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„Alkaline Phosphatase functionality and utilisation in *Alteromonas mediterranea*“

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Table of contents

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Abstract

Alkaline phosphatases are the main enzymes driving the regeneration of phosphate from organic matter in the ocean. Especially in regions where inorganic phosphate is low, they are crucial for marine microbes to overcome P-limitation. Multiple enzymes contribute to alkaline phosphatase activity, but their regulation and characteristics, as well as their distribution and functionality within the microbial community remain poorly understood. Here, we investigated how P-limitation and varying organic P-substrates affect phosphomono-, -di- and -triesterase activity in *Alteromonas mediterranea*. Previously, most studies focused on phosphomonoesterase when assessing phosphatase activity. We demonstrate that phosphomono- and diesterase activities were within a similar range, but displayed distinct kinetic characteristics, particularly under P-stress. Phosphotriesterase activity was detected in all cultures, despite the absence of annotated phosphotriesterases in *Alteromonas mediterranea*. This supports findings by Srivastava (2021) which showed that an alkaline phosphatase (PhoA) from *Alteromonas mediterranea* exhibits promiscuous phosphotriesterase activity. Low levels of phosphomono-, -di- and -triesterase activity were observed despite the presence of sufficient phosphate, regardless of the P-substrate. This implies that specific isozymes, such as PafA, function as constitutive phosphatases, enabling bacteria to constantly hydrolyse dissolved organic phosphorus and avoid an energy-costly phosphate-stress response. Our results suggest that measured alkaline phosphatase activity in cultures as well as in the environment originates from multiple enzymes that function together and provide microbes with diverse ecological strategies that can be adapted to changing environmental conditions. Further studies are necessary to improve our understanding on phosphate remineralisation and its implications on the phosphorus cycle and other biogeochemical fluxes.

Introduction

Phosphorus is an essential nutrient for life. It is vital for cell structure as it is a key component of phospholipids that form cell membranes; it facilitates energy transfer (e.g. through ATP) and forms the phosphate ester backbone of DNA and RNA (Davis & Mahaffey, 2017; De Duve, 1991). Inorganic phosphorus (P*i*) enters the ocean through various external sources, such as rivers, groundwater, atmospheric deposition, weathering of rocks, erosion, and human activities. However, the majority of phosphorus in the ocean that sustains microbial life is constantly transferred between dissolved and particulate pools by microbial processes, creating the marine phosphorus cycle (Karl, 2014; Paytan & McLaughlin, 2007). Vast regions of the ocean are limited in P*i*, such as the northwest Atlantic, northwest Pacific, and southwest Pacific, as well as the Mediterranean Sea (Duhamel et al., 2010). This limitation is expected to intensify in the future and, combined with ocean acidification may have considerable impacts on microbial communities, potentially affecting their productivity, nitrogen-fixing capacity, and other ecological services (Duhamel et al., 2021; Lin, 2023; Zhang et al., 2019). Thus, local recycling of phosphorus is crucial for sustaining community performance and supports approximately 90% of gross primary production in the ocean (Karl, 2014).

Dissolved organic phosphorus (DOP) forms the largest fraction of total phosphorus available in the water column, accounting for approximately 80% of total dissolved phosphorus in surface waters. It is a complex mixture of substrates that are released through excretion and decomposition processes of various aquatic organisms, including phytoplankton, zooplankton, and other members of the microbial community (Arnosti, 2014; Cotner & Biddanda, 2002; Karl & Björkman, 2002; Liang et al., 2022). DOP consists of a variety of organic phosphorus compounds, among which phosphoesters are the most abundant, accounting for 80–85% of total DOP, followed by phosphonates, which constitute 5–10%. Other forms such as inorganic and organic polyphosphates make up the remaining 8–13% (Diaz et al., 2018; Young & Ingall, 2010). The prevalence of phosphoesters is related to their abundance in living organisms, where they are synthesized and incorporated into essential biomolecules (Kolowith & Benner, 2001). Phosphomonoester bonds are for instance found in nucleotides and phosphosugars, while P-diester bonds are present in many phospholipids and nucleic acids (Diaz et al., 2018; Young & Ingall, 2010). In particular, P-diesters in the form of RNA and DNA have been reported to comprise approximately 12.9% of total DOP (Sakano & Kamatani, 1992).

The abundance of different phosphoesters in the water column is influenced by environmental factors and the present microbial community. From the limited data available, it appears that phosphomono- and diesters are present at similar levels (Monbet et al., 2009; Suzumura et al., 1998; Yamaguchi et al., 2019). Phosphotriesters, on the other hand, are only found to a lesser extent in biological systems. Yet, with the current influx of plastic waste and plasti-

cisers, anthropogenic phosphotriesters are entering the ocean. As a result, there is a growing interest in the microbial utilization of synthetic plasticisers, which contain phosphate in triester-bonds. Recent studies have demonstrated that certain microorganisms are able to metabolise synthetic phosphotriesters to some degree, but further research is necessary to assess potential impacts on plastic degradation (Despotovi et al., 2023; Kanehisa et al., 2008; Vila-Costa et al., 2019).

A majority of dissolved organic matter (DOM) is not directly available to microbes, since bacteria are generally unable to transport most of the materials larger than 0.6 kDa across their cell walls. This size limitation is exceeded by a considerable fraction of DOP; environmental DNA, for instance, typically consists of 6 to 23 kDa (Weiss, 1991). Some bacteria overcome this limitation through complex pathways that allow them to passively take up extracellular DNA. However, a majority of bacteria use extracellular enzymes to break down larger fragments into hydrolysed products that can then be further metabolised (Dell'Anno & Corinaldesi, 2004; Lennon, 2007; Paytan & McLaughlin, 2007). The most prominent enzymes catalysing this process are alkaline phosphatases (APases). They are produced by almost all organisms, including bacteria, archaea, and phytoplankton, and are ubiquitous in the marine environment (Karl, 2014; Karl & Björkman, 2002). Several factors, including substrate concentration, pH, and other environmental conditions, influence APase activity; however, our knowledge of those remains limited (Karl & Björkman, 2015).

It is widely accepted that enzymatic activity is regulated by end product concentrations: low concentrations lead to increased activity and *vice versa* (Chrost, 1990; Thomson et al., 2019). Thus, APase production in the ocean has long been interpreted simply as a response to low P*i* levels, enabling organisms to access DOP and avoid limitation, and several studies have even used APase activity as an indicator of phosphorus stress (Björkman & Karl, 2005; Dyhrman & Palenik, 1999; Dyhrman & Ruttenberg, 2006; Hoppe, 2003; Sebastián et al., 2004; Vila-Costa et al., 2019; Y. Yang et al., 2018). However, high levels of APase are often observed alongside increased P*i* concentrations in marine environments (Baltar et al., 2016, 2019; Hoppe & Ullrich, 1999; Lil et al., 1998). Moreover, many studies have observed an increase in APase activity with depth despite high P*i* levels, a phenomenon which has been termed the "APase paradox". Researchers have explained this paradox by speculating that APase was overexpressed to cleave the carbon backbone of phosphoesters from DOP and thereby help microbes overcome carbon limitation (Colman et al., 2005; Hoppe & Ullrich, 1999). Recent studies by Thomson (2019) contradicted this theory and hypothesized that high levels of APase accumulate in deeper layers since a large fraction of total APase is extracellular and solar radiation does not degrade enzymes as fast in the dark ocean. Altogether, recent findings have revealed that APase regulation is subject to more complex mechanisms, which are poorly understood but vital for understanding DOP remineralisation and its

links to the carbon and nitrogen cycle in the ocean (Karl, 2014; Lidbury et al., 2022; Srivastava et al., 2021).

The phosphate (Pho) regulon refers to the coordinated expression and synthesis of vital phosphorus-cycle proteins. It is initiated by a two-component system including the *PhoB* and *PhoR* transcription factors and is conserved across a wide range of bacteria. It is generally assumed that the Pho regulon upregulates selected Pho enzymes in response to low P*i* concentrations to degrade DOP and counteract P*i* limitation. Presumably, the presence of DOP substrates does not inhibit the activation of the pho regulon; hence P*i* limitation does not necessarily indicate phosphorus limitation. However, assumptions about the phosphorus stress response in marine bacteria are mostly solely built on laboratory-based models using *Escherichia coli*, where the Pho regulon consists of 31 genes that are arranged in eight separate operons (Karl & Björkman, 2015; Wanner, 1993). The most prominent enzymes contributing to APase activity belong to four distinct APase gene families: *phoA*, *phoD*, *phoX*, and *pafA* (Lidbury et al., 2022). The majority of bacteria possess at least one of these gene families. However, there are multiple other proteins, including nucleases, nucleotidases, phosphokinases, phospholipases, phosphomutases and nucleotidyl cyclases and transferases, that are presumably also controlled by the Pho regulon and share the ability to hydrolyse phosphoesters (Hoppe, 2003; Karl, 2014; Knowles, 1980).

Several studies have identified a specific P*i* concentration under which a starvation response is induced. However, the reported concentrations of this threshold, as well as the P*i*-stress response, exhibit considerable variations among different marine microbes (Karl, 2014; Sebastián et al., 2004).

