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Utilization of Selected Dissolved Organic Phosphorus Compounds by Bacteria in Lake Water under Non-limiting Orthophosphate Conditions

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Abstract

This study presents results on the availability of various organic P compounds for bacteria from mesotrophic Lake Constance. The rates of hydrolysis of all tested compounds added to the analyzed lake water samples did not correlate with assimilation of liberated inorganic P. β-glycerophosphate and AMP were the most efficiently hydrolysed by bacterial phosphohydrolytic enzymes. The highest specific P uptake was found in water samples supplemented with nucleotides. The fastest increase in bacterial numbers was observed in water samples enriched with DNA, RNA, ATP and phytin. Analysis of discrepancies between rates of hydrolysis, specific P uptake and bacterial growth rates in samples enriched with various organic P compounds suggested that bacterial phosphatases participated substantially in processes of dissolved organic carbon (DOC) compound decomposition in lake water, whereas 5'-nucleotidase was mainly responsible for bacterial P demand.

Keywords: bacteria, orthophosphate, phosphate esters, nucleotides, nucleic acids, P uptake, organic C uptake, phosphatases, 5'-nucleotidase

Introduction

Very few dissolved organic phosphorus (DOP) fraction constituents (i.e. glucose-6-P and glycerol-3-P) can probably be assimilated directly in limited quantities by bacteria [1, 2]. Generally, P combined in esters becomes biologically available as orthophosphate ions (Pi) after enzymatic hydrolysis of phosphate esters outside the cell cytomembrane. For mobilization of P from DOP pool aquatic microorganisms developed at least two different enzymatic Pi^- regeneration systems [3].

The first - "adaptative" system is activated relatively fast (by induction/derepression) during Pi limitation periods and is based on the activity of nonspecific phosphomonoesterases - alkaline phosphatases (APA) that are produced by almost all members of the plankton community, including bacteria, algae, fungi and zooplankton [4, 5, 6, 7, 8]. Since APA activity, in general, is strongly inhibited by Pi and thus dependent on periodically changing inorganic P concentrations in the environment, its participation in P regeneration processes in lake water is restricted to the short time intervals of Pi

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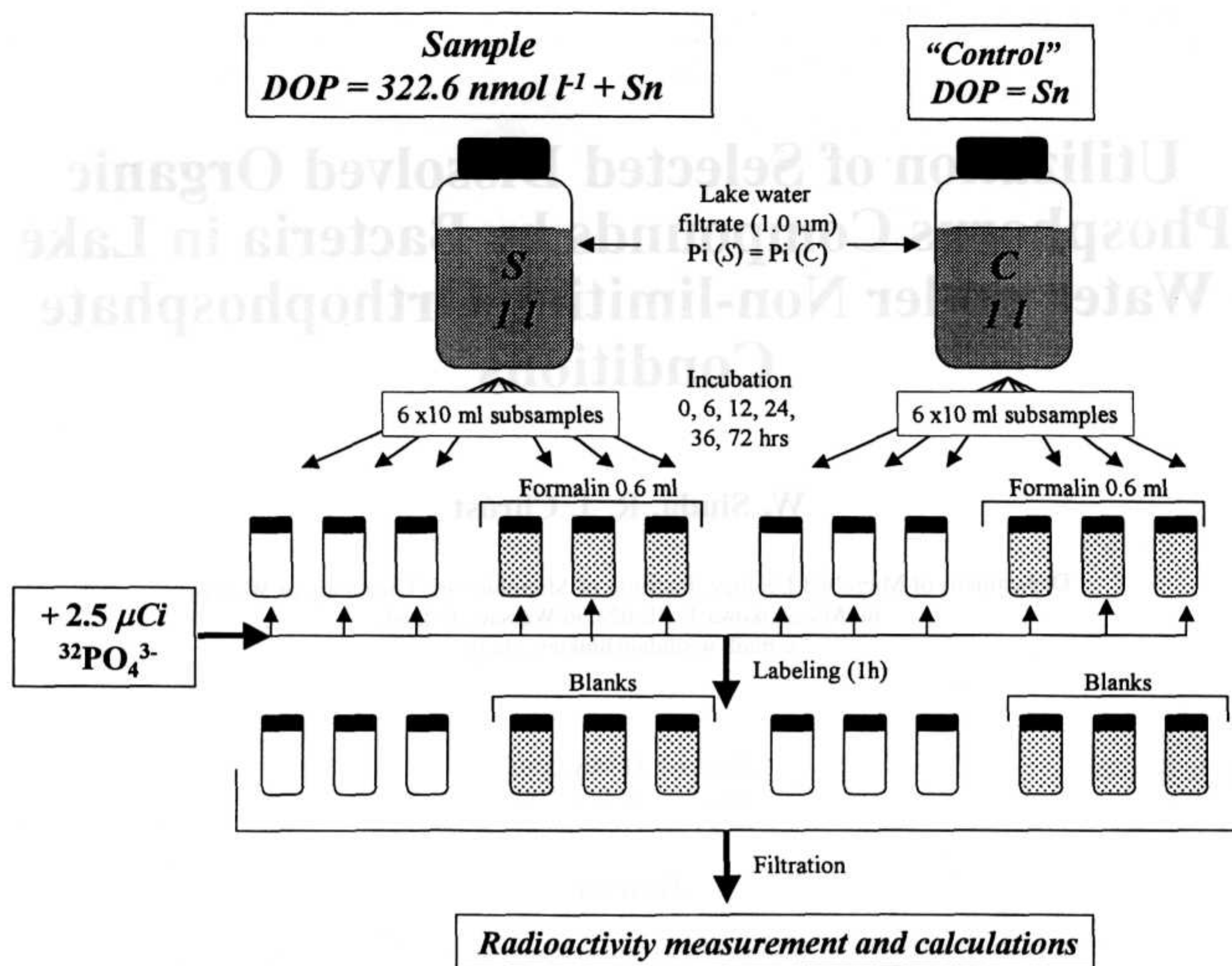

