype dynamics of *phoX* and *bpp* (Fig. 3).

Using the predicted amino acid sequences, alignments of each marker (using the Multiple Sequence Alignment soft- ware MUSCLE) and reference sequences of phosphatases (*phoX* and *phoD*) and *bpp* phytases were analyzed and trees were constructed with neighbor-joining and maximum likeli- hood algorithms (PhyML 3.0) [42]. Functions Bdist.seqs^ and Bcluster^ (using the furthest neighbor algorithm) of the plat- form for bioinformatic analyses MOTHUR v.1.33.3 [43] were used to group sequences into OTUs and to explore diversity within each marker (nonparametric richness estimator *Chao*, Shannon diversity index, as well as coverage, are reported in Table 1). The exploration of the sequence sets of each micro- bial consortia and season (stratification or circulation) was used to construct Venn diagrams showing the overlap and turnover of the markers explored.

Lake Alchichica AP (*phoX* and *phoD* partial gene-nucleotide sequences) from microbialites and bacterioplankton can be consulted in GenBank under the accession numbers KF891484–KF891515, KF891517–KF891828, and

KF891830–KF891882. PhoD-like transcripts (nucleotide and predicted amino acid sequences) were identified using the non- redundant (NR) database of NCBI BLAST. Sequences obtained have been submitted to the GenBank database under accession numbers KT763020–KT763031. Sequences shorter than 200 nucleotides are compiled in fasta archives (Online Resources 2, 3, and 4). Their affiliation and general features were explored through searches that correspond to the particular features of each set of sequences (see transcript sequences analyses in Online Resource 1).

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Table 2 Online Resource 1. Primers and PCR amplification conditions for each marker

Marker	[Primer] (mM)	$MgCl_2$ (mM)	PCR conditions
phoX	0.4	1.4	Denaturalization (95 °C for 5 min) 35 cycles (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min); spacefinal extension (72 °C for 10 min)
phoD	2	2	Denaturalization (94 °C for 3 min) 35 cycles (94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min); final extension (72 °C for 7 min)
BPP	1	2	Denaturalization (95 °C for 4 min), 8 cycles (95 °C for 30 s, 57 °C for 30 s, diminishing 1 °C each cycle and 72 °C for 30 s); 27 cycles (95 °C for 30 s, 48 °C for 30 s, and 72 °C for 5 min)

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[39]

[15]
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spaceDatabase Searches and Structural Determination of PhoX and BPP transcripts

PhoX transcripts and BPP sequences were explored manually (using and modifying the default search parameters of HMMER, nBLAST, and pBLAST algorithms) [44–46] to confirm the identity of the aforementioned sequences. A de- tailed description of the strategies followed to perform these analyses can be consulted in Online Resource 1. For se- quences less than 18 amino acids in length (some phoX tran- scripts), we decided to analyze the linear predisposition of the amino acids in the sequence to form regular secondary struc- tures [47]. Due to sequence length limitations, we considered the overall hydrophobicity, using the scale of Kyte and Doolittle [48] of a linear string of amino acids [49–51].

System's Water Balance

The water balance of Lake Alchichica was modeled by García Martínez [52] using a continuity algorithm. Alchichica water balance was estimated in an annual basis using primary and secondary data [53]. Primary data were obtained through di- rect measurements (e.g., wells), while secondary data were obtained from reported literature and databases. The water balance considered the following climatic data: temperature, precipitation, evaporation, real (ETR) and potential (ETP) evapotranspiration, as well as the surface area of the lake, altitude, and static level [52].

Phosphorus Mass Balance

A stationary state was assumed ($\Delta P/\Delta T = 0$) because Lake Alchichica's trophic status has exhibited relatively low varia- tion in SRP, TP, or SiRP concentrations in recent decades [54–56], and a whole system P balance was performed. Data for the estimation of the phosphorus mass balance included primary and secondary data. Phosphorus balance is defined by the equation provided below as the difference between P in- puts and outputs in a time basis to estimate the difference in magnitude between in- and outflows [57].

$$\Delta M\,(\mathrm{P})/\Delta t = \Sigma(\mathrm{P\ inputs} - \mathrm{P\ outputs}) \pm \mathrm{P\ Net\ Internal\ Processes\ (PNIP)} \quad (1)$$

where $\Delta M(P)/\Delta t$ is the mass change of P within the system and was evaluated numerically from the volume-weighted concen- trations and volume data of the lake. P inputs were computed and estimated for each water source or outflow (Table 6).

Some potential P sources were not considered for the P mass balance due to the following reasons: (1) local sewage inflows or water withdrawal was considered negligible (there is no infor- mation or evidence about their existence), (2) net P internal flow space(no studies on P capture and release have been reported for the lake), and (3) P precipitation in OH-apatite was not considered since this mineral is unsaturated in Alchichica water column (OH-apatite saturation index = −2.97) [25]. After these consid- erations, Lake Alchichica's P balance (Table 6) was constrained to the equation:

$$\mathrm{P\ Precipitation} + \mathrm{P\ Atmospheric\ deposition} + \mathrm{P\ Basin\ runoff} + \mathrm{P\ groundwater} = \mathrm{P\ Sedimentation} \quad (2)$$

To estimate the ratio of total P mass/P sedimentation flux (Table 6), lake's total P mass was calculated using the concen- tration on TP in each layer of the lake times the volume of each layer.