Taken together, recent findings suggest that knowledge about the regulation and characteristics of the individual enzymes contributing to APase activity, as well as on their distribution and functionality within the microbial community, is limited. Additionally, under natural conditions, it is common to find low concentrations of P*i* but high levels of DOP. A recent study by Lidbury (2021) demonstrated that PafA is expressed independently of P*i* concentrations, which allows bacteria to constantly facilitate DOP hydrolysis and avoid an energetically costly P-limitation reaction (Lidbury et al., 2022; Sunden et al., 2016). Moreover, it is generally accepted that the four main APase gene families (*phoA, phoD, phoX*, and *pafA*) function primarily as phosphomono- or -diesterases. However, certain APase isozymes display substrate promiscuity, meaning that they can hydrolyse a variety of compounds beyond their primary substrate. PhoA from *Alteromonas mediterranea* for instance exhibits primary phosphomonoesterase activity, as well as promiscuous phosphodiesterase, phosphotriesterase and sulfatase activity (Srivastava et al., 2021). This substrate promiscuity varies between the different isozymes, as does their substrate affinity, their kinetic properties, and their response to environmental factors.

Having multispecific enzymes may allow microorganisms to access a wider range of organic phosphorus sources and influence remineralisation processes. Regardless, research on alkaline phosphatase activity has mostly focused only on phosphomonoesterase, while phosphodiesterase and other promiscuous activities remain largely unexplored (Thomson et al., 2020).

Altogether, this suggests that APase isozymes are not redundant, but rather function together and provide different ecological strategies of DOP utilisation that allow bacteria to adapt to environmental conditions, and may affect major biogeochemical fluxes in the ocean. This flexibility could be particularly advantageous for fast-growing bacteria like *Alteromonas*. Members of this genus are heterotrophic r-strategists, which exhibit rapid growth under favourable conditions and can account for up to 30% of relative bacterial abundance (Pedler et al., 2014; Tada et al., 2011). *Alteromonas mediterranea*, a deep-sea ecotype isolated from the deep Mediterranean basins, contains PhoA, PhoD, and PafA proteins (Ivanova et al., 2015).

Despite the critical role of alkaline phosphatase in the ocean, little is known about the role that the individual isozymes play in the marine microbial community, how they respond to different phosphorus conditions and what promiscuous properties they possess. This study is a first attempt to investigate the impact of phosphorus substrate dynamics on the functional role of APase in marine bacteria. We incubated *Alteromonas mediterranea* under six different phosphorus conditions, three with different DOP substrates and three with increasing P*i* limitation. In early stationary phase, we measured phosphomono-, -di- and -triesterase and estimated three kinetic parameters: the cell specific maximum velocity (V_{max}), the Michaelis-Menten constant (K_M), and the ratio of cell-specific V_{max}/K_M, which assesses catalytic efficiency (Azúa et al., 2003; Healey, 1980). We hypothesise that the individual substrates and P-limitation induce a respective reaction and affect the APase isozymes differently, and expect this to be reflected in the kinetic characteristics of phosphomono-, di- and triesterase.

Material and methods

Cultivation and maintenance of *Alteromonas mediterranea*

Alteromonas mediterranea strain Adriatic-2/DE/Deep Ecotype (Ivanova et al., 2015) was purchased from the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ No.: 17117) and routinely cultivated in artificial seawater. The main salts for the medium were prepared according to the Aquil medium formula (Berges & Harrison, 2005), with the exception that the hydrous and anhydrous salt solutions were autoclaved separately and then combined after cooling. Nutrients, vitamins, and trace metals were added individually, as described below.

The standard positive control culture contained 50 µmol L^{-1} of carbon in the form of glucose, NH_4Cl (10 µmol L^{-1}), KH_2PO_4 (1 µmol L^{-1}), vitamin B_{12} (0.37 nmol L^{-1}) and the following trace metals: $MnCl_2$ 4 H_2O (5 nmol L^{-1}), $FeCl_3$ 6H_2O (2 nmol L^{-1}), $NiCl_3$ 6 H_2O (12 nmol L^{-1}), $ZnSO_4$ 7 H_2O (9 nmol L^{-1}), Na_2SeO_3 (2.3 nmol L^{-1}) and Na_2MoO_4 2 H_20 (105 nmol L^{-1}). Trace metal concentrations used in this study reflect the highest levels observed in the open ocean (Bruland & Lohan, 2003). Stock cultures (approximately 6 million cells ml^{-1}) were frozen with 25% glycogen (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and stored at -80°C. Prior to starting a new experiment, frozen bacterial stocks were grown in artificial seawater, prepared as mentioned above. Cell abundance was tracked, and the experimental cultures were inoculated to a starting cell density of 2.5 x 10^5 cells L^{-1}.

For the main experiment, 700 ml of artificial seawater were divided into washed (1% HCl and Milli-Q water (Merck Millipore)) and combusted (450 °C, 4 hours) 1L glass bottles. Vitamin B_{12} and trace metals were added to the bottles and samples were incubated at 15°C shaking at 100 rpm for 50 hours. Nutrients were added following the stochiometric ratio C:N:P 50:10:1 for heterotrophic bacteria as described in Fagerbakke (1996) to final concentrations of 500, 100 and 1 µmol L^{-1} of carbon, nitrogen and phosphorus, respectively. Cultures were incubated with six different treatments in biological triplicates. Three cultures were given varying levels of P*i* limitation, while the other three cultures contained different organic phosphate substrates. A schematic diagram of the experimental setup is shown in Fig. 1.

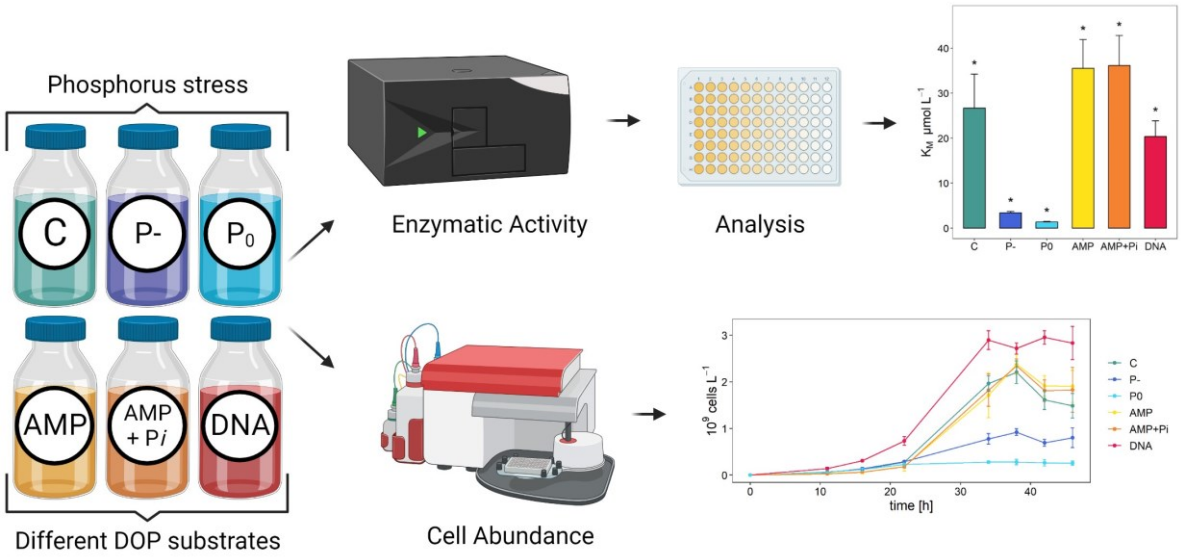

Fig. 1: Schematic diagram of the experimental setup created with BioRender.com.

Testing bacterial growth with different DOP substrates

Three cultures were grown on different DOP substrates, namely Adenosine-monophosphate (AMP) and DNA, as the sole phosphorus source. In the AMP culture, 1 µmol L^{-1} of phosphorus was added in the form of Adenosine-monophosphate (CAS: 149022-20-8, Sigma-

Aldrich). 1 µmol L^{-1} of AMP ($C_{10}H_{14}N_5O_7P$) already includes 10 µmol L^{-1} of carbon and 5 µmol L^{-1} of nitrogen. Thus, to maintain a C:N:P ratio of 50:10:1, only 40 µmol L^{-1} of carbon in the form of glucose and 5 µmol L^{-1} of NH_4Cl were added. The AMP+P*i* culture was prepared similar to the AMP culture, but with an additional 1 µmol L^{-1} of KH_2PO_4. The third culture (*DNA*) used DNA as a phosphorus source.

Substrate DNA was extracted from previous *Alteromonas mediterranea* cultures according to the following protocol: Bacteria were harvested via centrifugation (1000 rpm 10 min, Ultracentrifuge Optima XE, Beckman Coulter), redissolved into 180 µl ATL buffer (1% SDS, 2 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris, pH 8) and incubated with Protein Kinase K (CAS: 39450-01-6, Sigma-Aldrich) on a shaking heat block at 56°C. One hour later, MgCl (1 mmol L^{-1}), NaCl (2 mmol L^{-1}) and 1 µl of Glycogen (20 µg $µl^{-1}$; UltraPure™ Glycogen, Invitrogen™) were added and the mixture was vortexed. Next, 400 µl of ice-cold ethanol (99%) were added and the mixture was vortexed, incubated at -20°C for one hour and centrifuged at 14k rpm at 4°C for 45 minutes. After discarding the Ethanol and redissolving the DNA pellet in Sigma water (Sigma-Aldrich), the DNA was purified using the QuickClean 5M PCR Purification Kit (GenScript) and quantified following the instructions of the Qubit dsDNA BR (Broad Range) assay kit through a Qubit fluorometer (Qubit 2.0 Fluorometer, Invitrogen™). DNA was added to the culture to a final concentration of 1 µmol L^{-1} of phosphorus.