Fig. 1. Flow chart of the experiment on the utilization of the selected dissolved organic P compounds by bacteria from the surface water of Lake Constance. S - sample; C - control; Sn - concentration of natural DOP compounds in tested samples.

depletion during the summer stratification period in the photic zone of the lake [9]. The second system, exclusively bacterial, includes cooperation of various types of nucleases and 5'- nucleotidase (5'-nase), which liberates Pi from nucleic acids. Utilization of P from DNA or RNA by lake microplankton must be preceded by common endo- and exonuclease action resulting in the liberation of nucleoside 5'-monophosphates (5'-nucleotides) hydrolysed subsequently by 5'-nase. Moreover 5'-nase can also release Pi from nucleoside 5'-di- or triphosphates originated from sources other than DNA or RNA and, similarly as in marine waters [10], present in limited quantities in freshwater environments. Although all 5'-nucleotides can be hydrolysed by both APA and 5'-nase it seems that the role of APA in their decomposition is marginal [9]. The mechanism of Pi regeneration mediated by nucleases and 5'-nase is not affected by inorganic P and probably is "constitutive" in the majority of aquatic environments.

Considering the fragmentary and indirect proof published in the last few years one can speculate that Pi liberation via nucleases/5'-nase mechanism is more efficient than that mediated by APA. It was also suggested that nucleic acids and their degradation products can be the basic source of regenerated orthophosphate in aquatic ecosystems [9, 11, 12, 13, 14,15, 16, 17, 18, 19]. However, it is still extremely hard to confirm definitely this hypothesis by direct experiment or calculation. Problems with quantification of the contribution of both systems to the total regenerated Pi supply on a whole lake scale are caused mainly by lack of direct and reciprocally comparable methods for determination of P fluxes between various P pools. Since "step by step" calculation of the mean rate of P regeneration from nucleic acids is difficult and not fully comparable to estimated "maximal potential" APA substrate degradation rates, we applied a modification of the "pulse chase" technique to compare the importance of nucleic acids and other organic P compounds as a P source for aquatic bacteria. The principle of this method consisted in analysis of the differences between specific (calculated per single cell) orthophosphate uptake by bacteria in natural water samples (controls), and the same samples enriched with tested organic P compound. We expected that this methodology should, at

least in theory, permit the quantification or comparable estimation of the final effect of various organic P assimilation processes without arduous analysis of intermediate steps.

The main aim of this study was to elucidate the different mechanisms of degradation of various DOP compounds in lake water and to analyze the relationships between the rates of DOP decomposition, Pi assimilation and growth of aquatic bacteria. Additionally, the value of selected classes of DOP compounds as nutrient and energy sources for aquatic microorganisms was quantified.

