MASTERARBEIT | MASTER'S THESIS

Titel | Title

Alkaline Phosphatase Activity in Response to Changing Environmental Factors

verfasst von | submitted by

Klara Klöffer BSc

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 833
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Ecology and Ecosystems
Betreut von | Supervisor:	Assoz. Prof. Dr. Silvia Bulgheresi Privatdoz.

Table of Contents

Acknowledgements

I would like to express my deepest appreciation to Silvia Bulgheresi for supervising for my thesis, and to Federico Baltar for continuous guidance and support. A special thanks to Daniel Martinovic Saavedra for introducing me into this interesting topic, supporting me in the lab and the data analysis and never getting tired of all my questions. I would like to thank the fungi group for the nice working environment in the lab and meetings. Lastly, I want to thank my boyfriend, my family, and my friends for always believing in me.

Abstract

Alkalische Phosphatasen (APasen) sind mit die wichtigsten Enzyme im marinen Phosphorkreislauf. APasen hydrolysieren gelöste organische Substanzen (DOM) und liefern wertvolles Phosphor für das marine Ökosystem. Durch den Klimawandel kommt es zur Versauerung der Ozeane, was durch anthropogenen Einfluss weiter angetrieben wird. In dieser Studie wird der Einfluss verschiedener Umweltfaktoren auf die Aktivität der APasen getestet. Zuerst wurde untersucht welche Rolle die Substitution von Metall-Cofaktoren für APasen spielt, welchen Einfluss verschiedene Temperaturen in Kombination mit variierender Phosphatverfügbarkeit auf die enzymatische Lebensdauer haben, sowie welche Auswirkungen unterschiedliche pH-Werte auf die enzymatische Funktion der APasen haben. Dafür wurden fünf verschiedene APasen PafA, PhoD, PhoX und PhoX_OM unter verschiedenen kontrollierten Laborbedingungen getestet. Die Ergebnisse zeigen, dass Metall-Cofaktoren von APasen nur beschränkt substituiert werden und die Aktivität jeder APase eine komplexe Beziehung zwischen Temperatur und Phosphatverfügbarkeit aufzeigt. Darüber hinaus konnte gezeigt werden, dass mit sinkendem pH die Aktivität der Enzyme abnimmt. Im Hinblick auf den fortschreitenden Klimawandel liefert dies Hinweise darauf, dass die fortschreitende Versauerung der Ozeane die APase-Aktivität zunehmend beeinträchtigt und dies somit Auswirkungen auf den marinen Phosphorkreislauf hat. Diese Untersuchung unterstreicht die Wichtigkeit, die komplexe Dynamik von Metall-Cofaktoren, Temperatur, Phosphorverfügbarkeit und verschiedene pH-Werte auf die Aktivität von APasen näher zu verstehen. Sie zeigt, die Empfindlichkeit von APasen auf Umweltveränderungen auf und den weiteren Forschungsbedarf für ein tieferes Verständnis für die Auswirkungen auf APasen und somit auch auf den Phosphorkreislauf im Ozean.

Abstract

Alkaline phosphatases (APases) are arguably the most important enzymes in the marine phosphorus cycle. The major contribution of APases to the phosphorus cycle is the hydrolysis of dissolved organic matter (DOM). Ocean acidification is driven by anthropogenic influences, that have been shown to affect the dynamics of the marine ecosystems. In this study, we investigated different factors and their influence on APase activity. We analyzed the role of metal cofactor substitution, the influence of different temperatures and varying phosphate availability on enzymatic lifetime, as well as the impact of pH shifts on enzymatic function. PhoA, PafA, PhoD, PhoX and PhoX_OM were tested under controlled laboratory conditions. Our results show only a limited substitution of different metal cofactors. We show that there is a complex relationship between temperature and phosphate availability for each tested APase. Furthermore, we were able to show a clear pattern of decreasing activity with decreasing pH, indicating that the progressing ocean acidification affects APase activity and thus also have an impact on the marine phosphorus cycle. With this investigation we further emphasize the importance of understanding the complex dynamics of metal availability, temperature, as well as the influence of different phosphate concentrations and acidification on APases. These findings show the sensitivity of APases to environmental changes and emphasize the further need for further research, as a deeper understanding will be crucial for predicting the impact of ocean acidification on the phosphorus cycle.

Introduction

The element phosphorus (P) is one of the most important macronutrients as it is required by all forms of life. Weathering of continental rock and soils is the beginning of the biogeochemical cycle of P. The runoff is assimilated by microorganisms and plants, which make the P bioavailable as it moves through the food chain. Lastly, decomposers recycle the P, as they break it down to dead inorganic matter, which is then returned to the soil (Berthold et al., 2019). The runoff and erosion of soils is not only cycled in the terrestrial environment but is also introduced in rivers. Here P gets further transported into lakes and eventually into the ocean as it mainly arrives in a particulate form (Ruttenberg, 2014). The P input into the ocean is limited, for which reason, even only small manner of atmospheric deposition can be substantial in coastal areas (Berthold et al., 2019). P has no significant gaseous state as other macronutrients such as nitrogen or carbon, therefore, to make it biologically available, the biota is also dependent on sporadic inputs. This presence or absence of biologically available P influences the biota as it can limit biomass production (Dyhrmann & Ruttenberg, 2006).

Within water bodies, the importance of the aquatic P-cycle becomes more evident. Many biotic and abiotic processes take place in the aquatic P-cycle, and it has shown to be a central component of the global P-cycle (Ruttenberg, 2014; Mackey et al., 2019). Additionally, the aquatic P-cycle is also the most active part of the global P-cycle. Here it is tightly linked to the carbon cycle and other elemental cycles, for example metal cycles (Karl, 2014; Duhamel, 2021). All these cycles are influenced by the uptake of anthropogenic carbon dioxide, which leads to ocean acidification (Jiang et al., 2019). Microbial organisms also heavily rely on P, primarily in its inorganic form. P is integral to cellular structures, in the storage and transmission of genetic information, metabolism and regulatory processes (Karl & Björkman, 2001; 2015).

In coastal areas there is input of P which the microorganisms remove from the euphotic surface layer. Diving deeper and more far into the open ocean, microorganisms need then to regain phosphorus through particle respiration (Duhamel, 2024). As cells die, both upon grazing as well as of viral infections, P-containing components are released

into the environment which are then available as dissolved organic matter (DOM). In phosphate depleted ecosystems like surface waters and tropical/subtropical gyres dissolved organic phosphorus (DOP) is the main source of P for microbial growth (Karl & Björkman, 2015). Sometimes in some areas the DOP:Pi ratio can exceed 100, especially when a phytoplankton bloom emerges (Wu et al., 2000).

Microorganisms are capable of assimilating DOP, which is a complex mixture of organophosphoesters, including phosphomonoesters (monoesters), phosphodiesters (diesters), organic phosphoanhydrides and organophosphonates (Karl, 2014; Karl & Björkman, 2015). Phosphate-esters hold with 75-80% the main part of DOP in marine environments, which is closely followed by phosphonates (Lin et al., 2016; Bell et al., 2020; Clark et al, 1999). The biogeochemical cycles are driven by microbes (Falkowski et al., 2008). Assimilation and degradation of DOP is done with the help of enzymes. The most important enzyme in the marine P-cycle is the Alkaline Phosphatase (APase). APase is an enzyme which can be found from primitive bacteria to vertebrates (Sharifian et al., 2018). This prominent enzyme is also present and utilized in many microorganisms, as bioinformatic analysis showed that APase gene sequences are present throughout the global ocean (Luo et al., 2009). APases are essential for recycling of DOP and consequently providing P to organisms. Therefore, the sustaining of the P-cycle in marine environments is assured as phosphorus limitation is overcome by converting DOP to inorganic Pi and vice versa (Karl, 2014). Many microorganisms can use sinking particles as a P source through extracellular hydrolysis, making the decay of phytoplankton a highly bioavailable source for organic phosphorus (Feng et al., 2018). The regulation and function of seemingly redundant APase genes is still a topic to be further investigated (Zhang et al., 2022). Phosphohydrolases primarily break down monoesters, making phosphatases essential in that process. Additionally, it has been shown by Srivastava et al. 2021, that APases not only hydrolyze monoesters as previously thought, but they also show diesterase, triesterase and sulfatase activity. Hydrolysis of phosphate esters is primarily catalyzed by cell surface-bound ectoenzymes, which also include APases (Azam et al., 2007). The total extracellular enzyme activity includes both cell-bound and dissolved cell-free enzymes, with a substantial proportion attributed to cell-free enzymes. Unlike cell-bound enzymes, these cell-free enzymes operate independently

of living cells, performing their functions in the external environment. A major part of the enzymatic activity is linked to the dissolved fraction (Baltar, 2018A).

The families of APase are defined based on their structural, functional, and evolutionary characteristics. Variations in active site architecture may contribute to an affinity of different cofactors. As these enzymes are present in many areas, APases also have a significant ecological and evolutionary implication. APases show three different traits: the differentiation in subcellular localization and substrate specialization, a compensatory regulation, and functional complementation (Duhamel, 2024). There are different types of phosphatases. Alkaline phosphatases and acid phosphatases. Alkaline phosphatases are currently more abundant and extensively studied, with an optimum pH range between 8.5 – 9.5, as aquatic ecosystems still tend to be more alkaline. However, also acid phosphatases exist, which have their optimum pH range between 4 – 6 (Hoppe, 2003). Acid phosphatases can be found among organisms such as marine parasites, where there are considered as virulent factors (Salinas et al., 2011).