Results

Genetic Diversity of Bacterial P Utilization Genes

Microbialite and bacterioplankton DOP utilization genes showed affiliation with Actinobacteria (*phoD*), Alphaproteobacteria (*phoX*, *phoD*), Betaproteobacteria (*phoD*), Gammaproteobacteria (*phoD* and *bpp*), and Bacteroidetes (*bpp*).

Partial phytase sequences recovered from environmental DNA of Lake Alchichica's bacterioplankton and microbialites (~200 bp) were similar to *bpp* phytases from other environ- mental studies [15] and affiliated with annotated genomic se- quences (3-Phytase) of Bacteroidetes (Flavobacteriales), Beta, and Gammaproteobacteria (Fig. 2). Phytase (*bpp*) predicted amino acid sequences corresponded to protein domain 3- Phytase BPP (Pfam PF02333, InterPro IPR003431).

Alchichica's *phoX* partial sequences (~650 bp) showed phylogenetic affiliation with Alphaproteobacteria and Actinobacteria from aquatic environmental samples of different trophic states [10, 19]. Amino acid predicted sequences corresponded to pro- tein families COG3211: PhoX, Pfam 05787 (residues E273 and E873, Protein Data Bank 3ZWU_A), and to the Domain of Unknown Function (DUF) DUF839. This domain has been di- rectly associated to a large family of proteins that are able to metabolize phosphorus (Interpro Database) [59], while in the Pfam Database [60], DUF839 is considered a featured domain of the proteins which are included as part of the family with the PhoX domain [61].

Lake Alchichica partial *phoD* sequences (~350 bp) affiliated with Alpha, Beta, and Gammaproteobacteria from soil studies [39, 40]. Most (96 %) of the *phoD* amino acid predict- ed sequences contained the calcium binding site (residues N215, N216; 2YEQ; of the Protein Data Bank).

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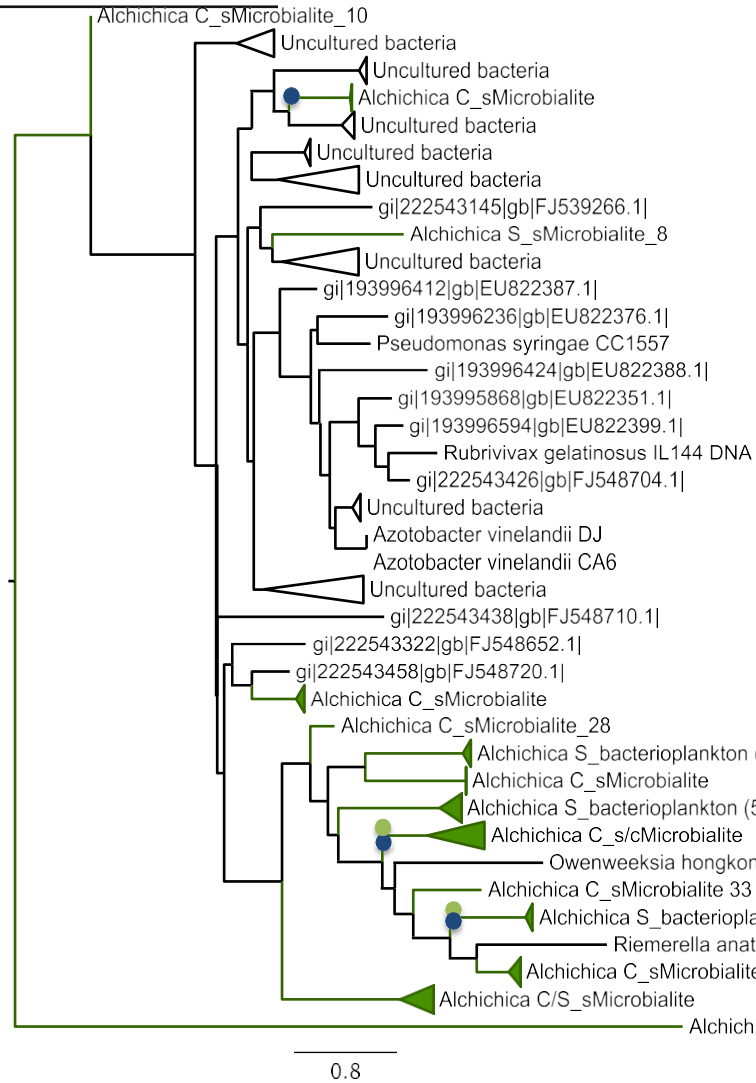

space**Fig. 2** Partial *bpp* sequences maximum likelihood topology. Alchichica Lake sequences (spongy and columnar microbialites and bacterioplankton); environmental studies *bpp* sequences are shown in *black branches* and genomic reference sequences are shown in *cyan*

space*color*. Branches show bootstrap testing over 1000 replicates (only bootstrap values >50 are shown). *Colored dots over clades* show the affiliation of BPP transcript sequences (*blue* for transcripts of 24 h and *green* for 18 h)