Methods

Determination of the Rate of Dissolved Organic P Compound Uptake by Bacteria

Studies were carried out on bacterial assemblages from the mesotrophic Lake Constance in south Germany (surface area 540 km^2, max. depth 250 m) during late autumn (15th October - 15th December). Concentrations of Pi and DOP in lake water varied from 32.3 to 161.3 nmol l^{-1} and from 80.6 to 216.1 nmol l^{-1}, respectively. Bacterial number oscillated around 7 x 10^6 cells ml^{-1} (from 5.5 to 9.8 x 10^6 cells ml^{-1}). Water samples (21) were taken from the surface layer (0.5 m depth) of pelagic zone of the eastern central part of the lake, filtered through 1.0 μm Nuclepore polycarbonate membrane filters and divided into two subsamples (Fig. 1). One subsample was enriched with tested organic P compound (P-glycerol-phosphate (β-Gl-P), glucose-6-phosphate (G-6-P), AMP; ADP; ATP; DNA; RNA or inositol hexaphosphate) to the final concentration 322.6 nmol P l^{-1}. The second subsample, without enrichment, was treated as a "control". In both subsamples Pi was adjusted (with taking into account orthophosphate contamination of each tested organic P compound) to the same concentration by adding a small volume of Pi stock solution. Pi : DOPadded ratio was always kept about 0.5. After Pi and DOP supplementation sample and "control" were incubated for 24-72 hrs (at 15°C, in the darkness). At To time and after selected time intervals (6, 12 and 24 hrs) 10 ml portions (6 from the "control" and 6 from the sample) were taken for Pi uptake rate measurements. Labeling was carried out directly in scintillation vials for 1 hour. Routinely, to 10 ml of lake water we added 0.3 ml of ^{32}Pi solution (total radioactivity in the sample was about 0.25 μCi ml^{-1}). Incubation was terminated by 0.6 ml of formalin (37%). Blanks were prepared identically as samples but fixed (5 min. before ^{32}Pi addition) with formalin (0.6 ml to 10 ml of sample). After incubation, 8 ml of each labeled replicate was filtered through a 0.2 μm filter. The rest (2 ml) was used for determination of the total radioactivity added to the sample. Filters with labeled material were washed twice with 5 ml portions of 10.0 mM KH_2PO_4. Additionally, at the same time as ^{32}Pi uptake measurements, Pi, DOP and bacterial number were determined in the controls and in the enriched samples.

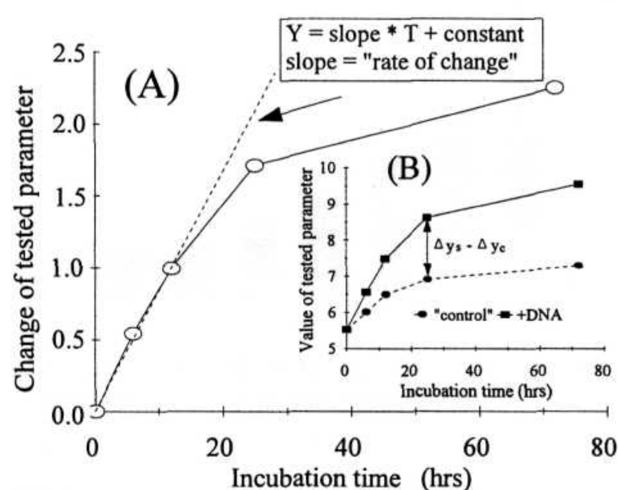

Fig. 2. (A) Graphical explanation of "rate of change" value calculation. Change of tested parameter Y= Δy_s - Δy_c was calculated from the experimental data presented in Fig. 2 (B) where Δy_s and Δy_c - increase of the value of measured parameter after given incubation time ΔT in the sample and in the "control", respectively.

Calculations

Changes of all tested parameters i. e. organic P concentration, bacterial number, total or specific Pi uptake (Pi uptake per bacterial cell) observed during incubation of samples supplemented with various DOP sources were quantitatively expressed as "rate of change". "Rate of change" value was related mathematically to the slope of F=Y(T) function, where: Y = change of tested parameter in sample (minus control) and T = incubation time (Fig. 2). For the calculations we assumed that during the first 12 hours of incubation all tested parameters changed linearly. Indeed, this method of presenting results did not permit for the most precise description of the temporal changes of DOP concentration, bacterial number or Pi uptake in analyzed samples during incubation. However, we were forced to this by the fact that commonly we had too few (only 3-4) data for describing them as rectangular hyperbola that apparently would better fit the observed data point arrangement.

Additional Analyses

Measurement of ^{32}P radioactivity was performed by a Beckman LS 800 scintillation counter (Cerenkov counting). Quench corrections were made by internal standardization. Bacteria were counted directly in epifluorescence microscope after staining (5 min. with di-amidino-phenylindole (DAPI, Serva) at 2.7 μM final concentration [20, 21]. Pi and DOP concentrations were determined spectrophotometrically using glass cuvettes 100 mm optical path length [22]. Sensitivity of the assay was 10 nmol P 1^{-1}.