As previously mentioned above APases are known to be promiscuous and show monoesterase activity, but also diesterase-, triesterase- and sulfatase activity. Revealing promiscuity and multifunctionality for APases across major families is crucial for the marine phosphorus cycle (Srivastava et al., 2021). This evidence of multifunctionality and different substrate affinities suggests diverse ecological roles for the specific APase families. Differential expression patterns of APase families across the phylogenetic branches emphasize the crucial role of dominant genera like *Alteromonas* (Saavedra et al., in revision). *Alteromonas* is a fast growing, cosmopolitan, heterotrophic gammaproteobacterium found abundantly at various ocean depths. In the North Pacific during phytoplankton blooms, *Alteromonas* displayed up to 62% of the total gammaproteobacterial abundance (Tada et al., 2011). The monoesterase activity of APase shows high efficiency at high substrate concentrations. Whereas at low substrate concentrations promiscuous activities of APase, like diesterase, triesterase and sulfatase activities become more efficient (Srivastava et al., 2021). To adapt to changing environmental conditions, microorganisms regulate their P uptake sources, rates and storage capacities (Popendorf & Duhamel, 2015).

APase genes have been thoroughly studied for their sequence structure and expression dynamics under varying conditions of phosphorus. These studies show that the active sites of APases bind different metal ions as cofactors as environmental conditions change, which influence the enzymatic activity (Luo et al., 2009; Lin et al., 2009; Srivastava et al., 2021).

The phosphorus metabolism is mainly regulated by the phosphate regulon (pho regulon). Regulation happens upon the sensing of the presence or absence of available phosphate in the environment and the adapted expression as a following response to P-limitation (Hsieh et al., 2010). Up to today, five APase gene families have been described: *phoA, phoD, phoX, pafA* and the newest family *psip1*. These families are distinguished by their function as monoesterases or diesterases. Distinction of these families is established by their substrate affinity and their native cofactor requirements (Sone et al., 1997; Wu et al., 2007; Yamane & Maruo, 1978; Yong et al., 2014; Duhamel et al., 2021; Torcello-Requena et al., 2024).

Monoesterases and diesterases differ significantly in their metal content (Duhamel, 2021). The presence of native iron and calcium ions for in the catalytic centre for a high activity is needed by PhoD and PhoX (Wu et al.,2007; Yong et al., 2014; Coleman, 1992; Ali & Hamaza, 2012). Whereas PhoA requires two native zinc ions as well as one magnesium ion at its active site (Wu et al., 2007). PafA is also known to be a zinc-dependent enzyme. Three of the AP families (PhoA, PhoD and PhoX) are often distributed within the cell of heterotrophic bacteria. PhoA can be found in the extracellular space, whereas PhoD in eukaryotes is mainly located in the endomembrane system (Dell'Aquilla et al., 2020; Zhang et al., 2020). Within the cytoplasm, PhoA holds a significant proportion (Luo et al., 2009) and contributes to the majority of total APase activity as an excreted enzyme. PhoD, an intracellular enzyme, contributes a minor fraction to the total APase activity. Interestingly, it has been shown that PhoA and PhoD are able to compensate for each other when one gets disrupted. Therefore, the uptake of DOP and the continuous maintaining of the status of the cell via different pathways is facilitated (Zhang et al., 2022).

In the cyanobacterium *Trichodesmium*, it has been shown, that PhoA and PhoX are expressed during Pi starvation. Here, they potentially provide complementary

functions which are crucial for survival (Orchard et al., 2012). Most APases are essential for survival in P-depleted environments, but some also function independent of phosphate availability. PafA, is known to be a phosphate insensitive APase, which can also be found in Bacteroidetes, which is abundant in marine (Lidbury et al., 2022) and freshwater environments (Zhao et al., 2023). PafA can provide carbon, energy, and phosphorus to marine flavobacteria. Additionally, it also releases bioavailable P for neighbouring microorganisms, regardless of the prevailing P availability (Duhamel, 2024).

Not only intracellular enzymatic activity of APases is present, but also mono- and diesterase activity occur in the cell-free extracellular environment, which is helpful when ambient phosphate concentrations are high (Su et al., 2023; Thomson et al., 2020; Thomson et al., 2019). For proteins there are several ways to be secreted. Interestingly, not all APases follow the same pathway. For example, PhoD contains a tat motif at the N-terminal region (Kageyama et al., 2011), and is therefore already completely folded inside the cytoplasm, which is also true for PhoX. Contrary to this, the enzymes PhoA and PafA follow the Sec-pathway, where the complete folding of the enzyme is only accomplished outside the cytoplasm. Therefore, PhoA and PafA are dependent on the presence of metal cofactors outside in the environment (Saavedra et al., in revision).

Because of the ubiquitous presence in diverse marine environments, including oligotrophic surface waters, deep seas, and coastal areas, the four different APases of *Alteromonas mediterranea* strain DE (deep ecotype) PhoD, PhoA, PhoX and PafA, were selected to investigate our findings (Ivars-Martínez et al., 2008; Srivastava et al., 2021). Additionally, another PhoX enzyme of *Pseudomonas* was tested, to gain more information regarding PhoX in general.

Even though, monoesterase, diesterase, phosphotriesterase and sulfatase activity for APases has been detected, we mainly focused in our study on the monoesterase and diesterase activity of the enzymes, as the hydrolysis of monoester bonds also exhibit the highest catalytic activity (Srivastava et al., 2021). APase activity is regulated by P availability and can serve as an indicator of microbial phosphate stress, DOP utilization efficiency (Su et al., 2023) and eutrophication levels (Singh & Pandey, 2023).

Although the multifunctionality and enzymatic promiscuity of APases have been extensively studied, their ecological relevance regarding their gene expression, enzymatic dominance, and interactions with pollutants in natural environments remains largely unexplored (López-Canut et al., 2011; Sunden et al., 2017; Pabis et al., 2016; Duarte et al., 2013). The ongoing increasing in anthropogenic CO_2 emissions is causing a decrease in pH of marine environments (Feely et al., 2004), which is predicted to potentially reduce APase activity (Weiss et al., 2018; Guo et al., 2023). Consequently, this decline in APase efficiency may limit the utilization of DOP, which hence could lead to a disruption of the P-cycle. In parallel, the anthropogenic excessive phosphorus input of fertilizers, which are the result of non-renewable rock phosphate mining, contributes to a negative influence on the marine and environment and the formation of dead zones. As is investigated these dead zones are expanding in number (Altieri & Gedan, 2015). This phosphorus overload promotes harmful algal blooms, which cause impaired water quality, reduced ecotourism, and negative impacts on fisheries (Smil, 2000). In response to P-stress, some microorganisms manage to substitute phospholipids in their membranes with phosphorus free lipids. However, cellular P can never be fully substituted (Van Mooy et al., 2009; Pasek, 2018).

Our investigation aimed to better understand how APase responds to changing environmental factors, in consideration of the continuing anthropogenic forces. We aimed to examine the enzymatic activity of APases under varying conditions as well as P-limitation. We assessed how temperature, and the presence of the inhibitor influences the lifetime of these enzymes. Additionally, we explored how different metal cofactors influence the enzymatic activity, as this could also have an influence on the coupling between the P-cycle and metal cycles. Furthermore, we investigated the influence of different pH levels on enzymatic activity, given that the ongoing ocean acidification is predicted to progress, unless harmful anthropogenic inputs are mitigated.

With these investigations, we sought to provide a deeper insight into possible adaptive mechanisms of APases and their important role in driving the P-cycle under future environmental conditions. This knowledge helps to gain a deeper understanding of

potential disruptions on biogeochemical cycles and further understanding of the resilience of marine ecosystems.

Materials and Methods

Artificial Seawater

To simulate conditions artificial sea water (ASW) was prepared following the Bigelow protocol (Sunda et al., 2005), except that only the Synthetic Ocean Water was prepared. The major nutrients, metal stock solution and mixed vitamin stock solution were excluded in this step, as we further investigated the different ratios of these components. Here it is important to note the high concentration of EDTA within the metal stock solution. When evaluating the presence of metal cofactors under conditions with a high EDTA concentration, it is critical to consider the potential for EDTA to chelate the metal ions and thereby sequestering them from the medium reducing their bioavailability. Therefore, we excluded EDTA in all experiments. The main salts, hydrous and anhydrous, were prepared separately, each autoclaved individually and combined after cooling. Different batches of ASW were prepared and adjusted to a specific pH level: 7.6, 7.9 and 8.1. Additionally, another batch was prepared at pH 8.1 with the addition of 1µM Pi in the form of NaH_2PO_4 H_2O. After pH adjustment, all batches were filtered again to ensure sterility and prevent contamination.

Kinetic measurements

Kinetic measurements of the APases were conducted in the presence of zinc in the form of $ZnSO_4$ and iron in the form of $FeSO_4$. Monoesterase and diesterase activity were again measured using MUF-P and bis-MUF-P. The product concentration was calculated by comparing the relative absorbance to that of a standard curve derived of known standard concentrations of 4-Methylumbelliferylphosphate, from now on MUF-Std, (ACROS Organics, Fisher Scientific GmbH, Austria) (10, 5, 1, and 0.5 µmol L^{-1}). Kinetic parameters, K_{cat}, K_M, and the catalytic efficiency K_{cat}/K_M were calculated with the corresponding enzyme concentrations using RStudio Version 2022.12.0+353. Each purified recombinant APase protein PhoA, PhoD, PhoX, PhoX_OM and PafA (100nM) (UniProtKB accession numbers F2G8N3, F2G754, T2DMD9, A0A1S8DKT0 and F2G994, respectively) prepared as described in Saavedra et al., (in revision), was

tested in a Greiner Bio-one 96-well non-binding microplate, with a final concentration of 1 nM, both with and without the presence of 100 nM zinc or iron. Monoesterase and diesterase activity were measured starting at 500 µM of 4-methylumbelliferyl (MUF)-phosphate and Bis 4-MUF phosphate, respectively. The concentrations were serially diluted by half each subsequent measurement. Samples were prepared in technical triplicates. ASW blanks without the addition of any enzyme or cofactor were used as a control. The microplate reader (TECAN, Infinite 200 POR) was used to measure the absorption values in volumes of 300 µL at excitation wavelength of 365 nm and emission wavelength of 445 nm. Data was recorded for 45 min at 5-min intervals.