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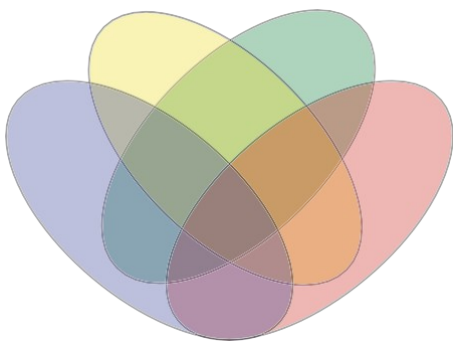

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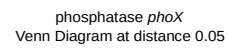

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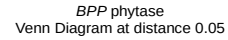

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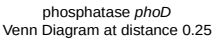

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Stratification.

spaceStratification. microbialites (7 phylotypes)

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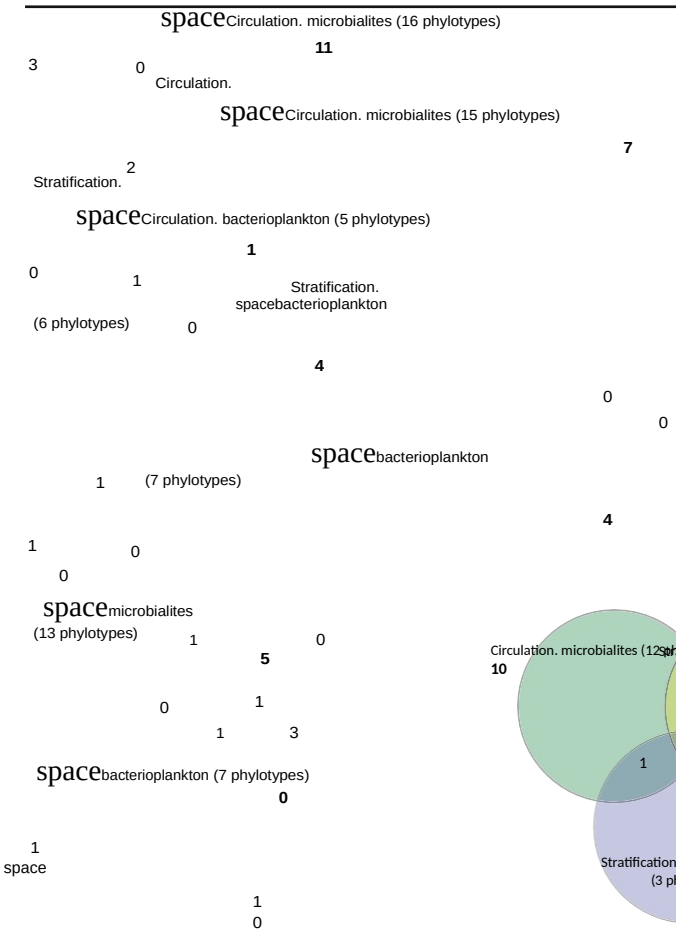

space Fig. 3 Venn diagrams showing the distribution of phylotypes of alkaline phosphatases *phoX* and *phoD*, and beta-propeller phytase *bpp* in microbialites and bacterioplankton of Lake Alchichica. The cutoff value

space for phylotype determination is shown, as well as the total number of phylotypes (*in parenthesis*) for each marker

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space Phylotype Distribution Between Microbial Assemblages (Microbialites and Bacterioplankton)

Alkaline phosphatases (*phoX*) exhibited the largest number of phylotypes (27), followed by *phoD* (23) and *bpp* (18). Diversity indexes indicate that overall, the three studied markers were more diverse in microbialites than in bacterioplankton (Table 1). Analysis of alkaline phosphatases *phoX* and *phoD* (cutoffs = 0.05 and 0.25, see Table 1) showed that some phylotypes are shared between microbialites and bacterioplankton and between lake circulation periods (strati- fication and circulation), while other phylotypes are unique (Fig. 3). The proportion of unique sequences was higher for *bpp* (72.4 %) followed by *phoX* (62.5 %) and *phoD* (52.2 %). Microbialites shared more *phoX* phylotypes within seasons than bacterioplankton. The proportion of *phoD* phylotypes shared between periods was the same in microbialites and bacterioplankton although microbialites exhibited a larger number of shared *phoD* phylotypes between periods (Table 3). Unlike alkaline phosphatases (that were present in microbialites and bacterioplankton during lake's circulation and stratification), *bpp* phytases were not found in the bacterioplankton during lake's circulation. Microbialites, on the other hand, contained four times more *bpp* phylotypes in circulation than during stratification. No phylotypes were common to all the *bpp* groups (Fig. 3).

The groups of sequences of *phoX*, *phoD*, and *bpp* showed a distinctive distribution pattern. The type of assemblage (microbialite or bacterioplankton) was more relevant than sea- sonality in the distribution of *phoX*. In contrast, seasonality distinguished *phoD* and *bpp* phylotype distribution. Bacterioplankton in circulation for *phoD* and microbialites in circulation for *bpp* were the groups that showed the highest number of unique phylotypes (Fig. 3).

A differential distribution of markers was explored by cal- culating the ratio of unshared phylotypes: shared phylotypes among assemblages (Table 4). Ratios indicate that exclusivity of DOP utilization phylotypes is favored in microbialites com- pared to bacterioplankton. A differential pattern was observed where *phoD* showed the lowest values for both assemblages, and *phoX* the highest ratio (Table 4).