Alteromonas mediterranea under P*i* limitation

In the P*i*-limitation cultures, KH_2PO_4 was used as a phosphorus source. To ensure comparability with the DOP cultures, carbon and nitrogen were added in the form of 40 µmol L^{-1} as glucose, 5 µmol L^{-1} of NH_4Cl and 1 µmol L^{-1} of Adenosine. The control (C) culture consisted of 1 µmol L^{-1} Adenosine, 40 µmol L^{-1} of carbon (as glucose), 5 µmol L^{-1} of NH_4Cl and 1 µmol L^{-1} of KH_2PO_4. The phosphorus limited culture (P-) was prepared like the control culture, but with only 0.1 µmol L^{-1} of phosphorus. No phosphorus was added to the phosphorus-depleted culture (P_0), but all other parameters were the same as for the control culture.

Controls and preliminary experiments with *Alteromonas mediterranea*

To test substrate concentrations, a preliminary experiment was conducted using 200 ml cultures in biological duplicates. Samples were prepared using a similar procedure as described in the main experiment section above (Fig. 1), with the exception that a vitamin mixture, prepared according to Aquil media instructions, was used instead of solely adding vitamin B_{12}. The experiment included the following four treatments, which were prepared as described in the main experiment section: Control (C), AMP, DNA, P-, and P_0 (Fig. 1).

As controls, four additional cultures were grown in previously combusted 75 ml glass tubes, containing 40 ml of medium. The positive "*Control"* culture contained Adenosine (1 µmol L^{-1};

CAS: 58-61-7, Acros Organics), carbon (40 µmol L^{-1}; as glucose), NH_4Cl (5 µmol L^{-1}) and KH_2PO_4 (1 µmol L^{-1}). The *Glucose* culture was grown without Adenosine to test whether the C:N mixture of Adenosine, glucose, and NH_4Cl would produce comparable results to a simple glucose:NH_4Cl 5:1 ratio (carbon 50 µmol L^{-1} (as glucose), NH_4Cl 10 µmol L^{-1} and KH_2PO_4 1 µmol L^{-1}). The first negative control (*No Cells*) was designed to test the medium for contamination by using the same media composition as the positive control culture, but without adding cells. The second negative control (*No Nutrients*) consisted only of *Alteromonas mediterranea* in artificial seawater without any supplements.

Bacterial abundance measurements

Samples were preserved with glutaraldehyde (2% v/v final concentration) every 3 to 6 hours and stored at -20°C until analysis. Bacterial abundance was measured through flow cytometry using an Accuri™ C6 Plus Flow Cytometer (Becton Dickinson, Franklin Lakes, USA). To enumerate bacteria, samples were diluted (1:10) with TE buffer (1 mmol L^{-1}, autoclaved). Samples were stained with SYBR® Green I (Sigma-Aldrich) 1x and incubated in 500 µl reactions in the dark at room temperature for ten minutes. Samples were analysed at medium flow rate (0.24 µl s^{-1}) for 2 minutes and counts were measured at 488 nm wavelength.

Determination of enzyme kinetic parameters for phosphomono-, -di- and -triesterase

Alteromonas mediterranea cultures were tested for phosphomono-, di- and -triesterase activity using the p-nitrophenol based substrates p-nitrophenylphosphate disodium salt hexahydrate, Bis(p-nitrophenyl) phosphate sodium salt, and Paraoxon-methyl, respectively. All chemicals were purchased from Sigma-Aldrich and appropriate stocks were prepared in Milli-Q water and filtered through 0.2 µm. Upon enzymatic hydrolysis, chromogenic p-nitrophenol is released from the substrates, leading to a chromophoric signal, which can be measured at a wavelength of 405 nm (Srivastava et al., 2021).

To calculate the concentration of released p-nitrophenol, measured relative enzymatic activity of the samples was compared to a standard curve. p-Nitrophenol (ACROS Organics, Fisher Scientific GmbH, Austria) diluted in artificial seawater was used for the standard curve at 10, 1, 0.5, and 0.1 µmol L^{-1}. All samples were prepared in technical triplicates and artificial seawater blanks were used as a control. Phosphatase activities of the cultures were assessed during early stationary phase after 42 hours of incubation (Fig. 1). Enzymatic activity was measured using a microplate reader (TECAN, Infinite 200 PRO) in 300 µl reactions incubated in Greiner Bio-one 96-well non-binding microplates. Samples were analysed at three time points, right after incubation (t0), at 45 and 150 minutes and were incubated in the dark at room temperature. Substrate concentrations were chosen based on preliminary experi-

ments. For phosphomono- and -diesterase, 10 – 0.977 µmol L^{-1} of p-Nitrophenylphosphate and Bis-p-nitrophenylphosphate were used, respectively. For phosphotriesterase 20 – 0.02 µmol L^{-1} of Paraoxon-methyl were added. The initial rate constants were calculated for each replicate using the starting linear slope of each substrate concentration and fitted into a linear regression.

Statistical analyses and visualization

Michaelis–Menten calculations of K_M and V_{max} were obtained using a non-linear regression leaning on the script from Huitema & Horsman (2018). For cell specific V_{max}, significance between groups was tested. To check normality and homogeneity of variance a Shapiro-Wilk normality test and a Bartlett test of homogeneity of variances was performed respectively. Differences in cell-specific V_{max} between treatments was assessed using an analysis of variance (ANOVA) followed by a Tukey's Honestly Significant Difference (Tukey-HSD) post hoc test. All statistical analyses and visualizations were performed using R version 4.2.2.

Results

Phosphorus limitation inhibits growth of *Alteromonas mediterranea*

Two 40 ml negative controls, one without cells (*No Cells*) and one without nutrients (*No Nutrients*), grew from a starting concentration of 2.5 x 10^5 cells L^{-1} to a maximum cell density of 2.6 x 10^6 and 8 x 10^7 cells L^{-1}, respectively (Fig. 2). This confirms that nutrient stock solutions were not contaminated and that cells grew exclusively on the nutrients provided. The *Glucose* and the Control (C) cultures displayed comparable growth and reached similar cell densities after 42 hours (1.3 x 10^9 and 1.9 x 10^9 cells L^{-1}, respectively). Thus, incorporating Adenosine in the culture medium, in addition to glucose and NH_4Cl in the Control (C), did not inhibit growth as compared to compared to the *Glucose* culture.

In the main experiment, the 700 ml cultures started with 2.5 x 10^5 cells L^{-1}. After 42 hours of incubation the cultures had entered stationary phase with measured values ranging from 3.1 x 10^9 cells L^{-1} in the DNA culture to 2.2 x 10^8 cells L^{-1} in the P-depleted (P_0) culture. The DNA culture reached a higher cell density compared to all other treatments (Fig. 3), while growth on AMP, AMP+P*i* and P*i* (C) was on a similar level. Before the main experiment, a preliminary experiment was conducted in 200 ml volumes. There, no noticeable difference was observed between cultures that were incubated with DNA versus those incubated with AMP or P*i* (Fig. 4).

Reducing phosphorus concentrations resulted in decreased cell densities in the main experiment (Fig. 3), the 40 ml control experiment (Fig. 2) and the 200 ml preliminary experiment (Fig. 4). In the main experiment, the phosphorus-limited cultures (P-) reached 8 x 10^8 cells L^{-1} on average, while the P-depleted cultures (P_0) reached 2.6 x 10^8 cells L^{-1} (Fig. 3).

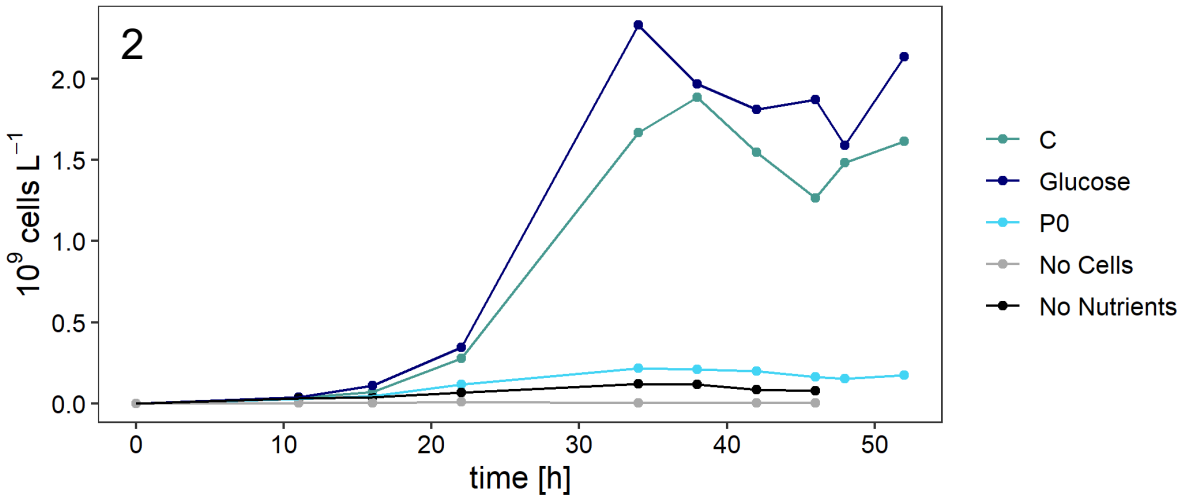

Fig. 2: 40 ml control cultures were grown simultaneously to the main experiment in Fig. 3. The *Glucose* culture was incubated without Adenosine as a comparison to the Control (C) culture. The negative controls were grown with no cells (*No Cells*) or no nutrients (*No Nutrients*), respectively. The P_0 culture was grown without the addition of phosphorus.