Cofactor Experiment

The impact of six different metal cofactors on the enzymatic activity was assessed. Each purified recombinant protein PhoA, PafA, PhoD, PhoX and PhoX_OM were tested for monoesterase and diesterase activity in presence of 1 nM cofactor of iron ($FeSO_4$), zinc ($ZnSO_4$), manganese ($MnCl_3$), cobalt ($CoCl_2$), copper ($CuCl_2 \cdot 2H_2O$), or nickel ($NiCl_3$). The relative enzymatic activity was again compared to a standard curve of MUF-Std at 200, 100, 25, and 5 nmol L^{-1}. For each APase a Greiner Bio-one 96-well non-binding microplates was filled with a final concentration of 1 nM APase, 200 µM of MUF- P or bis-MUF-P and 100 nM of each metal cofactor in ASW at pH 8.1. All samples were prepared in technical quadruplicates. ASW blanks without the addition of any enzyme or cofactor were used as a control. Additional blanks of only enzyme and only substrate in ASW were also measured.

The absorption values were measured with a microplate reader (TECAN, Infinite 200 PRO) in volumes of 300 µL at excitation wavelength of 365 nm and emission wavelength of 445 nm. Three consecutive measurements at t_0, 30 min and 60 min were taken.

Half lifetime and Lifetime

Half-lifetime and Lifetime of APases was investigated in presence of 1 µM Pi and under P-limited (P-lim) conditions in the dark at three different incubation temperatures 4°C,

14°C and 24°C. The APases were incubated in 1.5 mL Eppi-Tubes in ASW in the presence of all required cofactors and trace metals. The concentrations are based on the highest concentrations observed in the open ocean $MnCl_2$ (5 nM), $FeCl_3$ (2 nM), $NiCl_3$ (12 nM), $ZnSO_4$ (9 nM), Na_2SeO_3 (2.3 nM) and Na_2MoO_4 (105 nM) (Bruland et al., 2014).

P-monoesterase and P-diesterase activity were measured using the saturating concentration of 200 µM MUF-P or bis-MUF-P. The concentration of the products was determined by comparing the relative absorbance to the absorbance of a standard curve of known standard concentrations of MUF-Std (200, 100, 50, and 10 nmol L^{-1}). Each APase was tested in a Greiner Bio-one 96-well non-binding microplate, with a final concentration of 30 nM enzyme. Samples were prepared in technical triplicates. ASW blanks were used as a control. The absorption values were recorded in volumes of 300 µL at excitation wavelength of 365 nm and emission wavelength of 445 nm on a microplate reader (TECAN, Infinite 200 PRO). Data was recorded for three different timepoints t_0, 45 min and 90 min. The measurements took place periodically for 60 days.

Influence of pH

The influence of three different pH conditions (pH 8.1, 7.9 and 7.6) was examined, and along with the effect of pH 8.1 in the presence of 1 µM Pi and metal cofactors. Measurements for the five purified recombinant APases – PhoA, PhoD, PhoX, PhoX_OM and PafA (100nM) took place at room temperature. A 96-well deep well plate (2 mL capacity per well, SP Bel-Art) was prepared to assess the enzyme response at different pH levels. ASW was distributed across the wells. Metal cofactors were added to enhance the enzyme stability. Zinc at a concentration of 200 µM was added to PhoA and PafA, while iron at 200 µM was added to the enzymes PhoD, PhoX and PhoX_OM. Activity of APases were assessed based on hydrolysis of fluorogenic substrate analogs. To assess the monoesterase and diesterase activities, the fluorogenic substrates MUF-P and bis-MUF-P were used. Based on pretested kinetics, the relative substrate concentrations were determined at 200 µM. The relative enzymatic activity was compared to a standard curve of MUF-Std at 10, 5, 1, and 0.5

μmol L^{-1} carried out in a 100 μM Tris-Buffer. Greiner Bio-One 96-well non-binding microplates were filled with three technical replicates, consisting of 100 μM APase sample and 200 μmol of MUF- and bis-MUF-P in Tris buffer (100 μM), resulting in a final test concentration of 5 nM APase in a total volume of 300 μL. The absorption values were read in triplicates with a microplate reader (TECAN, Infinite 200 PRO) at an excitation wavelength of 365 nm and emission wavelength of 445 nm. Measurements were conducted at three time points: t_0, 45 minutes, and 90 minutes.

Analysis

Analysis of the enzymatic activity and visualization of the data of the APases, was done via RStudio Version 2022.12.0+353. Data of the kinetic experiment was fitted into a nonlinear regression. Michaelis-Menten kinetics and K_{cat} value evaluations with the corresponding enzyme concentrations were also obtained in RStudio.

Results

The results of this study are divided in four main sections. For every test the mono- and diesterase activity of the five different APases were tested: PhoD, PhoA, PafA, PhoX and PhoX_OM. First, the kinetic performance was tested, in presence of two different cofactors: iron or zinc. Second, we investigated the enzymatic activity in presence of six different metal cofactors, such as zinc, iron, nickel, cobalt, cupper, and manganese. Third, we observed the half-lifetime and lifetime activity of the enzymes. Lastly, we tested enzymatic activity of the enzymes in three different pH values in presence of specific cofactors and one additional batch which included the presence of Pi.

Kinetic performance in presence of zinc and iron

Kinetic performance of the enzymes was evaluated under identical conditions in the presence of zinc or iron using the substrates MUF-P and bis-MUF-P. Enzymatic activity was characterized by determining the specific kinetic K_{cat}, K_m and the catalytic efficiency ratio K_{cat}/ K_m expressed in nmol s^{-1} L^{-1}.

The relationships between MUF-P substrate concentration (µmol L^{-1}) and reaction velocity (nmol s^{-1} L^{-1}) in the presence of the metal cofactor zinc for all enzymes compared to their respective controls is illustrated in Fig.1. The data were fitted to the Michaelis-Menten equation. The reaction velocity increased for each enzyme with varying steepness, reaching an individual saturation plateau. Among the tested enzymes, PhoX exhibited the highest K_{cat} value of (2.45 ± 0.667) × 10^1 nmol s^{-1} L^{-1} at a substrate concentration of 62.5 µmol L^{-1}. In contrast, PafA displays the lowest K_{cat} value at (2.04 ± 0.0146) × 10^{-3} under the same substrate concentrations, which is also seen in the control. The control data generally adhered more closely to the Michaelis-Menten model.

In the presence of iron at a substrate concentration of 125 µmol L^{-1}, the monoesterase activity of both PhoX and PhoA display similar K_{cat} values at (1.76 ± 0.95) x 10^1 nmol s^{-1} L^{-1} and (1.78 ± 0.15) x 10^1 nmol s^{-1} L^{-1}, respectively. Generally, the presence of iron

enhances the data points to align more closely with the Michaelis-Menten model, compared to the respective control. Notably, PhoX shows a steeper increase in the reaction velocity in comparison to PhoA. PafA shows the lowest K_{cat} value at (1.78 ± 0.012) × 10^{-3} nmol s^{-1} L^{-1} but demonstrated a steep rise towards the saturation plateau. The data points for PafA, display a high scatter in the variability of the measurements, which is less under control conditions (Fig. 2). PhoX_OM exhibits, an early steep increase in reaction velocity, both in presence of zinc and iron. The enzyme reaches its saturation plateau at 15 µmol L^{-1}, indicating a lower substrate conversion efficiency for MUF-P (Fig. 1 and Fig. 2).

The relationship of phosphodiester activity between the substrate concentration of bis-MUF-P (µmol L^{-1}) and the reaction velocity (nmol s^{-1} L^{-1}) in the presence of either cofactor zinc or iron for the enzymes PhoD, PhoX_OM and PafA are shown in Fig. 3. Additionally, again the individual control conditions are also shown. In presence of the highest K_{cat} value of 2.18 ± 0.1 nmol s^{-1} L^{-1} is displayed by PhoD, which is slightly reduced compared to control conditions at 2.45 ± 0.18 nmol s^{-1} L^{-1}. Following closely, PafA achieves a K_{cat} value at 1.7 ± 0.064 nmol s^{-1} L^{-1}. In contrast PhoX_OM shows the lowest K_{cat} value at (1.05 ± 0.0283) × 10^{-1}. All three enzymes reach their saturation plateau at a high substrate concentration of 500 µmol L^{-1}.

When tested with iron, the K_{cat} values for all enzymes are lower compared to their respective controls. The highest K_{cat} under control conditions can be seen in control conditions for PafA at 1.82 ± 0.074 nmol s^{-1} L^{-1}. PhoX and PhoA also display diesterase activity in the presence of each cofactor. However, their activity follows a linear relationship with substrate concentration rather than the Michaelis-Menten model. Therefore, the data is not shown in Figure 3.