Results

Assimilation of phosphorus by bacteria from two groups of organic P compounds was investigated. The first group included DOP compounds that presumably could be hydrolyzed directly (ATP, ADP AMP) or indirectly by both 5'-nase and APA. The second group of DOP substrates comprised APA, but not 5'-nase substrates (β-Gl-P, G-6-P and phytin). Compounds selected for the investigations also differed distinctly from each other in degree of complexity (nucleic acids and others), composition of the elements (presence or absence of N atoms) and (C:N:P) ratio.

All tested DOP compounds distinctly stimulated bacterial growth. Supplementation of lake water samples with substrates even poorly utilized by the bacteria like AMP or β-Gl-P caused distinct (> 30%) increases in bacterial numbers during 24 hrs of incubation, whereas in the same time in all un-ammended controls bacterial numbers increased only slightly (< 20%). Growth of aquatic, free living bacteria was most efficiently supported by nucleic acids (Fig. 3). Rates of bacterial number increases in ATP and G-6-P containing samples were about 30-50% lower than in samples enriched with nucleic acids. AMP, ADP and β-G1-P additions caused the slowest growth of natural bacterial populations. It was about 70-80% lower than that found for DNA or RNA, and 50-70% lower than that achieved after supplementation of the sample with ATP or G-6-P. Although phytin seemed to be a generally good substrate (comparable to G-6-P or ATP) its utilization by bacteria was preceded by a relatively long (24 hrs) lag phase.

The results of the investigations on the rates of hydrolysis of the tested DOP compounds are shown in Fig. 4. AMP and β-Gl-P were the most efficiently hydrolyzed enzymatically (or perhaps directly taken up?) by bacteria, whereas in samples supplemented with nucleic acids the slowest disappearance of P from DOP pool was observed. The comparison of the data presented in Fig. 3 and Fig. 4 shows that substrates that the most efficiently stimulated bacterial growth were hydrolyzed relatively slowly. Inversely, although AMP and β-Gl-P were hydrolyzed (or directly taken up?) rapidly they increased bacterial growth rates less distinctly than nucleic acids that were degraded 4 times more slowly.

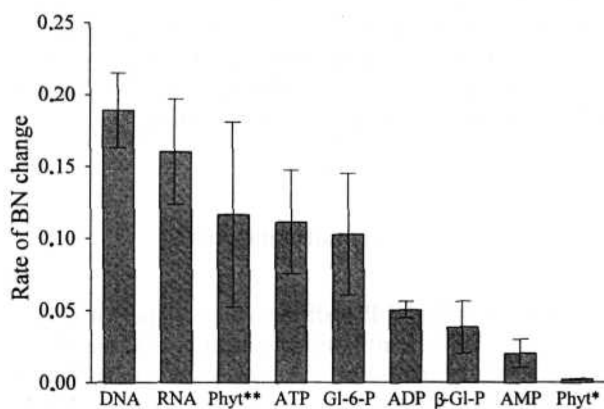

Fig. 3. The rates of bacterial number (BN) increase in samples from surface water (lm depth) of Lake Constance enriched with tested DOP substrates. * - values calculated for first 22 hrs of incubation, and ** - for period of intensive bacterial growth (from 22 to 57 hrs of incubation).

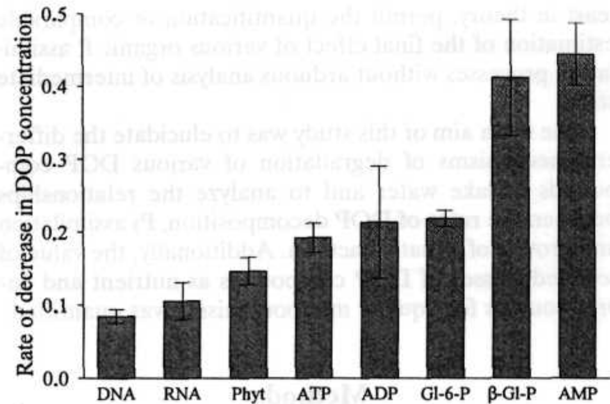

Fig. 4. Dephosphorylation of tested DOP substrates by bacteria from Lake Constance expressed as a rates in decrease of DOP concentration.

The increase in specific P uptake rates in lake water samples supplemented with tested DOP sources is presented in Fig. 5. Nucleotides (ADP, ATP and AMP) were the best P sources for the bacteria. Phosphorus from phosphomonoesters other than nucleotides (β-Gl-P, G-6-P) and more complex compounds like nucleic acids was assimilated considerably less effectively. In particular aquatic bacteria unexpectedly slowly took up phytin-P even after their adaptation to this substrate.