Transcription Patterns of phoX, phoD, and bpp Following a Diel Cycle

Alkaline phosphatases and phytases showed different expres- sion patterns (Fig. 4). PhoX transcripts exhibited a diurnal expression pattern, 40 % of the transcripts was recovered at sunrise (06:00 h) and 60 % at noon (1200 hours). PhoD tran- scripts showed a broad pattern of expression throughout the diel cycle, where 42 % of the transcripts corresponded to 0600 hours and 50 % to 1200 hours, although 8 % was recov- ered at midnight (none for 1800 hours). BPP phytase

space transcripts showed an afternoon pattern of expression where most of the transcripts were recovered at midnight (70 %) and 30 % at 1800 hours (Table 5, Online Resource 1).

Alkaline phosphatase PhoD transcripts were very similar in length (approximately 369 bp) to the ones recovered from total DNA. The closest affiliation of PhoD transcripts corresponded to alkaline phosphatase D (*Bacillus subtilis*-- type), two of the PhoD transcripts affiliated to precursor se- quences of alkaline phosphatase D of *Pseudomonas stutzeri* (Gammaproteobacteria) and *Bhradirhizobium* sp (Alphaproteobacteria); both are bacterial genus known to be involved in denitrification and nitrogen fixation, respectively (Table 5, Online Resource 1). PhoX and BPP phytase tran- scripts were smaller than the genomic sequences recovered from total DNA (transcript sequences can be consulted in Online Resource 1, also an ad hoc scrutiny of each of these sequences to verify their structural features and the presence of protein domains).

PhoX transcript sequences affiliated to three main functional categories: signaling, DNA binding, and phos- phorus metabolism (see Online Resource 1); transcripts also associated to the PhoX domain, linker domains (like

the SH3 domains) [62–64], and to a domain that is still not functionally characterized, DUF839. By performing the hydrophobicity plot of our sequences [48], we found that some of the PhoX transcript sequences could, theoretically, form alpha-helices and loops, while the rest of them may be forming special arrangements of beta-sheets (see Online Resource 1). The PhoX domain is approximately 120 amino acids long and folds into a very partic- ular three stranded beta-sheet followed by three helices and a proline-rich region that it is located before a membrane-interaction loop and spans approximately eight hydrophobic and polar residues [64, 65].

Transcript of BPP sequences showed affiliation (with sig- nificant scores) to sequences identified in the NR database of NCBI [45]. Transcript sequences of BPP showed domains involved in phosphorous metabolism, some specifically be- longing to phosphoenolpyruvate carboxykinase (PEPCK), a protein that catalyzes the reversible decarboxylation and phos- phorylation of oxaloacetate to yield phosphoenolpyruvate and carbon dioxide using ATP or GTP for the phosphoryl transfer [66]. Notably, in one of Alchichica's sequences (bppSsMcDNAOh_17), the Domain of Unknown Function DUF4397 (http://pfam.xfam.org/family/PF05787) was detected; DUF4397 has not been yet associated to any specific role [60].

P Mass Balance

Lake Alchichica's P mass balance is summarized in Fig. 5. Atmospheric deposition was the highest (annual basis average was 4.94 ton P/year) among P inputs to Alchichica Lake

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space Table 3 Alkaline phosphatases *phoX* and *phoD* and beta- propeller phytase *bpp* phylotype distribution among microbial assemblages

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Marker	Total phylotypes	
	Phylotypes shared between assemblages	
	Phylotypes only in bacterioplankton	
	Total	Present in both seasons
	Phylotypes only in microbialites	
	Total	Present in both seasons
phoX	36	2
phoD	30	7
bpp	18	2

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(Table 6). The sum of all inputs (precipitation, basin runoff, groundwater, and atmospheric deposition) reached 5.26 ton P/year, which was almost 10 % higher than P sedimentation (4.79 ton P/year), the main output identified. Since lake TP mass (calculated integrating TP concentrations over the water column) was 4.33 ton, we estimated the P residence time in the water column in the range of 0.82–0.90 y (i.e., 9.8–
10.8 months). P mass balance at Alchichica is further discussed in relation to the lake's physicochemical conditions during stratification and circulation periods, in the BLake Alchichica's P Status^ section.

Discussion

Potential for DOP Remineralization

The potential for DOP remineralization found in Lake Alchichica's microbialites and bacterioplankton concur with those obtained by Sebastián and Gasol [67], who identified (using microautoradiography combined with in situ hybridi- zation) a stronger response to P stimulation/starvation of Gammaproteobacteria and Bacteroidetes over other microbial groups (in bacterioplankton samples of an environment where P starvation was presumably prevalent). Jorquera et al. [68] also described the relevant abundance and diversity of *bpp* phytases in Gammaproteobacteria and in some Bacteroidetes genus such as *Flavobacterium*. Although Bacteroidetes (Flavobacteriales) has not been recognized as an abundant

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phylum in Alchichica's microbialites [32], this group may be a key participant in DOP transformation.

PhoD and BPP transcript sequences were associated with DNA sequences from Alchichica assemblages that showed their closest affiliation with Proteobacteria and Actinobacteria (PhoD) and Gammaproteobacteria and Bacteroidetes (BPP). PhoD transcripts of 0600 hours were significantly affiliated to sequences of Gammaproteobacteria (mRNA 6h_14 and mRNA 6h_7 of Table 5; Online Resource 1; E values = e-142 and e-175).