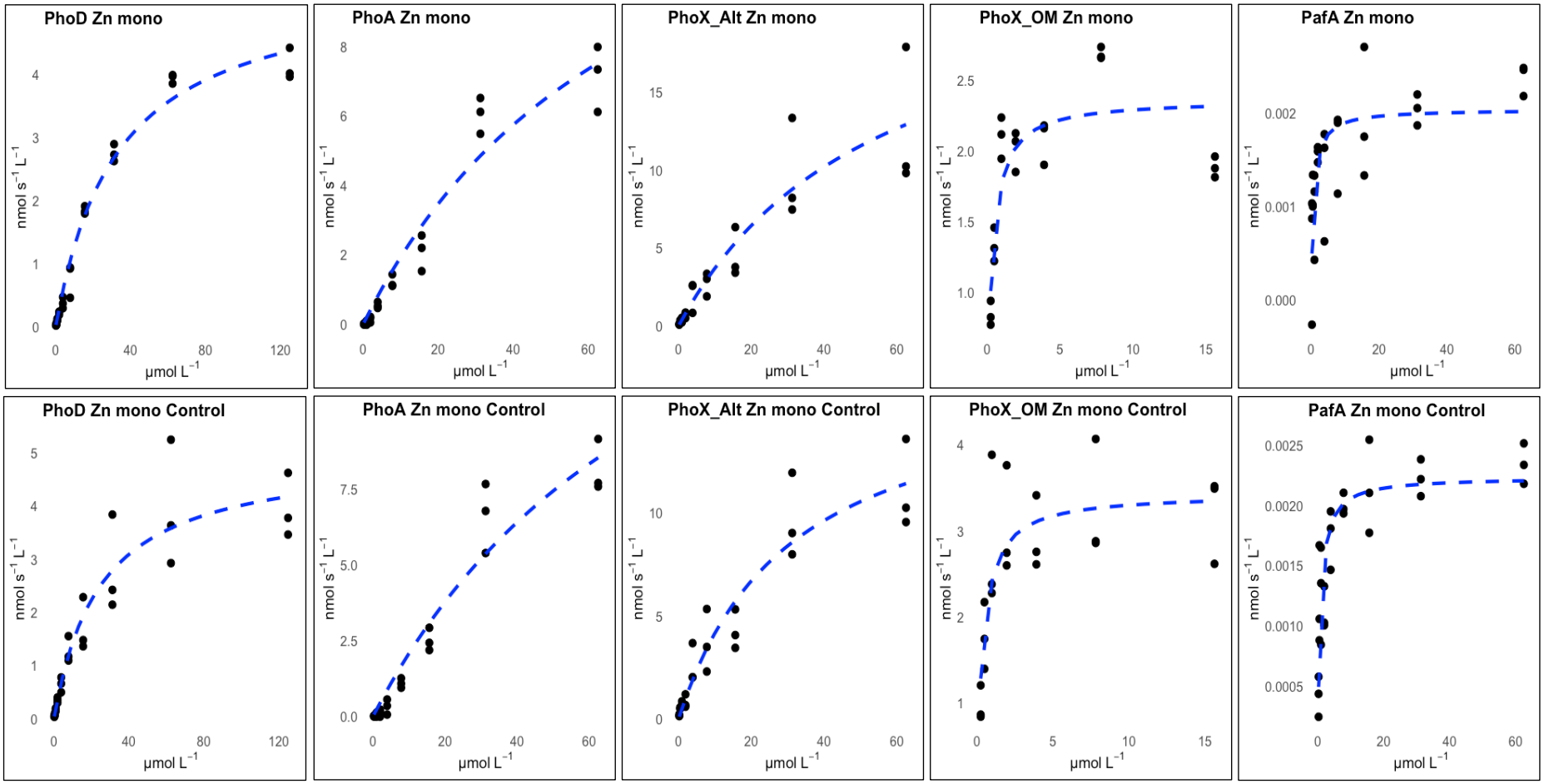

Fig. 1. Enzyme specific hydrolysis rates of the phosphomonoester MUF-P (µmol L^{-1}) in presence of cofactor $ZnSO_4$ (100nM) and the corresponding control conditions of PhoD, PhoA, PhoX, PhoX_OM and PafA in nmol s^{-1} L^{-1}. Dots represent the average of technical replicates and blue dashed line the Michaelis-Menten curve.

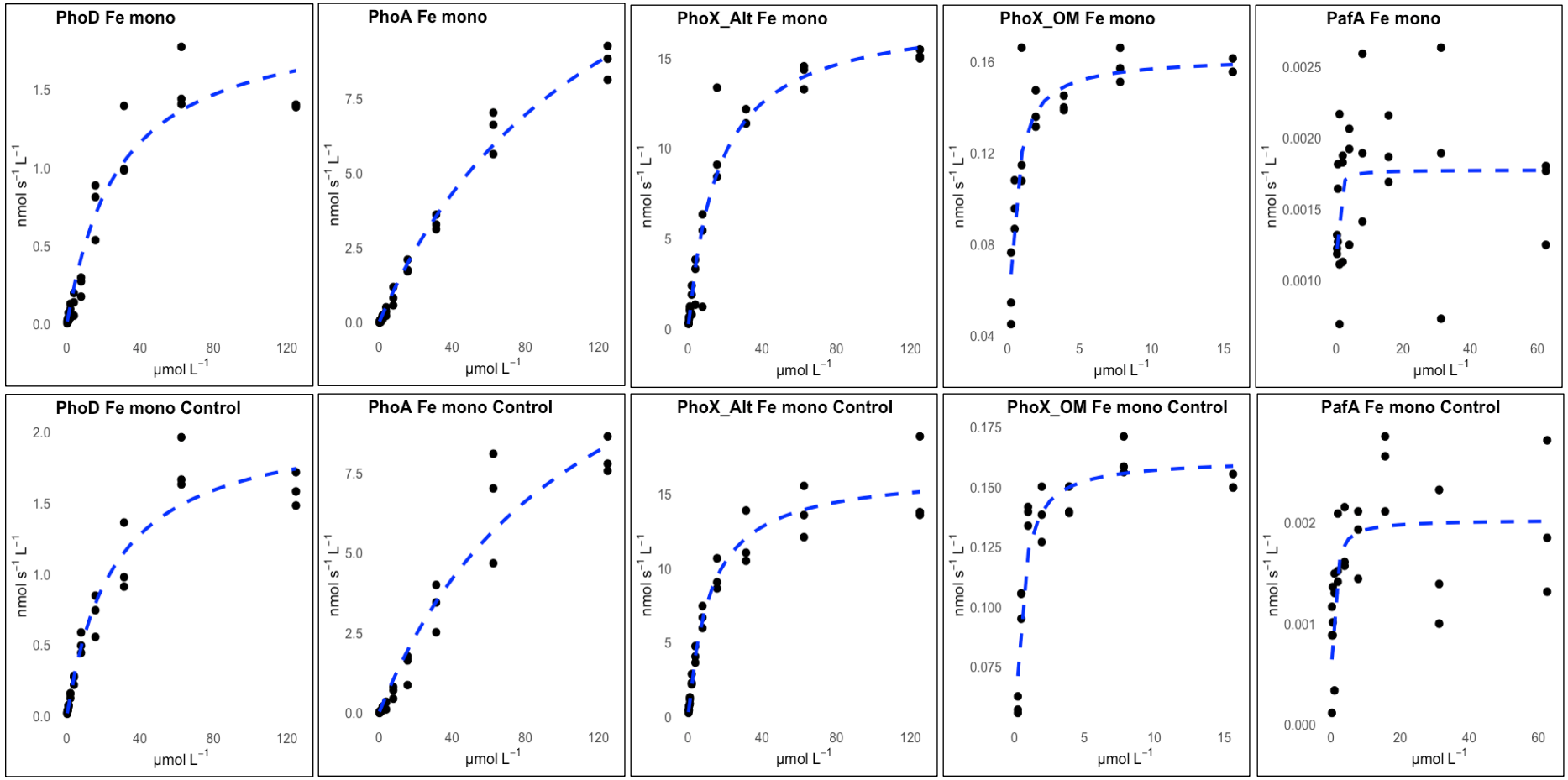

Fig. 2. Enzyme specific hydrolysis rates of the phosphomonoester MUF-P (µmol L^{-1}) in presence of cofactor $FeSO_4$ (100nM) and the corresponding control conditions of PhoD, PhoA, PhoX, PhoX_OM and PafA in nmol s^{-1} L^{-1}. Dots represent the average of technical replicates and blue dashed line the hydrolysis curve.