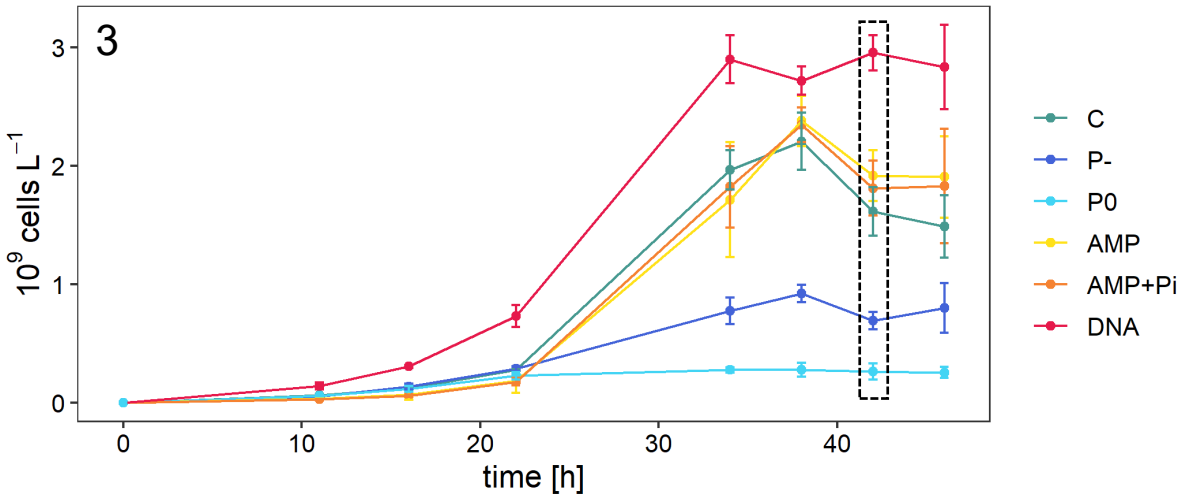

Fig. 3: *Alteromonas mediterranea* cultures on various organophosphorus substrates (AMP, DNA and AMP+P*i*), under phosphorus-limitation (P-), with no added phosphorus (P_0) and under optimal conditions (C). Results presented are the mean values of biological triplicates, error bars represent the standard deviation. Cultures were incubated in 700 ml volumes and further enzymatic analysis was performed after 42 hours (indicated by the black dashed square).

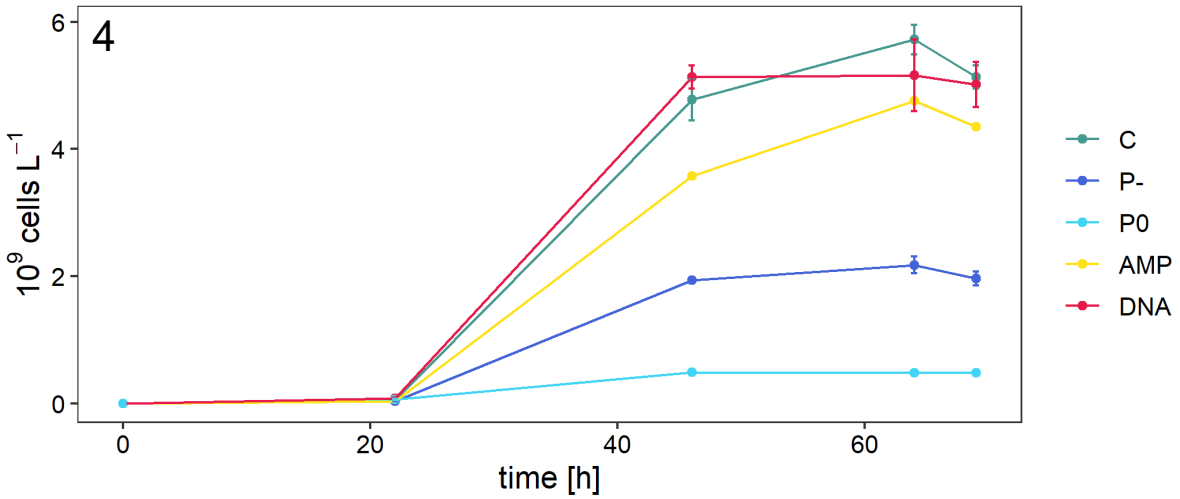

Fig. 4: Preliminary cultures were cultivated in duplicate 200 ml reactions, following a similar setup as described for the main experiment (Fig. 3).

Determination of enzymatic kinetic parameters

Phosphomono-, -di- and triesterase activities were detected for all six treatments. Phosphomonoesterase ranged between -0.007–2.231 fmol h^{-1} $cell^{-1}$ (mean ± SE: 0.405 ± 0.012 fmol h^{-1} $cell^{-1}$), phosphodiesterase between -0.003–0.960 fmol h^{-1} $cell^{-1}$ (mean ± SE: 0.051 ± 0.0065 fmol h^{-1} $cell^{-1}$) and phosphotriesterase activity between -0.375–0.403 fmol h^{-1} $cell^{-1}$ (mean ± SE: 0.05 ± 0.026 fmol h^{-1} $cell^{-1}$) (Fig. 5). Negative rates are not realistic, so when detected they are associated to the detection limit and variability of the method.

All cultures that were not P-limited (C, AMP, AMP+P*i*, and DNA) displayed similar hydrolysis rates (Figs. 5a, 5b and 5c), whereas values increased with decreasing phosphorus concentrations (Figs. 5A, 5Band 5C). Phosphotriesterase hydrolysis rates were lower compared to those of phosphomono- and -diesterase and did not exhibit clear Michaelis-Menten kinetics (Figs. 5C and 5c).

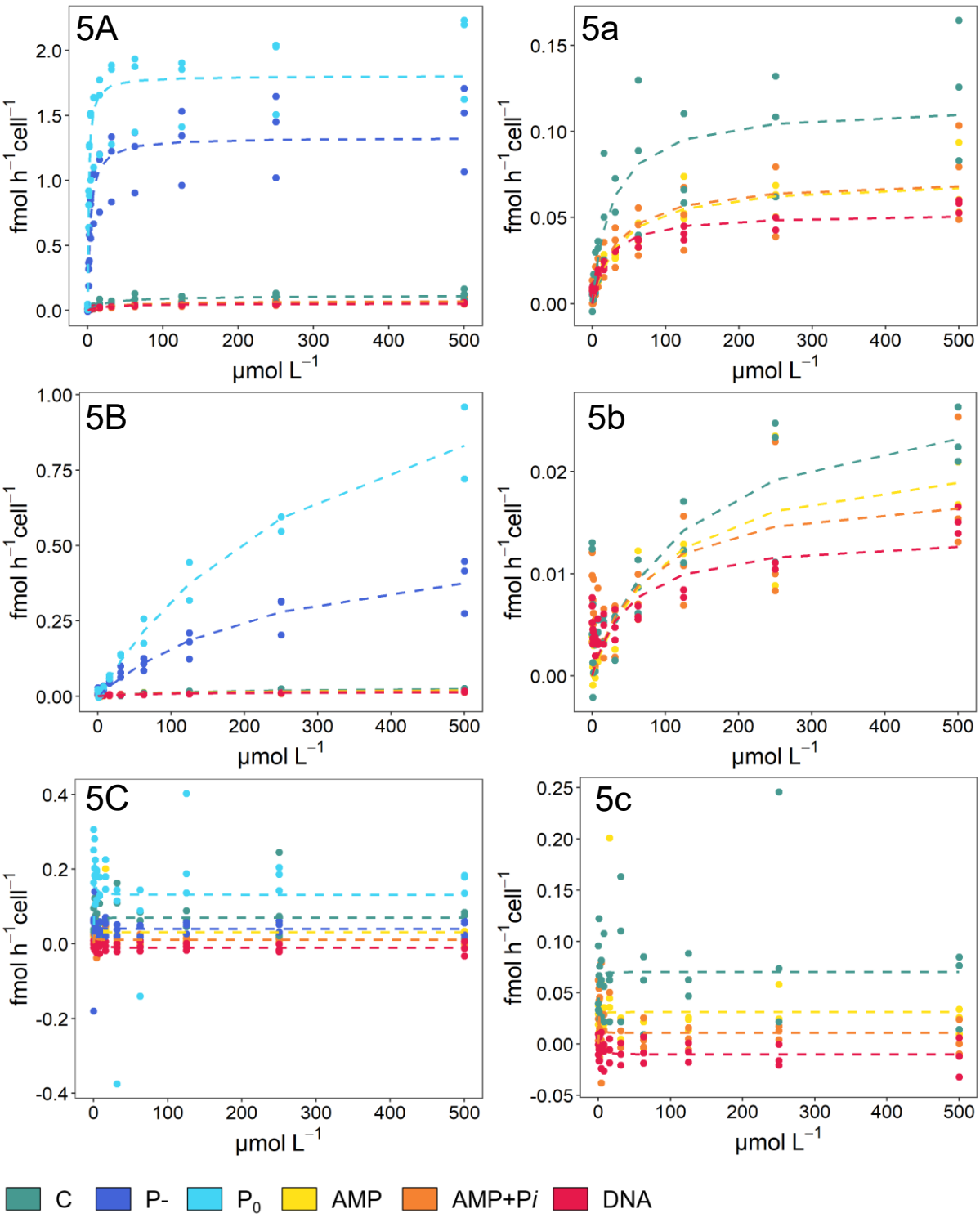

Fig. 5: Cell-specific hydrolysis rates of phosphomono-, -di- and triesterase (represented by (A), (B) and (C), respectively) for the six *Alteromonas mediterranea* cultures. Plots (a), (b) and (c) provide a detailed ("zoom in") overview of the unlimited treatments (C, AMP, AMP+P*i* and DNA). Dots represent the average of technical triplicates and dashed lines the hydrolysis curves.

Table 1: Michaelis-Menten parameters for phosphomono-, -di- and triesterase activity.