Phylotype Dynamics and Turnover

The type of microbial assemblage defined the abundance of DOP utilization genetic markers including *phoX*, *phoD*, and *bpp* when metabolic potential for DOP utilization of bacterioplankton (of the whole water column) was compared with that of microbialites. Although a functional

parallelism was shown, results indicate that the potential of bacteria (as defined by OTU numbers) to transform DOP through AP *phoX*, *phoD*, and *bpp* phytases was higher in microbialites than in bacterioplankton (Fig. 3, Table 1). In general, the bacterioplankton of the stratification period shared more phylotypes with microbialites than the bacterioplankton of the circulation period.

Although we studied only three molecular markers associated to DOP utilization, here, we explore some ideas about the meaning of these phylotype dynamics-patterns. While microbialites showed a higher potential (higher genetic diversity) than bacterioplankton to remineralize DOP through APs, the difference was markedly higher in microbialites to remineralize phytate through BPP phytase. Microbial assemblages harboring these markers showed change (phylotype replacement or seasonality) in a timespan as short as the seasonal cycle of

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Table 4 Ratios of *phoX*, *phoD*, and *bpp* unshared phylotypes of Microbialites

	Unshared/shared phylotypes	Normalized by coverage	Unshared/shared phylotypes	
phoX	4.5	4.8	8.0	8.6
phoD	0.3	0.3	2.0	2.1
bpp	0.5	0.6	6.0	7.0

Normalized by coverage: each microbial assemblage: shared phylotypes among assemblages

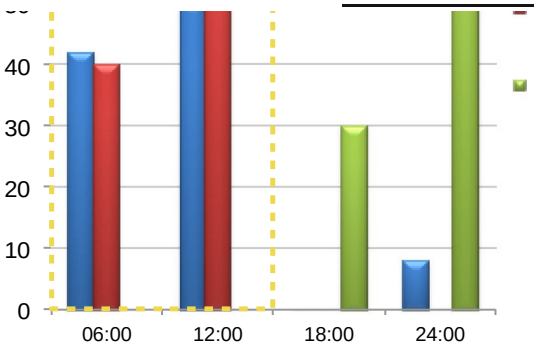

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Fig. 4 Expression patterns of DOP utilization genes in a diel sampling scheme

ever, bacterioplankton showed a more dynamic phylotype turnover (Table 4). This result may be coupled to their contrasting lifestyles: free-living vs. benthic consortia.

PhoD and BPP transcripts were obtained only during stratification, suggesting a more intense P starvation condition for the bacterial communities in this period (a circulation to stratification. The divergence of the groups *phoD*-Bacterioplankton and *bpp*-microbialites (of circulation) stands out overall, suggesting a faster dynamics of these markers, compared to *phoX*. In contrast, *phoX* dynamics did not show a marked difference among assemblages or periods. APs and phytase exhibited phylotype overlap (between assemblages and periods), as seen in the Venn diagrams (Fig. 3). These relationships suggest not only a structural connection between microbialites and bacterioplankton assemblages but also between the littoral and the pelagic zone of the system. Unchanged (unshared) phylotypes constitute therefore the genetic reservoir (resilient stock) for DOP transformation (Table 4). Bacterioplankton seems to harbor overall a minor genetic potential than microbialites (for DOP utilization); how-

Table 5 Online Resource 1. Characteristics of the PhoD, PhoX, and BPP transcripts recovered and their closest affiliation in the general databases

Transcript	Expression pattern	Season	Length (bp)
PhoX			
alpsSsMcDNA6h_2	0600	S	369
alpsSsMcDNA6h_003	0600	S	369
C_alpsSsMcDNA6h_7	0600	S	369
alpsSsMcDNA6h_10	0600	S	369
alpsSsMcDNA6h_14	0600	S	369
alpsSsMcDNA6h_16	0600	S	369
C_alpsSsMcDNA6h_19	0600	S	363
alpsSsMcDNA12h_5	1200	S	369
alpsSsMcDNA12h_6	1200	S	369
alpsSsMcDNA12h_007	1200	S	369
alpsSsMcDNA12h_11	1200	S	369
alpsSsMcDNA12h_12	2400	S	357
PhoX			
phoXcDNA.S6h.29	0600	S	24
phoXcDNA.S6h.32	0600	S	44
phoXcDNA.S12h.20	1200	S	24
phoXcDNA.S12h.22	1200	S	58
phoXcDNA.S12h.23	1200	S	25
NCBIBPP (3-phytase)			
bppSsMcDNA18h_1	1800	S	93
bppSsMcDNA18h_3	1800	S	93
bppSsMcDNA18h_4	1800	S	93
bppSsMcDNAOh_2	1800	S	114
bppSsMcDNAOh_3	2400	S	118
bppSsMcDNAOh_6	2400	S	63
bppSsMcDNAOh_15	2400	S	604
bppSsMcDNAOh_16	2400	S	57
bppSsMcDNAOh_17	2400	S	114
bppSsMcDNAOh_18	2400	S	54

[a] Sequence fragment affiliation corresponds to the results in the Protein Data Bank (PDB) and in the nonredundant database of the National Center for Biotechnology