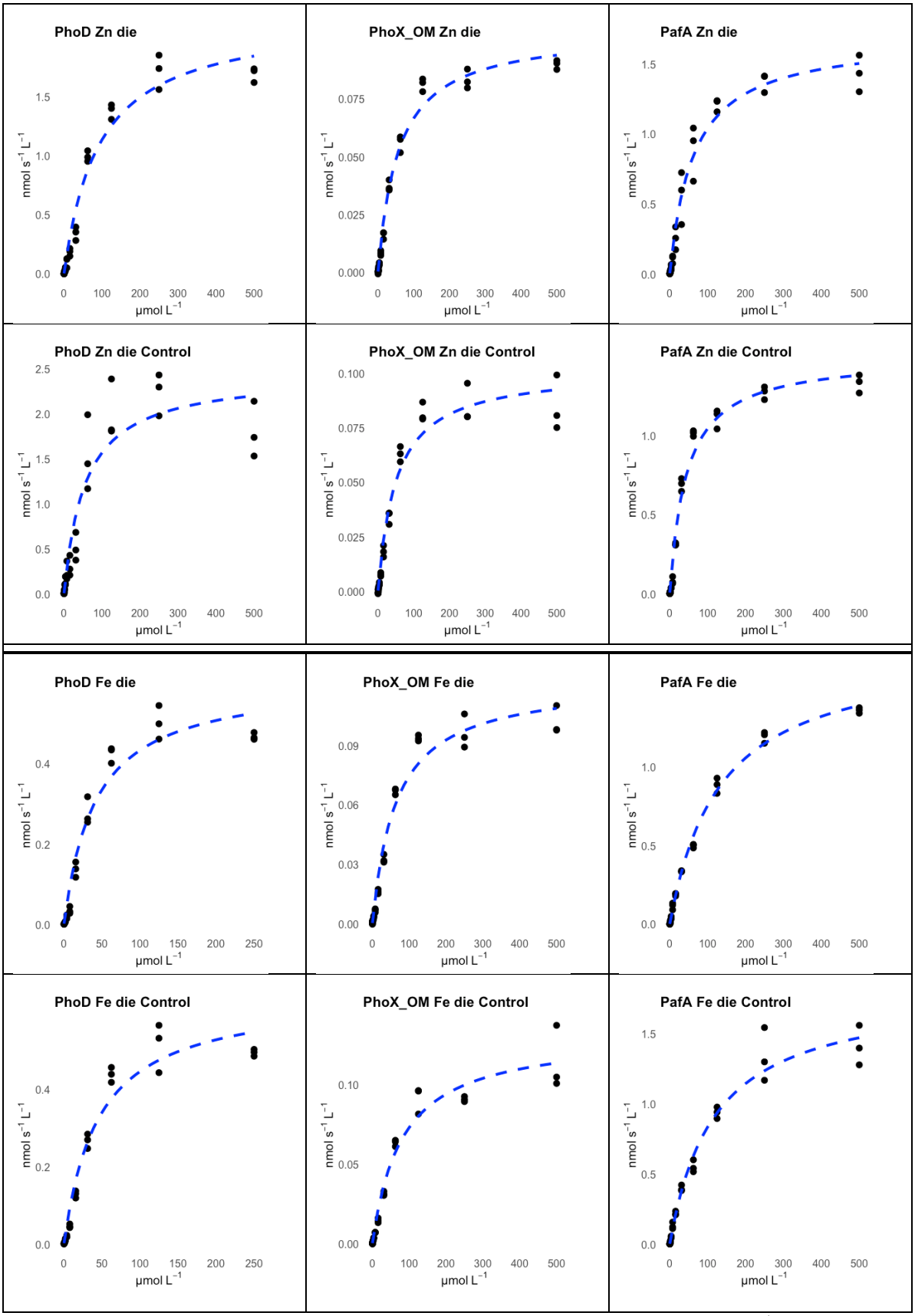

Fig. 3. Enzyme specific hydrolysis rates of the phosphodiester bis-MUF-P (µmol L^{-1}) in presence of cofactor $ZnSO_4$ (100nM) or $FeSO_4$ (100nM) and its corresponding control for PhoD, PhoX_OM and PafA nmol s^{-1} L^{-1}. Dots represent the average of technical replicates and blue dashed line the Michaelis-Menten curve.

Table 1 summarizes the Michaelis-Menten kinetic parameters (K_{cat}, K_M and K_{cat}/K_M) for the enzymes presented in Figures 1-3. As mentioned above, each enzyme was evaluated by comparing the effects of the metal cofactors iron and zinc, to their respective control conditions. This approach ensures that the influence of each metal cofactor is assessed independently. As the experimental measurements were conducted on 96-well plates, the variability between different plates led to in inconsistent absolute values. Therefore, comparisons are only investigated within the same plate.

The addition of metal cofactors influenced the kinetic performance for all enzymes, although the magnitude and direction of the effect varied by enzyme and cofactor.
When examining the K_{cat} values, most enzymes showed relatively stable activity in the presence to their respective controls. However, a notable exception was PhoX, which exhibited a 1.4-fold increase in the presence of zinc 24.5 ± 6.7 s^{-1}.

The K_M values showed greater variability between the treatments. For instance, PhoX displayed a 1.75-fold higher substrate affinity under control conditions compared to the presence of zinc. The most relevant difference was observed for PafA in the presence of iron, where the K_M decreased by a factor of 4.7 to (1.10 ± 0.072) × 10^{-1} nmol L^{-1} compared to the control conditions (5.27 ± 0.21) × 10^{-1} nmol L^{-1}, indicating a higher substrate affinity in presence of iron.

The ratio K_{cat}/K_M provides a more comprehensive of how the metal cofactors influence enzyme efficiency. For PhoA, the catalytic efficiency remained unchanged, as indicated by similar K_{cat}/K_M values regardless of the presence or absence of zinc. Similarly, the presence of zinc showed only a low difference in catalytic efficiency for PafA, contrary to the presence of iron. Some enzymes displayed a decreased efficiency with metal cofactors. PhoD exhibited slightly reduced efficiency with both zinc and iron. Similarly, PhoX_OM showed lower catalytic efficiency when in presence of iron, while PhoX displayed reduced efficiency with zinc.The most pronounced negative effect was observed for PhoX in presence of iron, as efficiency decreased by a factor of 3.5. Under control conditions, the K_{cat}/K_M ratio was 1.41 ± 0.2 nmol s^{-1} L^{-1}, whereas in the presence of iron, it decreased to (4.68 ± 0.97) × 10^{-1} nmol s^{-1} L^{-1}.

Substrate	Cofactor	K_{cat} (s^{-1})	K_M (nmol L^{-1})	K_{cat}/K_M (nmol L^{-1} s^{-1})
PhoD P-monoesterase	$FeSO_4$	1.99 ± 0.18	28.6 ± 6.3	(6.97 ± 1.66) x 10^{-2}
	Control	2.1 ± 0.13	25.7 ± 4.4	(8.18 ± 1.5) x 10^{-2}
	$ZnSO_4$	5.53 ± 0.23	33 ± 3.5	(1.67 ± 0.19) x 10^{-1}
	Control	5.01 ± 0.44	24.8 ± 5.9	(2.02 ± 0.52) x 10^{-1}
PhoA P-monosterase	$FeSO_4$	17.8 ± 1.5	125 ± 17.8	(1.43 ± 0.24) × 10^{-1}
	Control	15.7 ± 2.6	110 ± 310	(1.43 ± 0.47) × 10^{-1}
	$ZnSO_4$	16.7 ± 3.7	76.1 ± 26.4	(2.19 ± 0.9) × 10^{-1}
	Control	20.9 ± 5.4	90.6 ± 35	(2.31 ± 1.07) × 10^{-1}
PafA P-monoesterase	$FeSO_4$	(17.8 ± 0.12) × 10^{-4}	(1.10 ± 0.072) × 10^{-1}	(1.61 ± 1.05) × 10^{-2}
	Control	(20.3 ± 0.15) × 10^{-4}	(5.27 ± 0.21) × 10^{-1}	(3.86 ± 1.58) × 10^{-3}
	$ZnSO_4$	(20.4 ± 0.15) × 10^{-4}	(7.57 ± 0.27) × 10^{-1}	(2.60 ± 0.60) × 10^{-3}
	Control	(22.4 ± 0.1) × 10^{-4}	(8.58 ± 0.19) × 10^{-1}	(2.70 ± 0.97) × 10^{-3}
PhoX_OM P-monoesterase	$FeSO_4$	(16.2 ± 0.41) × 10^{-2}	(34.9 ± 0.58) × 10^{-2}	(4.66 ± 0.78) × 10^{-1}
	Control	(16.2 ± 0.54) × 10^{-2}	(31.1 ± 0.4) × 10^{-2}	(5.21 ± 0.69) × 10^{-1}
	$ZnSO_4$	2.37 ± 0.11	(32.7 ± 0.8) × 10^{-2}	7.24 ± 1.79
	Control	3.44 ± 0.22	(34.9 ± 0.58) × 10^{-2}	(4.66 ± 0.78) × 10^{-1}
PhoX P-monoesterase	$FeSO_4$	17.6 ± 9.5	16.2 ± 2.7	(4.68 ± 0.97) × 10^{-1}
	Control	16.6 ± 6.7	11.8 ± 1.6	1.41 ± 0.2
	$ZnSO_4$	24.5 ± 6.7	56 ± 26.2	(4.37 ± 2.37) × 10^{-1}
	Control	17.1 ± 2.4	31 ± 9	(5.51 ± 1.78) × 10^{-1}

Table 1. Michaelis-Menten parameters for monoesterase activity of each enzyme with and without the presence of $ZnSO_4$ or $FeSO_4$. Data reported are enzyme specific K_{cat} (V_{max} divided by concentration of enzyme), the K_M Michaelis-Menten constant and the K_{cat}/K_M.

Table 2 presents the Michaelis-Menten parameters (K_{cat}, K_M and K_{cat}/K_M) for the phosphodiester activity of each enzyme which are shown in Fig. 3, both with and without the presence of each cofactor.