	K_M µmol L^{-1} P-monoesterase	K_M µmol L^{-1} P-diesterase	V_{MAX} fmol $cell^{-1}$ h^{-1} P-monoesterase	V_{MAX} fmol $cell^{-1}$ h^{-1} P-diesterase	V_{MAX} fmol $cell^{-1}$ h^{-1} P-triesterase
C	26.69 ± 7.55	133.72 ± 57.35	0.12 ± 0.04	0.03 ± 0	0.07 ± 0.04
P-	3.42 ± 0.28	254.09 ± 31.98	1.33 ± 0.3	0.57 ± 0.16	0.04 ± 0.02
P_0	1.34 ± 0.14	336.17 ± 53.06	1.8 ± 0.32	1.43 ± 0.36	0.13 ± 0.03
AMP	35.51 ± 6.42	103.4 ± 42.4	0.07 ± 0.02	0.02 ± 0.01	0.03 ± 0.02
AMP+Pi	36.16 ± 6.66	69.03 ± 38.21	0.07 ± 0.02	0.02 ± 0.01	0.002 ± 0.002
DNA	20.33 ± 3.5	47.16 ± 21.38	0.05 ± 0.005	0.01 ± 0.004	-0.01 ± 0.011

Data reported are the cell specific V_{max} (V_{max} divided by cell abundance), the K_M Michaelis–Menten constant and the V_{max}/ K_M (Fig. 3). No K_M values could be calculated for phosphotriesterase. Values are provided for the six treatments tested in the main experiment C (Control), P- (P-limited), P_0 (P-depleted), AMP (Adenosine-monophosphate), AMP+P*i* (Adenosine-monophosphate and P*i*) and DNA (DNA).

Lower P*i* concentrations result in higher V_{max} values

Cell specific V_{max} values were in a similar range for phosphomono- and -diesterase; however, consistently lower for phosphodiesterase (Figs. 6A and 6B). Decreasing P*i* concentrations resulted in increasing cell specific V_{max}, reaching the highest values in the P_0 culture for phosphomonoesterase (Table 1). No significant differences ($p < 0.05$, TukeyHSD post-hoc test) were found between samples that were not limited in P*i* (C, AMP, AMP+P*i* and DNA) for phosphomono- and -diesterase, regardless of the P-substrate (Figs. 6A and 6B). Measured phosphotriesterase activity was partly below the detection limit, and V_{max} values were substantially lower compared to those of phosphomono- and -diesterase. Still, a consistent trend of higher cell-specific V_{max} values with decreasing phosphorus levels could be observed, since the P_0 culture exhibited the highest V_{max} among all cultures ($p < 0.05$, TukeyHSD post-hoc test; Fig. 6C).

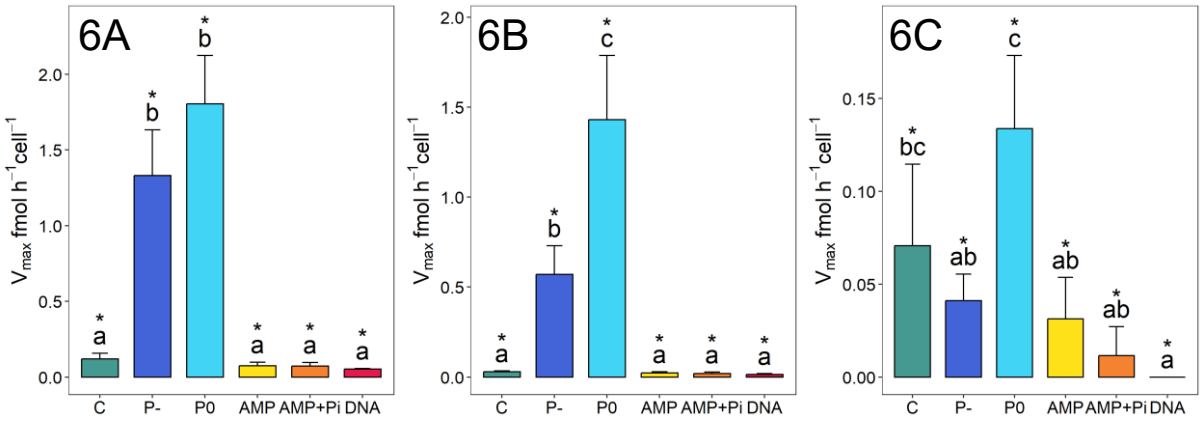

Fig. 6: Cell specific V_{max} values for phosphomonoesterase (A), phosphodiesterase (B) and phosphotriesterase (C) for the six treatments. V_{max} were calculated for each biological triplicate; bars represent averages and error bars standard errors. The lettering system indicates significant differences between treatments based on the Tukey test results. The same letter indicates no significant difference ($p > 0.05$), while different letters indicate significant differences ($p < 0.05$). Asterisks (*) show a

significant fit to the non-linear regression Michaelis-Menten model ($p < 0.05$) as described by Huitema and Horsman (2018).

P*i* limitation affects the K_M of phosphomono- and -diesterase differently

In general, phosphodiesterase had notably higher K_M values compared to phosphomonoesterase (Table 1). Due to technical limitations, it was not possible to determine the K_M values for phosphotriesterase. For phosphomono- and diesterase no significant difference between non P-limited samples (P+, AMP, AMP+P*i* and DNA) could be detected. With increasing P-limitation, the K_M value of phosphomonoesterase decreased. In contrast, phosphodiesterase showed a reverse trend, where K_M values increased with low P*i* concentrations (Fig. 7A and 7B).

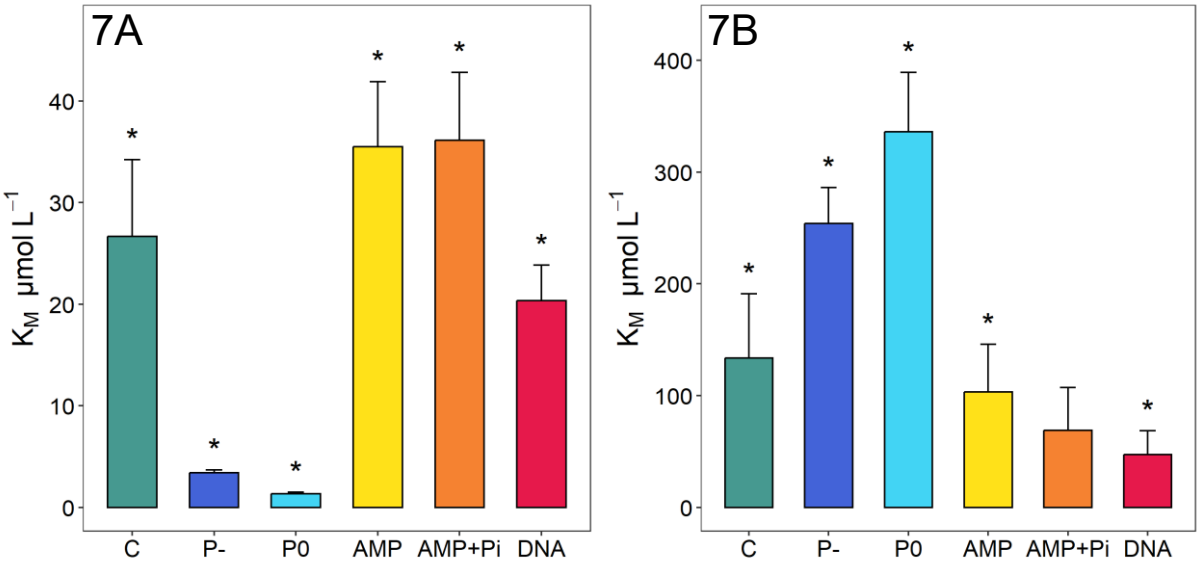

Fig. 7: Calculated Michaelis Menten constants (K_M) for phosphomono- and -diesterase. Bars show the K_M values calculated for all replicates of a treatment and error bars indicate the standard deviation Asterisks (*) represent significant fit to the Michaelis-Menten model ($p < 0.05$). All K_M values were significant ($p < 0.05$), except for the phosphodiesterase K_M of AMP+P*i*, where $p = 0.08$.

Cell-specific V_{max}/K_M increases upon limitation

Enzymatic efficiency was further analysed by assessing the initial slope of the Michaelis-Menten curve by calculating the ratio of cell-specific V_{max} to K_M. This was done by dividing the cell-specific V_{max} by the K_M for phosphomono- and -diesterase. The cell specific V_{max}/K_M was considerably higher for phosphomonoesterase (range: 0.002 ± 0.001 (AMP+P*i*) to 1.341 ± 0.4605 (P_0)) than for phosphodiesterase (range: 0.0002 (AMP) ± 0.0001 to 0.0042 ± 0.003 (P_0)) (Fig. 8). Along with the V_{max} and K_M, no significant difference could be detected between samples that were not limited in phosphorus (P+, AMP, AMP+P*i* and DNA), for both phosphomono- and -diesterase. The ratio of cell-specific V_{max}/K_M increased with P-limitation for both phosphomono- and -diesterase.