Information (NCBI). Predicted sequences of amino acids were used as templates

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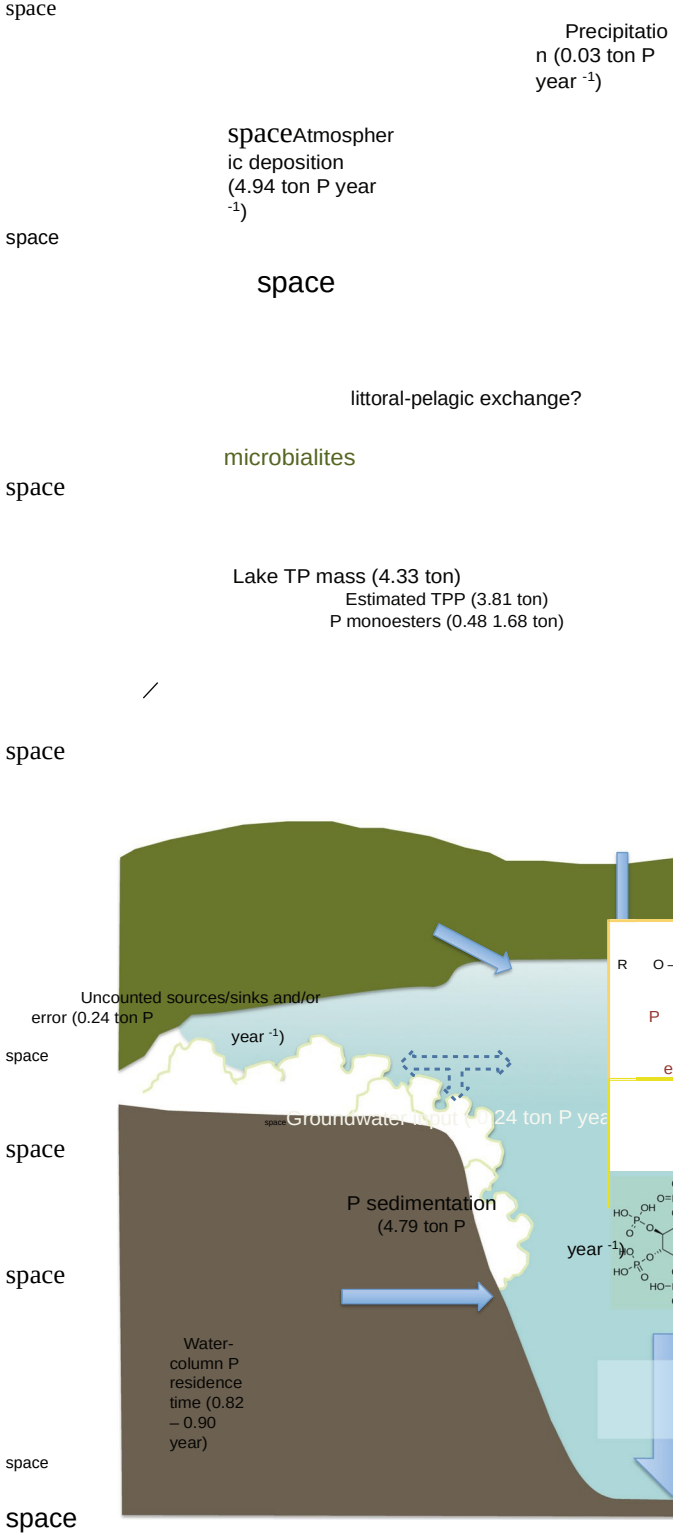

Fig. 5 Lake Alchichica phosphorus condition. The diagram shows the main phosphorus in- and outflows (from atmospheric deposition, basin runoff, groundwater, and sedimentation), lake total phosphorus (*TP*) mass, total particulate (*TPP*), P monoester fraction, and residence time

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and in an annual basis. *Boxes* enclose enzymatic mechanisms of alkaline phosphatases *phoX* and *phoD*, as well as beta-propeller phytase *bpp* to release inorganic phosphorus from dissolved inorganic phosphorus (DOP)

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showed mostly a daytime expression pattern compared to the overall evening expression of BPP. These patterns should corre- spond to peaks of activity of the community harboring such a metabolic potential. Although more effort should be done to understand the whole functional community, an indication of the activity of Gammaproteobacteria and Bacteroidetes in DOP utilization has been previously described in a low phos- phorus environment [67]. The identification of Lake Alchichica BPP transcripts affiliated to diazotroph genus *Scytonema hofmanni* also suggests that Cyanobacteria may be an important group in phytate degradation, as reviewed by Jorquera et al. [68]. The type and frequency to which Alchichica's sequences were associated to best-hit proteins into the functional categories of

Table 6 P mass balance in Lake Alchichica, inputs (positive sign fluxes) and outputs (negative sign)

Fluxes of P	(P ton/year)	% of P mass	Reference
Lake total P mass (tons)	4.33	100	This study
Precipitation	0.03	0.8	[52]
Basin runoff	0.05	1.1	[52]
Atmospheric deposition	4.94	114	[52, 58]
Groundwater	0.24	5.5	[25, 52]
Sedimentation	−4.79	−111	[27]
System P balance	0.47		This study

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general databases may reflect that affiliations are not completely at random, and these results are not false

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positives but structur- ally significant sequences (see Online Resource 1). DUF839 has been previously associated to *phoX* alkaline phosphatase [10, 19]; DUFs are frequently uncharacterized protein families found in the protein databases, and they seem to play important roles the clarification of DUFs' challenge.