For the enzymes PhoD, PafA and PhoX_OM, the K_{cat} values remained relatively stable across conditions, showing minimal variation between the presence of each metal cofactor and their respective controls. A similar trend was observed for the K_M values, though two notable exceptions emerged. For PafA, the presence of zinc resulted in a 1.4-fold decrease in substrate affinity. In contrast, PhoX_OM exhibited a 1.25-fold increase in substrate affinity when iron was present. The K_{cat}/K_M ratio revealed only low differences in the overall catalytic efficiency of the enzymes between cofactor and control conditions. However, the presence of zinc had a slight negative influence on

the catalytic efficiency of PafA, reducing it by a factor of 1.2. Similarly, PhoD exhibited a 1.8-fold decrease in efficiency in the presence of zinc. Both PhoA and PhoX also demonstrated diesterase activity. However, for these enzymes, the relationship of the substrate concentration of bis-MUF-P and the reaction-velocity was linear. Therefore, no Michaelis-Menten parameters from these enzymes, and their values are not included in Table 2.

Substrate	**Cofactor**	**K_{cat} (s^{-1})**	**K_M (nmol L^{-1})**	**K_{cat}/K_M (nmol L^{-1} s^{-1})**
PhoD P-diesterase	$FeSO_4$	$(6.13 \pm 0.03) \times 10^{-1}$	41.3 ± 6.4	$(1.49 \pm 0.24) \times 10^{-2}$
	Control	$(6.48 \pm 0.03) \times 10^{-1}$	45 ± 6.7	$(1.44 \pm 0.23) \times 10^{-2}$
	$ZnSO_4$	2.18 ± 0.1	92.5 ± 12.1	$(2.36 \pm 0.33) \times 10^{-2}$
	Control	2.45 ± 0.18	56 ± 13.2	$(4.37 \pm 1.08) \times 10^{-2}$
PhoA P-diesterase	$FeSO_4$	NA	NA	NA
	$ZnSO_4$	NA	NA	NA
PafA P-diesterase	$FeSO_4$	1.76 ± 0.035	133 ± 67	$(1.32 \pm 0.072) \times 10^{-2}$
	Control	1.82 ± 0.074	118 ± 127	$(1.55 \pm 0.18) \times 10^{-2}$
	$ZnSO_4$	1.70 ± 0.065	62.8 ± 7.5	$(2.70 \pm 0.34) \times 10^{-2}$
	Control	1.51 ± 0.045	43.9 ± 4.5	$(3.43 \pm 0.36) \times 10^{-2}$
PhoX_OM P-diesterase	$FeSO_4$	$(1.23 \pm 0.047) \times 10^{-1}$	64.7 ± 7.6	$(1.90 \pm 0.23) \times 10^{-3}$
	Control	$(1.32 \pm 0.06) \times 10^{-1}$	80.6 ± 10.7	$(1.64 \pm 0.23) \times 10^{-3}$
	$ZnSO_4$	$(1.05 \pm 0.028) \times 10^{-1}$	56.9 ± 4.9	$(1.84 \pm 0.17) \times 10^{-3}$
	Control	$(1.02 \pm 0.04) \times 10^{-1}$	50.8 ± 6.6	$(2.01 \pm 0.27) \times 10^{-3}$
PhoX P-diesterase	$FeSO_4$	NA	NA	NA
	$ZnSO_4$	NA	NA	NA

Table 2. Michaelis-Menten parameters for diesterase activity of each enzyme with and without the presence of $ZnSO_4$ or $FeSO_4$. Data reported are enzyme specific K_{cat} (V_{max} divided by concentration of enzyme), the K_M Michaelis-Menten constant and the K_{cat}/K_M.

Enzymatic activity in presence of different metal cofactors

Enzymatic activity of the five enzymes was assessed under identical conditions in the presence of six different metal cofactors (Co, Cu, Mn, Ni, Fe and Zn). Both monoesterase activity, measured using the substrate MUF-P, and diesterase activity, measured using bis-MUF-P, were analyzed.

The presence of the metals affected the monoesterase activity of all enzymes (Fig.4). For PhoA, the highest enzymatic activity was observed under control conditions (1.4 nmol s^{-1} L^{-1}) and in presence of iron (1.45 nmol s^{-1} L^{-1}). The other metal cofactors appeared to slightly inhibit its enzymatic performance. PhoD exhibits its highest enzymatic activity in the presence of cobalt and manganese, reaching 0.55 x 10^{-2} nmol s^{-1} L^{-1}. In Contrast, PhoX_OM showed the highest enzymatic activity in the presence of copper, with a rate of 0.88 x 10^{-2} nmol s^{-1} L^{-1}. PhoX displayed its highest activity under control conditions, while the addition of metal cofactors reduced its performance of the enzyme by approximately 25%. The enzyme PafA exhibited notably low activity in presence of iron, manganese, and copper, as indicated by negative values in Figure 4. The monoesterase activity of PafA showed an extremely low to no activity.

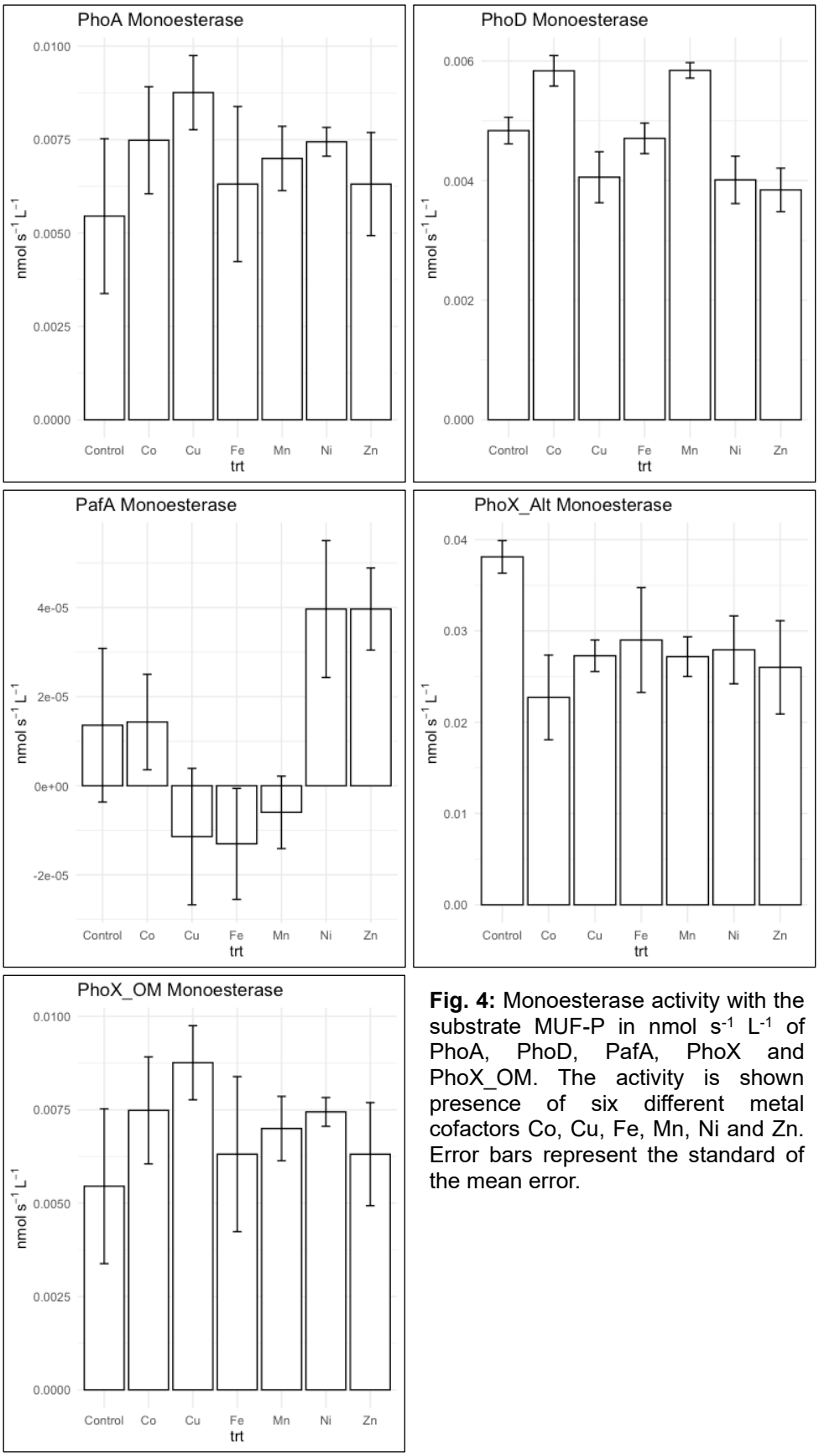

Fig. 4: Monoesterase activity with the substrate MUF-P in nmol s^{-1} L^{-1} of PhoA, PhoD, PafA, PhoX and PhoX_OM. The activity is shown presence of six different metal cofactors Co, Cu, Fe, Mn, Ni and Zn. Error bars represent the standard of the mean error.

Diesterase activity was detected for all enzymes, though the influence of metal cofactors varied (Fig.5). For PafA and PhoD, the highest enzymatic activity was observed under control conditions. Specifically, PafA exhibited a diesterase activity of approximately 0.175 x 10^{-1} nmol s^{-1} L^{-1} under control conditions, while the presence of metal cofactors, resulted in slightly reduces activity, ranging between 0.1 x 10^{-1} and 0.15 x 10^{-1} nmol s^{-1} L^{-1}. Similarly, PhoD displayed its highest activity at 0.23 x 10^{-1} nmol s^{-1} L^{-1}, with cobalt showing the most pronounced inhibitory effect, reducing activity to 0.16 nmol s^{-1} L^{-1}. In contrast, PhoX, PhoX_OM and PhoA exhibited increased activity in the presence of certain metal cofactors.