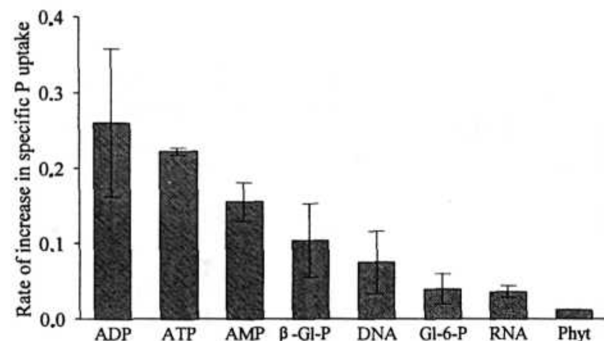

Fig. 5. The rate of increase in specific Pi uptake in water from Lake Constance supplemented with tested DOP compounds.

In the second part of our study we focused on the question whether the rates of organic P substrate hydrolysis determine the rates of specific Pi uptake and increase in bacterial number in tested lake water samples. Analysis of the results (Fig. 6) showed that enzymatic orthophosphate liberation was not tightly coupled to Pi uptake by bacteria. Hydrolysis of all tested organic P compounds fully covered bacterial demand for

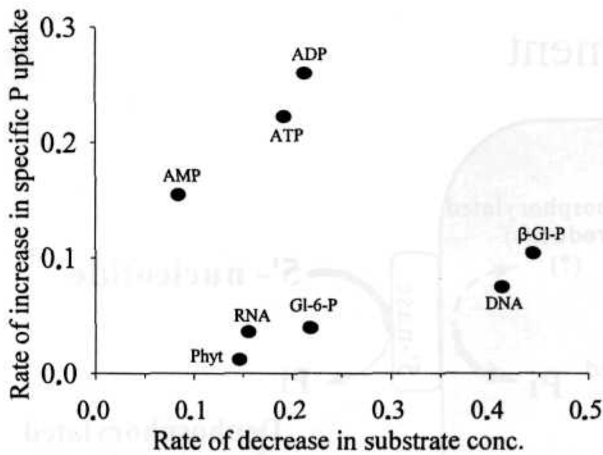

Fig. 6. Relationship between dephosphorylation of tested DOP compounds and specific P uptake by bacteria from surface water of Lake Constance. Values for phytin were calculated for first 22 hrs of incubation.

phosphorus in tested lake water samples and led to orthophosphate accumulation in the environment, which was positively correlated ($r = 0.72$; $n = 9$; $p < 0.02$) with the activity of phosphohydrolytic enzymes (Fig. 7). Although considering this fact one could presume that the increase of the hydrolysis rate of all investigated DOP compounds should not be correlated with the specific bacterial Pi uptake rate, it was rather unexpected that intensification of DOP decomposition (except phytin) might be negatively correlated ($r = -0.93$; $n = 8$; $p < 0.001$) with the increase of bacterial number (Fig. 8).

Apparently an increase in bacterial growth was not a consequence of an increase in specific orthophosphate uptake rates (Fig. 9). However, a more detailed analysis of the relationship between these phenomena revealed intriguing regularity. The development of bacteria was positively correlated with uptake of Pi originated from nucleic acids and nucleotides. However the rise of the rates of bacterial number increase in samples enriched with phytin, G-6-P and β-Gl-P resulted rather in diminution of orthophosphate assimilation rate.

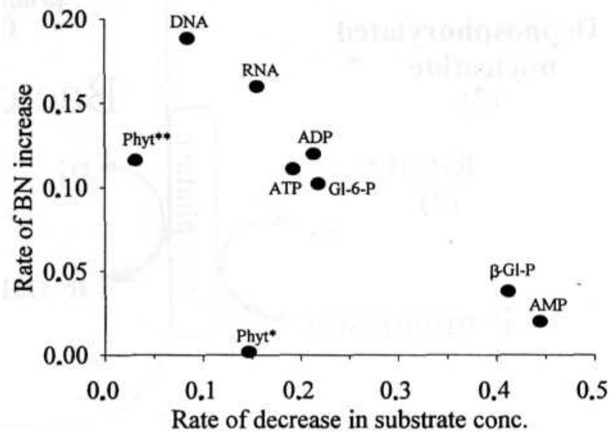

Fig. 8. Relationship between the rate of decrease in substrate concentration and the rate of increase of bacterial number in water samples taken from Lake Constance supplemented with tested organic P compounds. * - values calculated for first 22 hrs of incubation, and ** - for period of intensive bacterial growth (from 22 to 57 hrs of incubation).