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f the lake, including water col- umn stability and dissolved oxygen and nutrients, vary impor- tantly between circulation and stratification (Fig. 1). Although the littoral zone exhibits low availability of N and P (low P_{org} and DIN/SRP ratio <16), the littoral SRP concentration during the

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stratification period is almost half of the SRP concentra- tion duri in the wate (epilimn time; on may be a (epilimn developr the onse

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space Lake Alchichica's P balance shows that allochthonous P total input was 9.8 % higher than the TP exported to the sediments (Fig. 5).

This difference (or unaccounted P) could correspond to presently unknown sources or sinks of P, as well as to errors in the present estimates, or due to intra-annual heterogeneity. In any case, this P mass balance revealed that the magnitude of P fluxes is similar (between 8 and 10 % higher) to the lake's total P mass. The average time that each P atom spends in the water column of Lake Alchichica before it sediments (i.e., the P water column-residence time) ranges between 0.82 and 0.90 year, as estimated by the P mass/P flux ratio calculated using either total inputs or total outputs.

Temporal heterogeneity is a factor that should be consid- ered in this exploration, although at the moment, a dynamic water balance in Lake Alchichica is constrained due to the uncertainty on the lake's groundwater flows and particularly on their variability. During winter, dry and dusty conditions of the Mexican highlands favor higher atmospheric P deposition rates than during the rest of the year (see seasonal atmospheric dust deposition in Oseguera et al. [58]). On an annual basis, the circulation bloom of big-size diatoms [27] is assumed to be the main driver of organic matter sedimentation.

Alchichica Lake's TPP accounted for 3.67 and 3.94 tons of organic P in the water column during circulation and stratifi- cation, respectively. According to novel ^{31}P NMR spectrosco- py P fractioning studies, P monoesters would account for 11 to 44 % of TPP (in a productive system) [70]; using this range (in the absence of any similar measurements for Alchichica), the P monoesters fraction of the lake would range from 0.42 to 1.68 tons, a considerable size for a pool that would have to be remineralized by microorganisms [71] fitted with P extracel- lular enzymes (i.e., *phoD*, *phoX*, *phoA*, etc). Nevertheless, understanding the metabolic strategies to utilize this P fraction likely requires multiple approaches, from microscopic to community-scale research strategies. It is also important to assess the exchange of P, organic matter, and other nutrients from the littoral zone (where there are areas with high cover- age of macrophytes, epibenthic diatoms, and living microbialites) to the pelagic zone, as outlined by Ardiles et al. [27], who also reported benthic littoral phytoplankton species (pennate diatoms) in the central and deep areas of the lake. Finally, it is also remarkable that, likely because of the high P output flow associated to sedimentation, there is no experimental evidence of net P release from the sediments.

Final Remarks

Microbialite and bacterioplankton assemblages seem to exhib- it different strategies for DOP utilization (i.e., through alkaline phosphatases and phytases). A Bstock strategy^ favored in microbialites, focused on preserving a diverse community (a higher potential to utilize DOP, e.g., maintaining spaceproteobacterial participants) and a Bfast-change strategy^, overall observed in bacterioplankton, in which other (less abundant) phylotypes exhibit a higher turnover. Although microbialites harbor a higher genetic potential for DOP utili- zation (through *phoX*, *phoD*, and *bpp*), the resilience potential of each assemblage is still to be assessed and may be related to the time scale as well as to the magnitude of environmental changes. Under this scenario, we hypothesize that the water column communities may be more effective to face drastic environmental change, although their effective rate of DOP transformation may be lower (due to their lower phylotype diversity) than in their benthic counterpart.

Accounting for the area covered by microbialites in Lake Alchichica, and considering their extensive genetic potential and functional capabilities for DOP utilization, both assem- blages (microbialites and bacterioplankton) may play an im- portant role in contributing to the availability of nutrient and trace metals and in maintaining a relatively high production in a system where allochthonous nutrient input is relatively low. This Befficient^ remineralization would help explain observa- tions that refer higher production features than expected for an oligotrophic system (with low P inputs). This evidence in- cludes high abundance of total picoplankton relative to low trophic state systems [30] and regular *Nodularia* blooms [33, 55].

DOP utilization genes/transcripts showed different dynam- ics in contrasting hydrological conditions (within microbialites and bacterioplankton). Our findings indicate that microbial groups have different responses to face P stimula- tion/starvation. This provides dynamic capacities (system's metabolic potential) to utilize P (DOP). It is relevant to ob- serve seasonality of microbial phosphorus utilization potential to understand transformations of other major biogeochemical cycles such as carbon and nitrogen.