For PhoX, enzymatic activity in the presence of nickel was comparable to the control, while all other metal cofactors slightly enhanced its enzymatic activity. PhoX_OM showed a similar trend, with zinc maintaining activity levels comparable to the control while all other metal cofactors led to increased enzymatic activity.
The most pronounced enhancement was observed for PhoA. The presence of manganese doubled its enzymatic activity, while cobalt and copper tripled the enzymatic activity relative to control conditions, both reaching approximately 0.09 nmol s^{-1} L^{-1}.

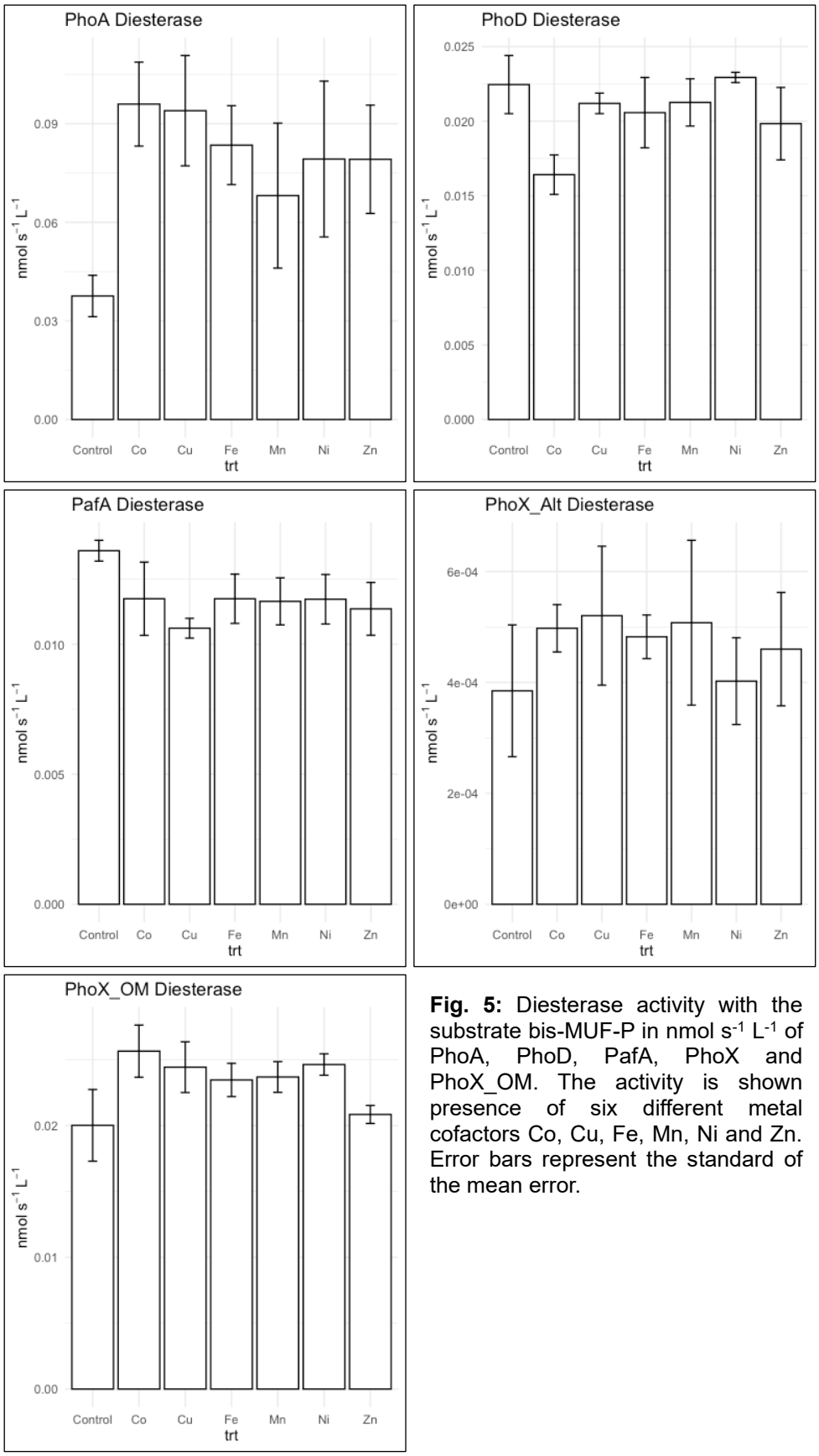

Fig. 5: Diesterase activity with the substrate bis-MUF-P in nmol s^{-1} L^{-1} of PhoA, PhoD, PafA, PhoX and PhoX_OM. The activity is shown presence of six different metal cofactors Co, Cu, Fe, Mn, Ni and Zn. Error bars represent the standard of the mean error.

Half Lifetime & Lifetime

The half-lifetime of an enzyme describes the time required for enzymatic activity to decrease to 50% of its initial performance. Figure 6 illustrates the half-lifetime of monoesterase activity of PhoA, PhoD, PhoX and PhoX_OM as well as the diesterase activity of these enzymes, including PafA. The monoesterase activity of PafA is left out, as the activity was minimal. The measurements were conducted at three different temperatures: 4°C, 14°C and 24°C in both ASW (control) and ASW with the addition on 1 µM Pi.

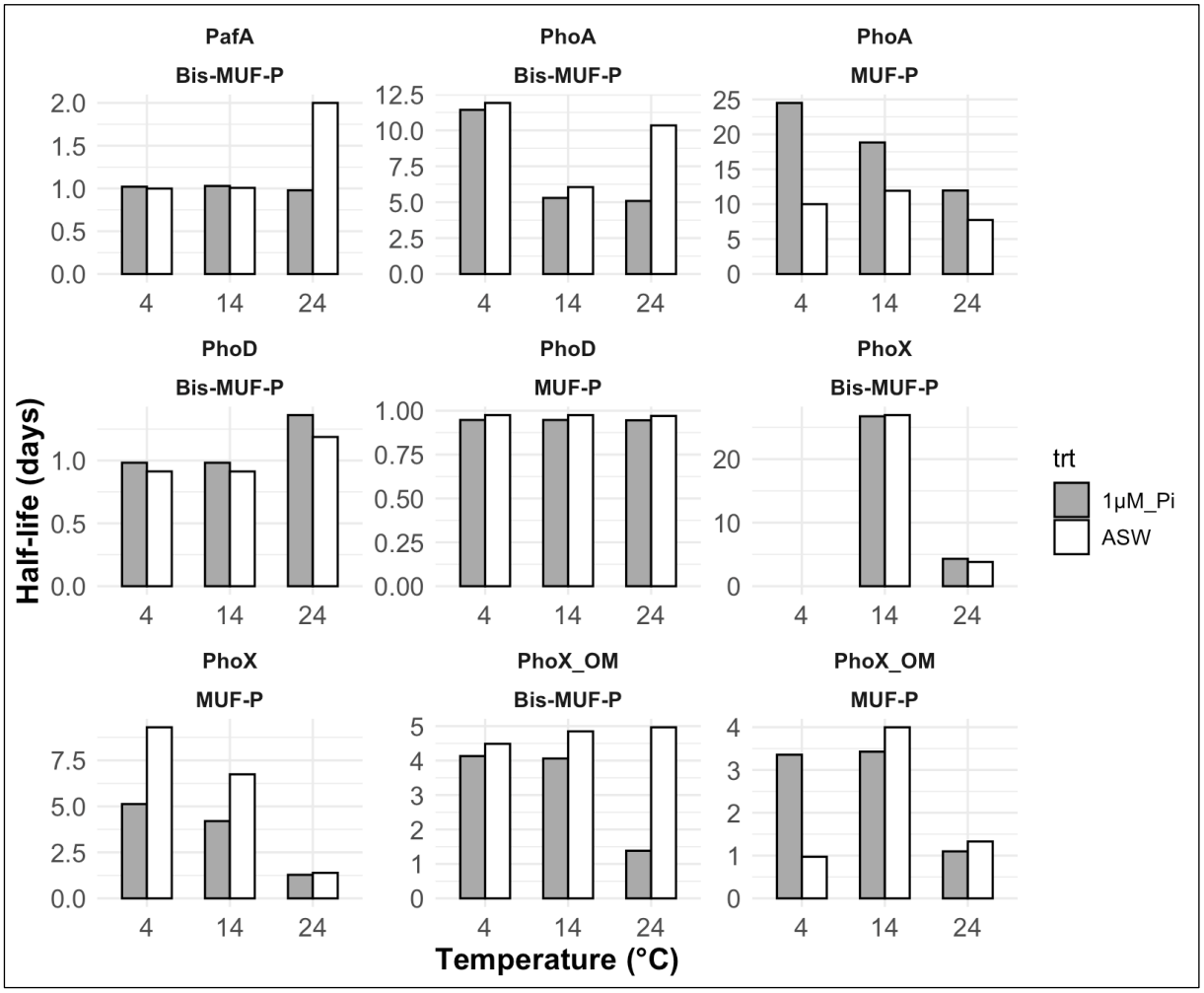

Fig. 6: Half-lifetime for PafA, PhoA, PhoD, PhoX and PhoX_OM with and without presence of 1µM Pi at 4°C, 14°C and 24°C. Half-lifetime shows the amount of time it takes for 50% of the enzyme initial activity to be lost.

For the monoesterase activity of PhoA, the presence of Pi notably increased stability. At 4°C, PhoA exhibited a half-lifetime of 25 days under additional Pi, which is 2.5 times longer than the control. This represents the longest half-lifetime observed among all enzymes. This trend of presence of Pi persisted at 14°C and 24°C, though the difference between presence of Pi and control conditions became less pronounced. PhoX diesterase activity shows at 14°C under both conditions the second highest lifetime of 24 days. At 24°C, PhoA had a half-lifetime of 12 days with Pi, compared to 8 days in the control.