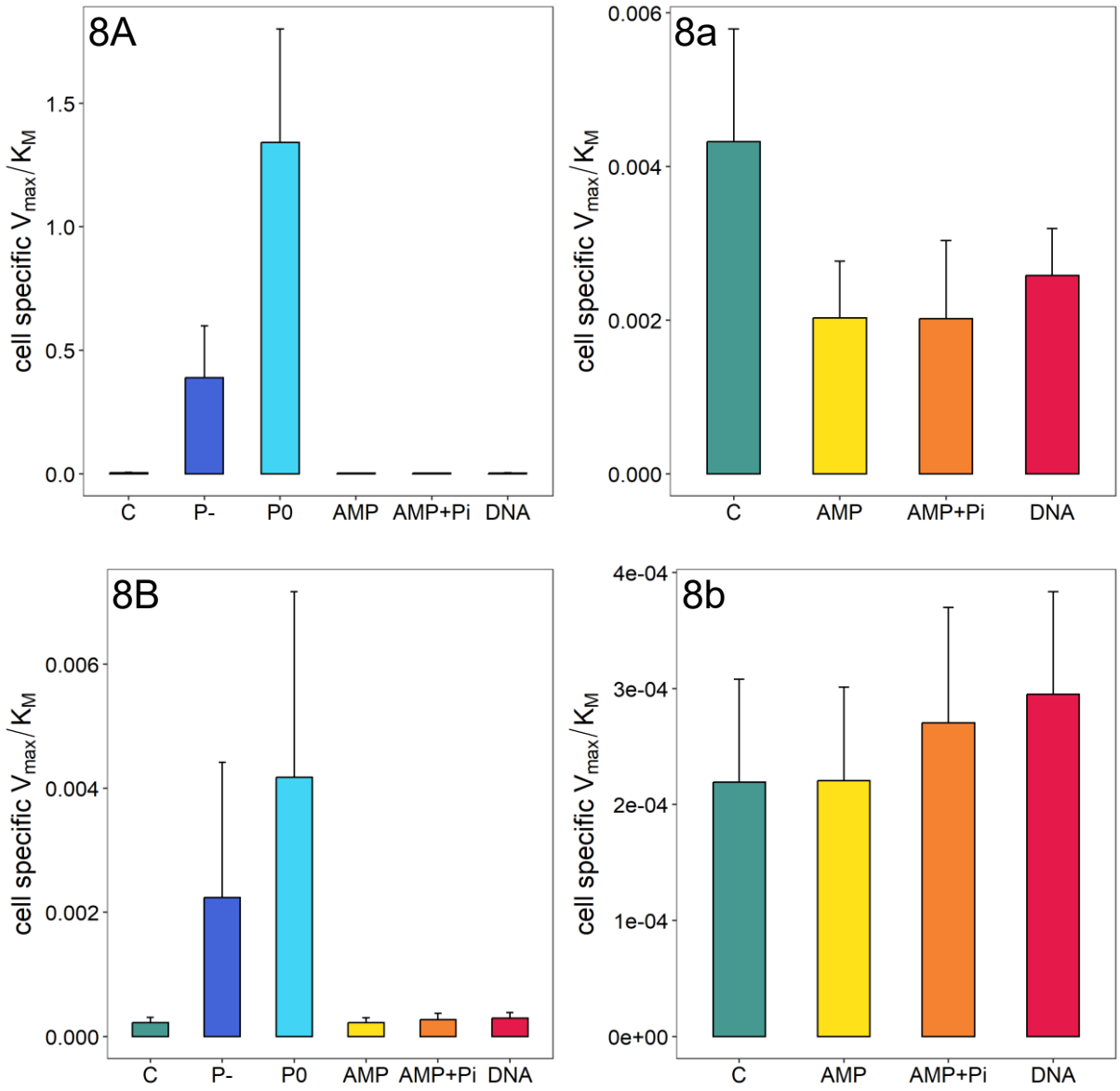

Fig. 8: Cell-specific V_{max}/K_M values calculated by dividing the cell specific V_{max} by the K_M for each of the six treatments for phosphomonoesterase (5A and 5a) and phosphodiesterase (5B and 5b). The graphs with lower case letters provide a more detailed insight into non P-limited cultures (C, AMP, AMP+P*i* and DNA).

Discussion

DOP supports microbial growth

Overall, our results show that *Alteromonas mediterranea* is capable of thriving on various DOP substrates as a sole phosphorus source. Cultures grown on AMP and DNA showed no inhibition compared to cultures grown solely on directly accessible P*i*. Reducing the relative amount of P*i* however, resulted in lower cell densities (Fig. 3). Previous research has shown that some marine microbes can use external DNA and nucleotides as a primary source of phosphorus and nutrition. However, there appears to be considerable variation in how different bacterial families and clades utilize DOP substrates, and not all species exhibit this ca-

pability (Dell'Anno & Corinaldesi, 2004; Diaz et al., 2018; Lennon, 2007; Pinchuk et al., 2008; K. Yang et al., 2022). For instance, the size of DOM alone can lead to the dominance of specific phylogenetic clades, influencing the composition of microbial communities (Lennon, 2007). Moreover, even closely related taxa can exhibit different DOM degradation rates and studies have observed considerable variability in growth efficiency among three *Shewanella* strains grown exclusively on DNA. These variations in DOP utilisation may be due to differences in the enzymatic repertoire, specific "phosphatase" activities, production rates or other utilization pathways of marine microbes (Lennon, 2007; Pinchuk et al., 2008).

In a study by Pinchuk (2008), *Shewanella oneidensis MR-1* reached maximum cell density faster when incubated solely on DNA as compared to P*i*, but the reason for this was not clear. This happened also in our study, *Alteromonas mediterranea* grew faster and to a higher cell density when grown with DNA compared to all other cultures in the main experiment (Fig. 3). However, preliminary data showed similar growth rates among all unlimited cultures (C, AMP, AMP+P*i* and DNA), including those using DNA (Fig. 4). The DNA was prepared, purified and quantified separately for each experimental run, thus differences in the DNA substrate may have been caused by sample handling or deviating concentration measurements. Thus, it is possible that the presence of nutrient-containing substrates in the kit solutions accounts for the observed increase in cell growth on DNA, rather than that DNA is metabolised more effectively than P*i* or AMP. It is worth noting that all samples in our study were prepared with adenosine as part of their nutrient supply, while DNA contains a mixture of adenosine, cytosine, thymine and guanine. However, it is unlikely that this difference in nucleotide composition caused such a significant change in growth.

Research conducted by Adams (2022) demonstrated that AMP and other DOP substrates supported similar growth of *Ruegeria pomeroyi DSS-3*. Our results build upon this discovery and represent the first evidence that *Alteromonas mediterranea* can thrive on DNA and nucleotides under open ocean resembling conditions. This supports previous studies which suggested that the majority of aquatic microbes use APases to hydrolyse phosphoesters and other forms of DOP to access nutrients. DOP and extracellular nucleotides are especially abundant after blooms and proteobacteria, which are typically opportunistic bacteria, tend to thrive under such conditions of excessive nutrient supply. Members, such as *Alteromonas* are well known to produce extracellular enzymes to hydrolyse released DOM and have been identified as dominant microbes thriving on extracellular DNA enrichments (Dell'Anno & Corinaldesi, 2004; Lennon, 2007; Pinchuk et al., 2008; Tada et al., 2011; K. Yang et al., 2022). Thus, proteobacteria and particularly *Alteromonas* play a crucial role in the remineralisation of DOP after blooms, yet knowledge on the pathways and enzymatic mechanisms is limited (Fandino et al., 2001; Pinhassi et al., 1999; Tada et al., 2011). Further research is

needed to comprehensively analyse how DOP is metabolised by different alkaline phosphatases and assess how this impacts the marine phosphorus cycle.

Extracellular proteins in *Alteromonas mediterranea* involved in phosphorus acquisition

Alteromonas mediterranea contains multiple extracellular proteins that are potentially involved in acquiring phosphorus from DOP, including two annotated extracellular nucleases (F2G2Z7 and F2GB28) and one annotated exonuclease/phosphatase (F2G4X2). In addition, it encodes three genes that are annotated as alkaline phosphatases containing signal peptides, indicating that they are extracellular.

One of these, F2G8N3, belongs to the PhoA protein family (pfam: PF00245), which has been found throughout the ocean and is induced by P*i* depletion (Berlutti et al., 2001; Horiuchi T, 1959; Wanner, 1996). The PhoA protein from *Alteromonas mediterranea* has been extensively studied by Srivastava (2021), who demonstrated that it exhibits promiscuous activity as a phosphodi- and -triesterase and as a sulfatase in addition to its primary role as a phosphomonoesterase. The F2G754 protein is a member of the PhoD family (pfam: PF09423), whose enzymes are known to function as both phosphomono- and -diesterases in many marine organisms. PhoD is the most abundant APase in marine bacteria and is activated by phosphorus starvation (Adams et al., 2022; Kageyama et al., 2011; Luo et al., 2009; Ragot et al., 2015). F2GD45 is also annotated as an APase, but it does not appear to belong to any known APase superfamily. The F2G994 gene is annotated as a phosphodiesterase in *Alteromonas mediterranea DE* and belongs to the PafA protein family (pfam: PF01663). PafA is typically associated with phosphodiesterase, but studies have shown that it actually exhibits greater activity as a phosphomonoesterase. In *Flavobacterium johnsoniae*, PafA is P*i*-insensitive and produces most of the constitutive APase activity (Lidbury et al., 2022).

The specific role of each APase isozyme in phosphorus acquisition and whether the concept of constitutive APase activity extends to other bacteria, such as *Alteromonas*, remains unclear. Previous studies suggested that having multiple APase isozymes allows bacteria to utilise diverse ecological strategies to acquire P*i* form the environment (Luo et al., 2009). However, knowledge on how different enzymes work together to avoid phosphorus starvation and to what extent non-APase genes are involved in both constitutive and inducible APase activity is limited.