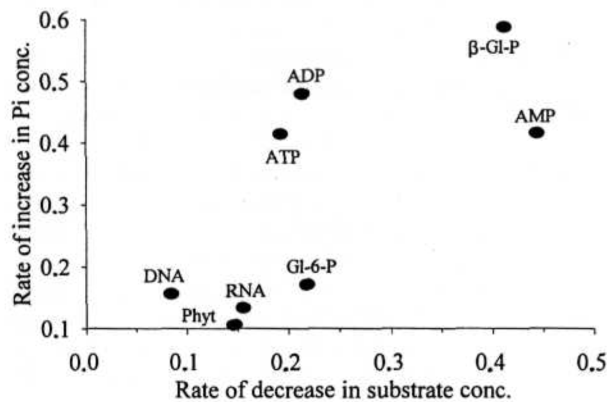

Fig. 7. Relationship between dephosphorylation of tested substrates by bacteria from Lake Constance and cumulation of orthophosphate in the environment. Values for phytin were calculated for first 22 hrs of incubation.

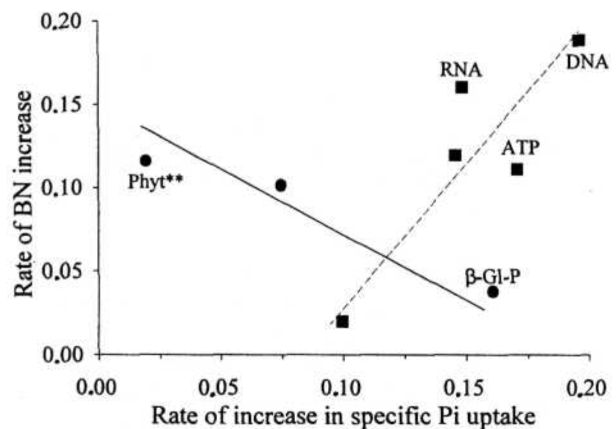

Fig. 9. Relationship between the increase of total Pi uptake rate and the rate of bacterial number increase in surface (lm depth) water of Lake Constance enriched with tested DOP compounds. ** - values calculated for period of intensive bacterial growth (from 22 to 57 hrs of incubation).