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ization genes *phoD*, *phoX*, and *bpp*; richness and diversity estimators; as well as coverage for each group (microbialites and bacterioplankton) and period

ff	*phoD* (sequences obtained = 180)				*phoX* (sequences obtained = 192)				*bpp* (sequences obtained = 58)			
	OTUs	Chao	Shannon (*H'*)	Coverage (%)	OTUs	Chao	Shannon (*H'*)	Coverage (%)	OTUs	Chao	Shannon (*H'*)	Coverage (%)
	23	58	2.51	80	16	30	2.28	88	15	33	2.3527	74
	15	20	2.22	92	14	17.3	2.21	92	10	20	1.95	86
	17	27.5	2.13	91	7	7	1.67	100	3	4	0.7963	71
	13	20.5	1.68	92	6	6	1.46	100	3	4	0.8	71
	5	8	1.56	33	7	7.25	1.15	97	–	–	–	–
	5	8	1.56	33	6	6	1.12	98	–	–	–	–
	9	14	1.56	83	6	16	1.04	72	3	3	0.7356	100
	7	7.33	1.44	93	6	16	1.04	72	3	3	0.74	100

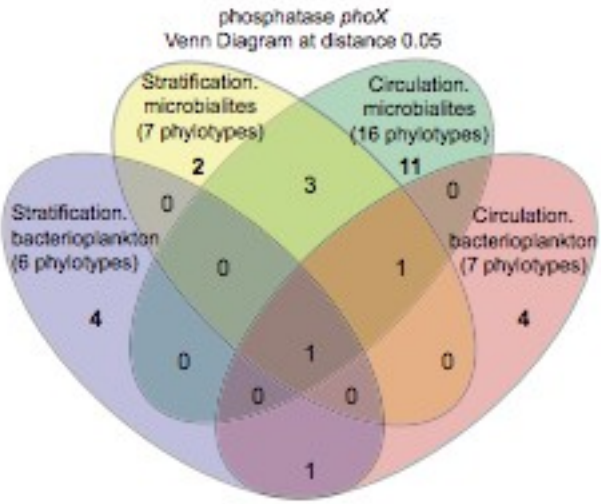

Fig. 3 Venn diagrams showing the distribution of phyl phosphatases *phoX* and *phoD*, and beta-propeller microbialites and bacterioplankton of Lake Alchichica

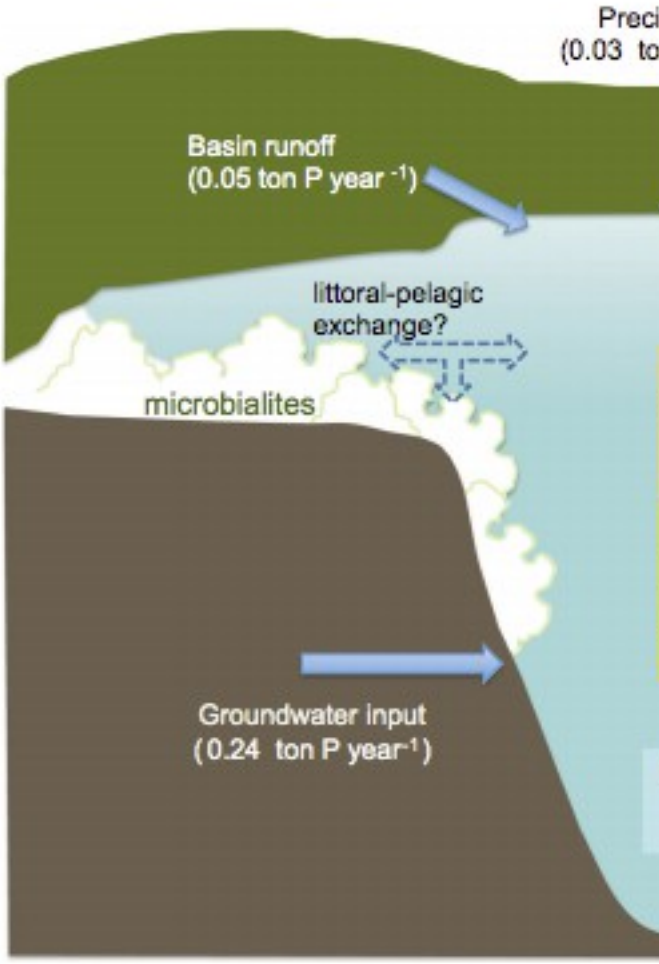

Fig. 5 Lake Alchichica phosphorus condition. The diagram shov main phosphorus in- and outflows (from atmospheric deposition, runoff, groundwater, and sedimentation), lake total phosphorus mass, total particulate (*TPP*), P monoester fraction, and residence

Table 4 Ratios of *phoX*, *phoD*, and *bpp* unshared phylotypes of each microbial assemblage: shared phylotypes among assemblages

	Ba
	Un phy
phoX	4.5
phoD	0.3
bpp	0.5

Table 3 Alkaline phosphatases *phoX* and *phoD* and beta-propeller phytase *bpp* phylotype distribution among microbial assemblages

Marker	Total phylo
phoX	36
phoD	30
bpp	18

Table 6 P mass balance in Lake Alchichica, inputs (posit fluxes) and outputs (negative sign)

Fluxes of P	(P ton/year)	% of P mass	Re
Lake total P mass (tons)	4.33	100	Th
Precipitation	0.03	0.8	[5
Basin runoff	0.05	1.1	[5
Atmospheric deposition	4.94	114	[5
Groundwater	0.24	5.5	[2
Sedimentation	−4.79	−111	[2
System P balance	0.47		Th