The overlooked role of promiscuous and constitutive APase activity in DOP hydrolysis

Previous studies have mostly focused on measuring phosphomonoesterase activity when assessing DOP hydrolysis and phosphorus depletion (Adams et al., 2022; Lidbury et al., 2022; Pinchuk et al., 2008). The reaction of promiscuous alkaline phosphatase activities as a response to changing environmental conditions has largely been overlooked, despite the fact that phosphatases partly also exhibit phosphodiesterase and on lower levels, phosphotriesterase and sulfatase activity (Lidbury et al., 2022; Srivastava et al., 2021). Our study provides an in-depth kinetic analysis of phosphomono-, -di- and -triesterase of *Alteromonas mediterranea* cultures that were grown on DOP and under P-stress conditions. Overall, our results are consistent with previous studies that have reported similar levels of phosphomonoesterase activity regardless of the type of organic phosphorus substrate used (P*i*, AMP, AMP+P*i* or DNA) (Adams et al., 2022; Pinchuk et al., 2008). Moreover, we now extend this finding to phosphodi- and -triesterase activity. In addition, we found that all three enzymatic activities exhibited comparable cell-specific V_{max}, K_M and cell-specific V_{max}/K_M values among cultures that were not limited in P*i* (P*i*, AMP, AMP+P*i* or DNA).

In the case of the "APase paradox", Hoppe and Ullrich (1999) found increased APase activity in the deep sea despite high P*i* concentrations and proposed that APase was upregulated to utilise the carbon backbone of DOP. However, no studies have demonstrated that APase is actually upregulated as a response to low carbon levels (Hoppe & Ullrich, 1999). We tested whether APase activity would increase if bacteria were exposed to excessive amounts of phosphorus but limited in carbon in the AMP+P*i* culture. However, providing the bacteria with AMP and P*i*, and therefore doubling the amount of phosphorus present, did not result in any considerable difference to the culture grown on AMP alone. Both cultures exhibited similar bacterial growth, Michaelis-Menten curves and V_{max} and K_M values showed similar patterns, suggesting that a comparable enzymatic response was obtained. Our findings thus contradict the theory that APase is upregulated in response to low carbon levels (Hoppe & Ullrich, 1999; Thomson et al., 2019). Instead, we support studies that suggested that higher levels of APase in the deep sea can be attributed to the accumulation of cell-free enzymes with long lifetimes (Thomson et al., 2019).

Enzyme production is typically regulated by the end-product concentration of an enzymatic reaction. Therefore, under low P*i* concentrations APase should be upregulated (Chrost, 1990; Thomson et al., 2019). Yet, cultures grown on DOP substrates as a sole P-source exhibit the same level of APase activity as those grown on P*i*, despite lacking directly available P*i*. This observation was puzzling since in cultures incubated with P*i*, direct uptake of P*i* would seemingly eliminate the need for enzymatic hydrolysis. However, when incubated with AMP or DNA, APase is essential for hydrolysing phosphoester bonds and making phospho-

rus accessible. Nonetheless, APase levels did not increase in DOP cultures, whereas substrate limitation led to a visible rise in enzymatic activity.

Lidbury (2021) demonstrated that specific phosphatase isozymes are constantly expressed, while others are only induced upon P-stress. Therefore, we propose that this strategy of sustaining a constant level of APase activity to extract P*i* from DOP under natural, non-stressed conditions is also employed by *Alteromonas* and potentially other bacterial species. In *Flavobacterium johnsoniae*, PafA appears to be the primary constitutive APase. Considering that *Alteromonas mediterranea* encodes an enzyme that belongs to the PafA family, we hypothesize that PafA may also be responsible for constitutive APase activity in *Alteromonas*. However, since the two proteins belong to different bacterial groups, their enzymatic properties might differ. Thus, further studies are needed to confirm whether PafA is also a major contributor to constitutive APase activity in *Alteromonas mediterranea* to provide insights into the mechanisms underlying its ability to access P*i* from DOP.

DNA is a polymer of nucleotides linked by phosphodiester bonds, which can be broken down by extracellular enzymes such as DNases, exonucleases, and phosphodiesterases. Since enzymatic activities were not increased in the DNA culture relative to other cultures that were not P-limited and grown on either phosphomonoesters (AMP) or directly accessible P*i*, we hypothesize that the enzymes responsible for constitutive APase activity in the unlimited cultures exhibit phosphodiesterase activity. The extent to which PafA causes phosphodiesterase activity and how other enzymes, such as DNases and exonucleases, contribute to constitutive phosphodiesterase activity is not yet clear. Furthermore, it remains uncertain how prevalent constitutive phosphodiesterase activity is within the microbial community. Additional studies are needed to comprehend the role of the different enzymes involved in DOP hydrolysis and the contribution of promiscuous activity. Moreover, understanding the enzymatic characteristics of each APase isozyme in *Alteromonas mediterranea* will enhance our knowledge of the diversity of phosphorus acquisition strategies in bacterial species and their ecological implications.

Putting phosphomono- and -diesterase activity into context

Phosphomonoesterase rates measured in our *Alteromonas mediterranea* cultures (71.2 to 139.1 nmol L^{-1} h^{-1} with 100 µmol L^{-1} pNPP) were elevated but similar to those measured in the Gulf of Trieste, a region that typically experiences seasonal P-limitation (Celussi et al., 2019; Celussi & Del Negro, 2012). However, they were considerably higher compared to rates detected in subantarctic waters and in the North Atlantic (Steen & Arnosti, 2011; Thomson et al., 2019). Nonetheless, cell specific phosphomonoesterase rates measured in our study are consistent with those reported by Adams (2022) for *Ruegeria pomeroyi* cultures grown on DOP substrates, including AMP.

Thomson (2020) conducted the first and only comprehensive analysis of phosphodiesterase activity in the open ocean. In our study, we discovered that hydrolysis rates of P-unlimited cultures (ranging from 15.82 to 27.22 measured with 125 µmol L^{-1} pNPP) were elevated but still fell within the reported range by Thomson (2020). Our study is the first to provide kinetic parameters for phosphodiesterase activity in marine bacterial cultures, as phosphodiesterase activity has been largely overlooked in the past. In general, hydrolysis rates and V_{max} values of phosphodiesterase were lower compared to those of phosphomonoesterase, but still within a similar range. This is consistent with findings of Thomson (2020), who reported that both activities were in a comparable range across the global oceans, although with varying ratios depending on geographic region and season.

Phosphotriesters were not common in biological systems before their industrial production and only few phosphotriesterases have been identified in nature. Consequently, research on phosphotriesterase rates across the ocean as well as on enzyme kinetic parameters is scarce (Despotovi et al., 2023; Vila-Costa et al., 2019). We demonstrate that *Alteromonas mediterranea* exhibits phosphotriesterase activity, irrespective of the source or concentration of phosphorus. Since *Alteromonas* do not encode a primary phosphotriesterase in their genome, any measured phosphotriesterase is likely to result from promiscuous APase activity. Our results indicate that phosphotriesterase activity is lower in comparison to phosphomono- and -diesterase. Due to the detection limit of lower substrate concentrations, hydrolysis curves for phosphotriesterase partly did not fit the Michaelis-Menten model, and no K_M values could be calculated. It was possible to estimate cell-specific V_{max} values, but they were generally lower than those for phosphomono- and diesterase, with low confidence, high standard deviations and variability between biological replicates.

Compared to K_M values measured by Srivastava (2021) for PhoA (the pure enzyme) of *Alteromonas mediterranea*, our study found lower K_M values for phosphomonoesterase in *Alteromonas mediterranea* cultures (approximately 3 times lower), and 3-4 orders of magnitude higher K_M values for phosphodiesterase. This indicates that APase activity measured in our study originates from several enzymes with different enzymatic properties, rather than solely from PhoA. As a result, the ratio of cell-specific V_{max}/K_M for phosphomonoesterase was generally higher compared to that of phosphodiesterase. The ratio V_{max}/K_M assesses the initial slope of the Michaelis–Menten curve and describes the ability of bacteria to hydrolyse substrate at low concentrations. Thus, our results indicate that phosphomonoesterase has a higher substrate affinity and catalytic efficiency compared to phosphodiesterase. Measured phosphodiesterase activity may originate from multispecific and promiscuous APase isoenzymes as well as from other proteins. Considering that APase is the most prevalent enzyme involved in DOP degradation, we hypothesize that the promiscuity of some isozymes contributes significantly to observed phosphodiesterase activity. Our results suggest that consid-

ering phosphodiesterase alongside phosphomonoesterase is crucial for accurately assessing nutrient fluxes and the P-limitation status of marine environments. Additionally, we highlight the impact of multispecific APase activity in DOM recycling and propose that its relevance may have been underestimated in the context of the marine phosphorus cycle.

Exploring APase responses: phosphomono-, -di-, and triesterases under phosphorus limitation

Our results indicate a significant increase in APase activities under phosphorus limitation, which supports the established concept that phosphatase activity is elevated in response to P*i* limitation (Duhamel et al., 2010; Hoppe, 2003; Su et al., 2023). Despite extensive research on the correlation between phosphomonoesterase and P*i* limitation, little is known regarding phosphodiesterase response. In this study, we demonstrate that similar to phosphomonoesterase, phosphodiesterase also increases upon low levels of P*i*. Additionally, it appears that promiscuous phosphotriesterase is also elevated under P-stress, since the P_0 culture, which had the strongest P-limitation, exhibited the highest rates of phosphotriesterase hydrolysis, and exhibited the highest V_{max}.