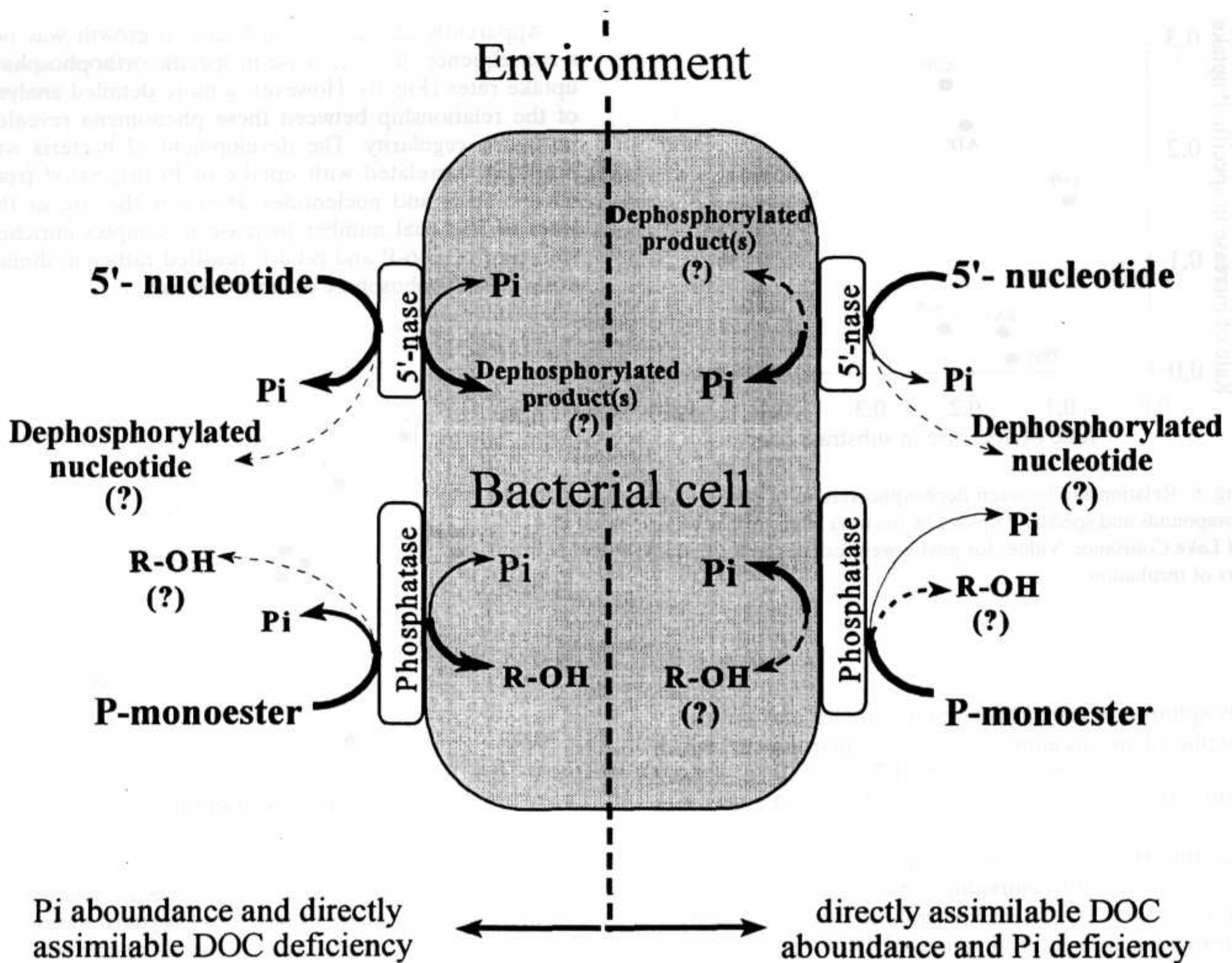

Fig. 10. Conceptual scheme of ecological functions of phosphatases and 5'-nase in environments characterized by various PO_4^{3-}/ DOC ratios. Solid arrows - processes well evidenced in the literature. Pools (?) and fluxes (dashed arrows) poorly known and not estimated quantitatively. For further explanations see text (Discussion).

Discussion

Although the role of phosphohydrolytic enzymes (especially APA) in assimilation of P from DOP by bacteria is generally accepted and relatively well documented [23, 24, 25, 26], our knowledge on other possible functions of these enzymes and final effects of their activity *in situ* is still scarce and inadequate. There are many reasons for such "one-way" thinking about the role of phosphohydrolases in the environment. For instance, it should be noted that the majority of observations on activity of APA reported in literature was carried out during summer stratification periods. Moreover, they were commonly focused on the photic zone of the lakes where bacteria were sufficiently supplemented with dissolved organic C and N compounds but strongly limited with orthophosphate. Under these conditions relationships between APA and DOP or orthophosphate concentrations are commonly clear and the most evident [27, 28, 29]. The second reason is deficient methodology that provides only indirect and often unequivocal results. The conclusions concerning DOP transformations by bacteria in lakes were mostly based on enzymatic or ^{32}P approaches. Unfortunately, although analysis of phosphohydrolase activities determined using standard enzymatic substrates (p-nitrophenyl-phosphate, methyl-umbelliferyl-phosphate) permits defining correctly maximal potential rates of orthophosphate liberation from DOP substrates under given conditions, it may be useless for estimation of the real demand of bacteria for phosphorus. Additionally, neither enzymatic nor radiochemical approaches (based on phosphomonoesters single labeled ^{32}P or ^{14}C as a substrates) allows finding what environmental factors incline bacteria to using DOP hydrolyzing enzymes preferentially for assimilation of orthophosphate, organic C or both of these substrates simultaneously from actually available organic P compounds [3].

Considering the fact that the suitability and perceptive possibilities of both standard enzymatic and ^{32}P-DOP uptake methods start to be limited on the present level of our knowledge on bacterial utilization of organic P substrates we decided to completely change our approach to

the subject during investigations discussed here. Namely, our experiments were carried out in late autumn, in the terminal phase of lake stratification. Low, but relatively stable orthophosphate concentrations in lake water suggested that bacteria in Lake Constance were not limited by Pi but their growth was mainly regulated by temperature and availability of organic carbon and nitrogen (Giide, personal comm.). Moreover, we did not focus on activity of phosphohydrolytic enzymes but rather on changes in bacterial growth rates, Pi (DOP) concentrations and rates of P uptake by microorganisms in lake water samples supplemented with various types of DOP substrates.

Since during the investigations rapid hydrolysis of every tested compound was observed (Fig. 4) we could conclude that under the ecological conditions described above both APA and 5'-nase mechanisms mediated assimilation of DOP by bacteria. Consequently, because bacteria were not limited by orthophosphate, which may be confirmed by rapid increase in orthophosphate concentration in samples enriched with each of tested compounds (Fig. 7) and by lack of correlation between increases of DOP hydrolysis and orthophosphate uptake rates (Fig. 6), one can presume that under non-limiting orthophosphate conditions at least part of bacterial phosphatases had to be synthesized constitutively, without the control of the Pi repression/derepression mechanism [30, 31]. Similar conclusions come from analysis of bacterial growth stimulation by various DOP sources (Fig. 3) and the relationship between increases in bacterial growth and substrate hydrolysis rates (Fig. 8). Nucleic acids that theoretically are not readily utilized (for their degradation two step enzymatic hydrolysis is required) or phytin (that needs at least 22 hours of adaptation of bacteria for its effective utilization) were the most favourable substrates for development of aquatic bacteria populations. Inversely, easily and fast hydrolysable 5'-nucleotides and P-Gl-P that were the best P source for the bacteria (Fig. 5) only slightly increased bacterial growth. Considering it, we hypothesize that an increase of some bacterial phosphorolytic activity in DOP-enriched lake water must be caused by reasons other than phosphorus deficiency and newly synthesized APA (and probably also 5'-nase) served for purposes other than just only Pi regeneration [29, 33, 34]. For instance, some of organic compounds liberated enzymatically from phosphate esters (glycerol or glucose) could be good energy sources used frequently for respiration processes [35, 36]. The others (nucleosides) could be directly incorporated by heterotrophic microorganisms into nucleic acids [37].

Limitation of bacterial growth by availability of easy utilizable organic carbon compounds seems to be the only logical explanation for the fact that the most intensive hydrolysis of added substrate was observed in samples in which the rate of bacterial number increase was the lowest (Fig. 8).

Assuming that bacteria under some circumstances can preferentially (in respect to Pi) utilize organic moieties liberated enzymatically from tested phosphate esters [32] one can propose that relatively slow decomposition (including dephosphorylation) of carbon rich compounds probably better supported bacteria with C than even fast degradation of substrates with low C content like P-Gl-P. Although this conclusion fits this assumption well, it does not elucidate why AMP (C:P molar ratio = 9:1) that disappeared very fast from the water samples did not cause distinct stimulation of bacterial growth. The most logical answer is provided by the analysis of the data points arrangement shown in Fig. 9. Although it may be only accidental, it may also suggest that different enzymatic systems were responsible for the decomposition and assimilation of both groups of tested DOP compounds by aquatic bacteria. The first, including nuclease and 5-nucleotidase, active towards nucleic acids, oligonucleotides and 5'-nucleotides that probably secured constant P "over-nutrition" of the microorganisms. The second, bi-functional consisted of phosphatases (and perhaps phytase) that by splitting off phosphoester bonds of G-6P, β-Gl-P? or phytin supported bacteria mainly with low molecular easy utilizable carbon compounds.

The conceptual scheme that summarizes our present knowledge on the role of phosphohydrolytic ectoenzymes of heterotrophic bacteria in lake waters and illustrates still unanswered questions is presented in Fig. 10. The results provided by the present investigations suggest that during periods of high concentrations of orthophosphate in lake water some non-specific P-monoesterases are probably still produced and display their activity. Additionally, it seems probable that similarly as was evidenced for 5'-nase [10, 11, 12], in the case of phosphatases non-repressible by orthophosphate some kind of metabolic coupling between DOP hydrolysis and Pi uptake can be expected. That coupling should be tight when orthophosphate concentration decreases and becomes loose when it starts to grow. Moreover, assuming that the mechanism by which aquatic bacteria couple hydrolysis of organic P compounds with assimilation of liberated product, is not limited only to orthophosphate but is more universal, one can suggest that it also regulates uptake of organic C moieties liberated enzymatically from nucleotides and P monoesters. Some literature data suggest that, for instance, uptake rates of Pi and glucosyl moiety of G-6P may be different and depend on environmental conditions [2, 32]. However, until now we have no evidence to support this hypothesis which may be definitely proved by application of double ^{32}P and ^{14}C labeled DOP substrates to further investigations of phosphohydrolase activity in aquatic environments.

Conclusions

1. Uptake of Pi by bacteria was generally not correlated with the rates of enzymatic hydrolysis of DOP com pounds.
2. In the presence in the environment orthophosphate concentrations as high as 160 nM, 5'-nucleotides were the best organic P source for the bacteria. Phosphorus from other phosphate esters (β-Gl-P, G-6P, phytin) and from more complex P compounds (DNA and RNA) was assimilated by bacteria considerably less efficiently.

3. Usually, carbon rich DOP compounds more effective ly stimulated bacterial growth than C poor P esters. Although phytin seems to be as good a substrate as G-6-P or ATP, for assimilation of this compound bac teria needed a relatively long (22 hrs) adaptation per iod.
4. Discrepancies between rates of hydrolysis, specific Pi uptake and bacterial growth in samples enriched with various DOP compounds suggest that bacterial phos phatases participated substantially in processes of bac terial DOC decomposition in lake water whereas 5'-nase secured mainly bacterial P demand.

Acknowledgements

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