However, phosphotriesterase V_{max} values were overall considerably lower and non-limited cultures showed a lower relative increase upon limitation compared to phosphomono- and -diesterase. This observation aligns with the relative scarcity of phosphotriester substrates and phosphotriesterases in the ocean. However, recent studies analysed the microbial utilization of synthetic phosphotriesters and revealed that certain marine bacteria are capable of using these, particularly under nutrient-limited conditions (Despotovi et al., 2023; Vila-Costa et al., 2019). Takahashi (2017) showed that PhoA and PhoX are essential for the degradation of anthropogenic triester-bonds in certain Alphaproteobacteria isolates. Even though these isozymes have not been shown to hydrolyze phosphotriester bonds, PhoA from *Alteromonas mediterranea* was recently reported to exhibit promiscuous phosphotriesterase activity (Srivastava et al., 2021; Takahashi et al., 2017). While synthetic triester bonds have recently increased in the water column, they still account for only 0.01% (±0.03%) of total P*i* (Vila-Costa et al., 2019). Nevertheless, the influx of anthropogenic phosphotriester compounds and the increase of P-limitation in the ocean may impact patterns of marine phosphorus utilization in the future. In such a scenario, promiscuous phosphotriesterase activity may be crucial for bacteria to adapt, given that APase is the most dominant enzyme in the marine phosphorus cycle.

V_{max} values of phosphomono- and -diesterase increase with decreasing P-concentrations; however, phosphodiesterase shows a stronger increase, even though its constitutive activity is lower. In particular, under P-limitation, phosphomonoesterase V_{max} values were only marginally increased compared to those of phosphodiesterase (1.3 and 2.3 times increased for

P- and P_0, respectively). However, under non P-limited conditions (C, AMP, AMP+P*i* and DNA), V_{max} values of phosphomonoesterase were approximately 3-4 times higher (Table 1; Figs. 6A and 6B). V_{max} is a measure of the maximum velocity of an enzymatic reaction, which is proportional to the amount of enzyme present in culture studies (Arnosti, 2011; Azúa et al., 2003). Thus, our findings show that the reaction velocity of phosphodiesterase is stronger increased compared to that of phosphomonoesterase. This indicates that phosphomono- and -diesterase activity are regulated separately, suggesting that phosphodiesterase may have a more critical role than previously thought in counteracting P*i* limitation, rather than just being coupled to phosphomonoesterase.

Generally, with decreasing phosphorus concentrations, phosphomonoesterase is characterized by high V_{max} values, low K_M values, and a high ratio of cell-specific V_{max}/K_M. However, kinetic parameters showed significantly different patterns for phosphodiesterase. For phosphodiesterase, hydrolysis curves were less steep initially and reached maximum velocity at higher substrate concentrations. Thus, while V_{max} values were in a comparable range, phosphodiesterase K_M values were notably higher compared to those of phosphomonoesterase. It was especially surprising that phosphodiesterase showed a reverse trend to phosphomonoesterase; i.e., K_M values increased with decreasing phosphorus concentrations (Fig. 7A and 7B). In general, high K_M values suggest low binding affinity to the substrate, indicating that high substrate concentrations are needed to achieve a high reaction velocity. Usually, as for phosphomonoesterase, low K_M values indicate that enzymes are expressed at low substrate concentrations. However, to achieve turnover of substrates under suboptimal conditions, organisms might compensate high K_M values by high V_{max} values, which seems to be the case for phosphodiesterase. The V_{max}/K_M ratio increases under P-limitation for both phosphomono- and -diesterase, indicating higher catalytic efficiency with P-stress. However, due to low phosphodiesterase K_M values, the V_{max}/K_M ratio increases more strongly under P-limitation for phosphomonoesterase than for phosphodiesterase. Particularly, under P-limitation the V_{max}/K_M ratio of phosphomonoesterase is notably higher than that of phosphodiesterase (174-fold and 321 times higher for P- and P_0, respectively). In contrast, in non P-limited cultures (C, AMP, AMP+P*i* and DNA), the V_{max}/K_M ratio for phosphomonoesterase is only 11-fold higher (Fig. 8). Thus, despite the strong increase in velocity for phosphodiesterase, higher K_M values result in a lower substrate affinity and an overall less efficient inducible reaction. Phosphomonoesterase is especially more efficient upon limitation, indicating that it has an overall different inducible reaction and is better adapted to P-stress.

Altogether, this demonstrates that phosphomono- and -diesterase act fundamentally different while being active at similar levels. However, the behaviour of phosphodiesterase has been barely studied before, despite the presence of multiple genes associated with phos-

phodiesterase activity and emerging studies on the enzyme promiscuity of widely distributed APase isozymes. We propose that variations in enzymatic response between phosphomono-, -di-, and triesterase are due to the distinct characteristics of the enzymes that contribute to total APase activity. The observed variability in the limitation response suggests that the individual isozymes possess different promiscuous properties and are regulated independently. It is not known how the different genes are affected by the pho regulon, what proteins contribute to constitutive and inducible APase activities and to what extent different enzymes display promiscuous activity. Further studies are necessary to understand the pathways behind APase activity to improve our knowledge of the variety of phosphorus acquisition strategies in microorganisms and their ecological implications. For instance, PhoD is limitation-inducible and functions as a phosphodiesterase in other members of the bacterial domains (Adams et al., 2022; Kageyama et al., 2011; Luo et al., 2009; Ragot et al., 2015). We thus hypothesize that the increase in phosphodiesterase may be correlated with PhoD. However, further studies are necessary to understand the function and regulation of the individual isozyme to assess their impact on DOM recycling and phosphorus fluxes in the ocean (Lidbury et al., 2022). Constitutive phosphomono-, -di- and triesterase activity and the intensity of the inducible response imply that promiscuous APase activities are crucial for bacteria to hydrolyse DOM and counteract P-limitation. Collectively our results expand the range of DOP substrates (phosphomono-, -di- and triesters) accessible in P-limited environments and should be considered when assessing P availability.

Conclusion

Previously, most studies primarily focused on phosphomonoesterase when assessing marine alkaline phosphatase activity. This project is among the first to provide enzyme kinetic parameters for phosphomono-, -di- and triesterase activity and provides insights into promiscuous properties and environmental influences on a marine bacterium cultured mimicking open ocean conditions. Despite lacking an annotated phosphotriesterase, *Alteromonas mediterranea* exhibits phosphotriesterase activity. Considering the increasing influx of anthropogenic triester compounds into the ocean, further studies are necessary to understand potential impacts of phosphotriesterase in the future. Our results demonstrate that *Alteromonas mediterranea* consistently expresses alkaline phosphatases, even in the presence of sufficient P*i*, which allows continuous hydrolysis of DOP substrates and prevents a P*i*-stress response as long as bioavailable substrates are present. This goes along with studies by Lidbury (2021), which revealed that the PafA isozyme produces constitutive APase activity. Given that *Alteromonas mediterranea* also possesses an enzyme from the PafA family, it can be further hypothesized that constitutive APase activity is derived from this particular isozyme. However, since current knowledge on the functions of the individual proteins is limited, further studies are essential to investigate and understand the contribution of other isozymes to constitutive APase activity. Phosphomono- and diesterase hydrolysis rates were within a similar range, but kinetic properties differed remarkably, which suggests that phosphodiesterase has been largely overlooked previously and should be considered when assessing phosphatase activity in the ocean. Collectively, our findings suggest that “APase activity” is a result of the complex interplay of different (iso)enzymes, each with diverse and currently unknown functions. This observation becomes crucial when evaluating DOP hydrolysis and its impact on the phosphorus cycle and other biogeochemical fluxes.

Zusammenfassung

Alkalische Phosphatasen zählen zu den wichtigsten Enzymen, die die Regeneration von Phosphat aus gelösten organischen Stoffen im Meer antreiben. Insbesondere in Regionen mit niedrigen Phosphatkonzentrationen, sind sie essenziell für marine Mikroben, um Phosphorlimitierung zu umgehen. Verschiedene Enzyme sind an der Phosphatase Aktivität mitbeteiligt, jedoch sind deren Regulation und Charakteristiken sowie ihre Verbreitung und Funktionalität innerhalb der mikrobiellen Gemeinschaft noch kaum verstanden. Hier haben wir untersucht, wie Phosphorlimitierung und verschiedene organische Phosphorsubstrate die Phosphomono-, -di- und -triesterase Aktivität von *Alteromonas mediterranea* beeinflussen. Bisherige Studien zu Phosphatase Aktivität fokussierten sich hauptsächlich auf Phosphomonoesterase. Wir zeigen, dass Phosphomono- und -diesterase Aktivitäten in einem ähnlichen Bereich liegen, aber unterschiedliche kinetische Eigenschaften aufweisen, insbesondere unter Phosphorstress. Phosphotriesterase Aktivität wurde in allen Kulturen gemessen, obwohl *Alteromonas mediterranea* keine annotierten Phosphotriesterasen besitzt. Dies bestätigt Studien von Srivastava (2021) die zeigten, dass *phoA* promiskuitive Phosphotriesterase Aktivität aufweist. Niedrige Phosphomono-, -di- und -triesterase Aktivitäten wurden trotz ausreichender Phosphatmenge und unabhängig vom jeweiligen Phosphorsubstrat beobachtet. Dies deutet darauf hin, dass bestimmte Isoenzyme, wie zum Beispiel PafA, als konstitutive alkalische Phosphatasen funktionieren, die es Bakterien ermöglichen, organisch gebundene Phosphorsubstrate konstant zu hydrolysieren und eine energieaufwendige Phosphatstress-Reaktion zu umgehen (Lidbury et al., 2022). Zusammengenommen zeigen unsere Ergebnisse, dass gemessene enzymatische Aktivitäten von unterschiedlichen Enzymen ausgehen, die zusammenarbeiten und es Mikroben ermöglichen, vielfältige ökologische Strategien zu verfolgen, die an wechselnde Umweltbedingungen angepasst werden können. Es bedarf weiterer Studien, um unser Verständnis über die Remineralisierung von gelöstem organischem Phosphor und dessen Auswirkungen auf den Phosphorkreislauf und andere biogeochemische Stoffflüsse zu verbessern.

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