For PhoX_OM monoesterase activity, presence of Pi also enhanced stability, particularly at 4°C, where the half-life increases to three days compared to one day under control conditions. Similarly, the diesterase activity of PhoD was also stabilized in presence of Pi. At 4°C and 14°C, the half-lifetime remained comparable between presence of Pi and control conditions, while at 24°C, the half-life increased to 1.4 days with the presence Pi, compared to 1.2 days in the control.

Generally, for some enzymes the presence of Pi shows a slightly negative effect on the half-lifetime. This could be seen for the monoesterase activity of PhoD and PhoX_OM, and the diesterase activity if PhoA, PhoX and PhoX_OM.
Temperature partially also influences the half-lifetime of some enzymes. Most of the enzymes a faster decay at higher temperatures can be observed. For example, the monoesterase of PhoA, PhoX and PhoX_OM follows this trend. But for the diesterase activity of PhoA and PhoD, this trend is not visible.

The following two figures (Figure 7 and 8) illustrate the monoesterase and diesterase activities of all five enzymes – PhoA, PhoD, PafA, PhoX_OM and PhoX – over a 60-day period.

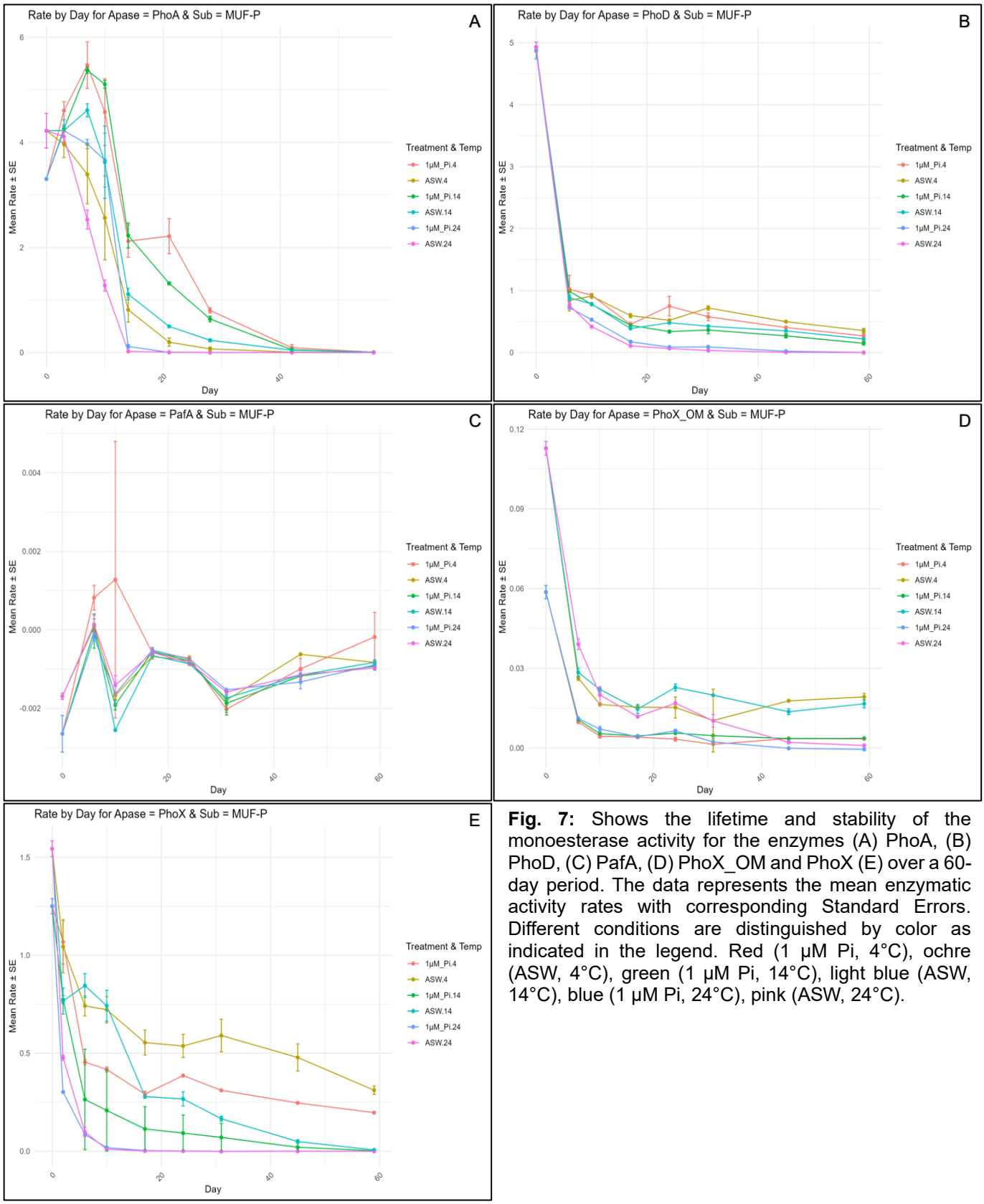

Fig. 7: Shows the lifetime and stability of the monoesterase activity for the enzymes (A) PhoA, (B) PhoD, (C) PafA, (D) PhoX_OM and PhoX (E) over a 60-day period. The data represents the mean enzymatic activity rates with corresponding Standard Errors. Different conditions are distinguished by color as indicated in the legend. Red (1 µM Pi, 4°C), ochre (ASW, 4°C), green (1 µM Pi, 14°C), light blue (ASW, 14°C), blue (1 µM Pi, 24°C), pink (ASW, 24°C).

The monoesterase activity of three enzymes, PhoD, PhoX_OM and PhoX exhibit a similar trend (Fig. 7). The first measurement reveals the highest enzymatic activity, with PhoD displaying the most pronounced activity across all conditions. At the start the mean activity rate of PhoD is approximately 4.9 nmol s^{-1}, which decreases sharply to ~0.7 – 1 nmol s^{-1} within the first 5 days. After the initial decline, the activity levels stabilize at a low plateau under the different conditions. Both conditions at 24°C exhibit the lowest enzymatic activity at 0.1 nmol s^{-1} compared to the lower temperature treatments. The monoesterase activity of PhoX_OM follows a similar trend. Under control conditions the activity begins at a higher rate (0.1 nmol s^{-1}), whereas in the presence of Pi, the activity starts at 0.06 0.1 nmol s^{-1}. Both conditions experience a sharp decline, stabilizing at a low plateau. However, control conditions consistently maintain higher activity levels compared to Pi conditions throughout the measurement period. The enzyme PhoX shows a comparable trend. However, the activity range between the different conditions is more pronounced, compared to the preceding enzymes. Overall, the highest enzymatic activity across the period of measurement is observed for the control conditions at 4°C. The enzyme PhoA shows an initial increase in activity after the first measurement. At the first measurement a clear distinction between the control and Pi conditions is observed. The enzymatic activity under ASW conditions the enzymatic activity initially starts at 4.3 nmol s^{-1}, while the Pi conditions start slightly lower at 3.3 nmol s^{-1}. Afterwards the Pi conditions show a greater increase in activity compared to the control conditions, followed by a more gradual decline in activity relative to the trends observed for other enzymes. The enzyme PafA shows the most irregular trend in monoesterase activity. Its activity begins at a negative rate and subsequently increases slightly, although the overall activity remains below zero throughout the measurement period.

The diesterase activity of the five enzymes over a 60-day period show similar trends as the monoesterase activity. PhoD shows sharp decline in activity between the first to the second measurement (Fig.8). Among the temperature conditions, the activity of PhoD at 24°C decreases most notably, stabilizing at a consistently low level for the period of measurements. In contrast, PafA initially shows higher diesterase activity at 24°C with 0.8 nmol s^{-1}, whereas the activity at 14°C and 4°C the activity begins at a slightly lower level at 0.65 nmol s^{-1}. After 5 days the activity decreases to approximately 0.25 – 0.35 nmol s^{-1} and remains at a similar level for the 60-day period. The diesterase

activity of PhoX_OM generally follows a trend comparable to PhoD and PafA. However, the control exhibits twice the activity compared to the presence of Pi. Under control conditions the enzymatic activity starts at 0.09 nmol s^{-1}, whereas in the presence of Pi the enzymatic activity displays only 0.05 nmol s^{-1}. This difference persists throughout the entire time span, with one notable exception. By the end of the 60-day period, the control conditions at 24°C decline to the same activity level as those measured in the presence of Pi. PhoA and PhoX display a different trend compared to the other enzymes. Initially, the activity of PhoA declines, similar to the other enzymes, but increases up to 0.51 nmol s^{-1} after 10 days to levels exceeding the first measurements at 0.4 – 0.5 nmol s^{-1}. Following this peak, the activity decreases to 0.02 -0.1 nmol s^{-1}, where it stabilizes. For PhoX, both conditions at 24°C and the presence of Pi at 14°C show a decline in activity after the first measurement, eventually stabilizing at similarly low activity levels. Under control conditions at 14°C the activity increases to 0.048 nmol s^{-1} before gradually declining throughout measurement period. In contrast, both conditions at 4°C show no decline, rather a stable activity. For the control conditions, the activity remains between 0.03 – 0.043 nmol s^{-1}, while in presence of Pi, it displays 0.022 – 0.032 nmol s^{-1}.

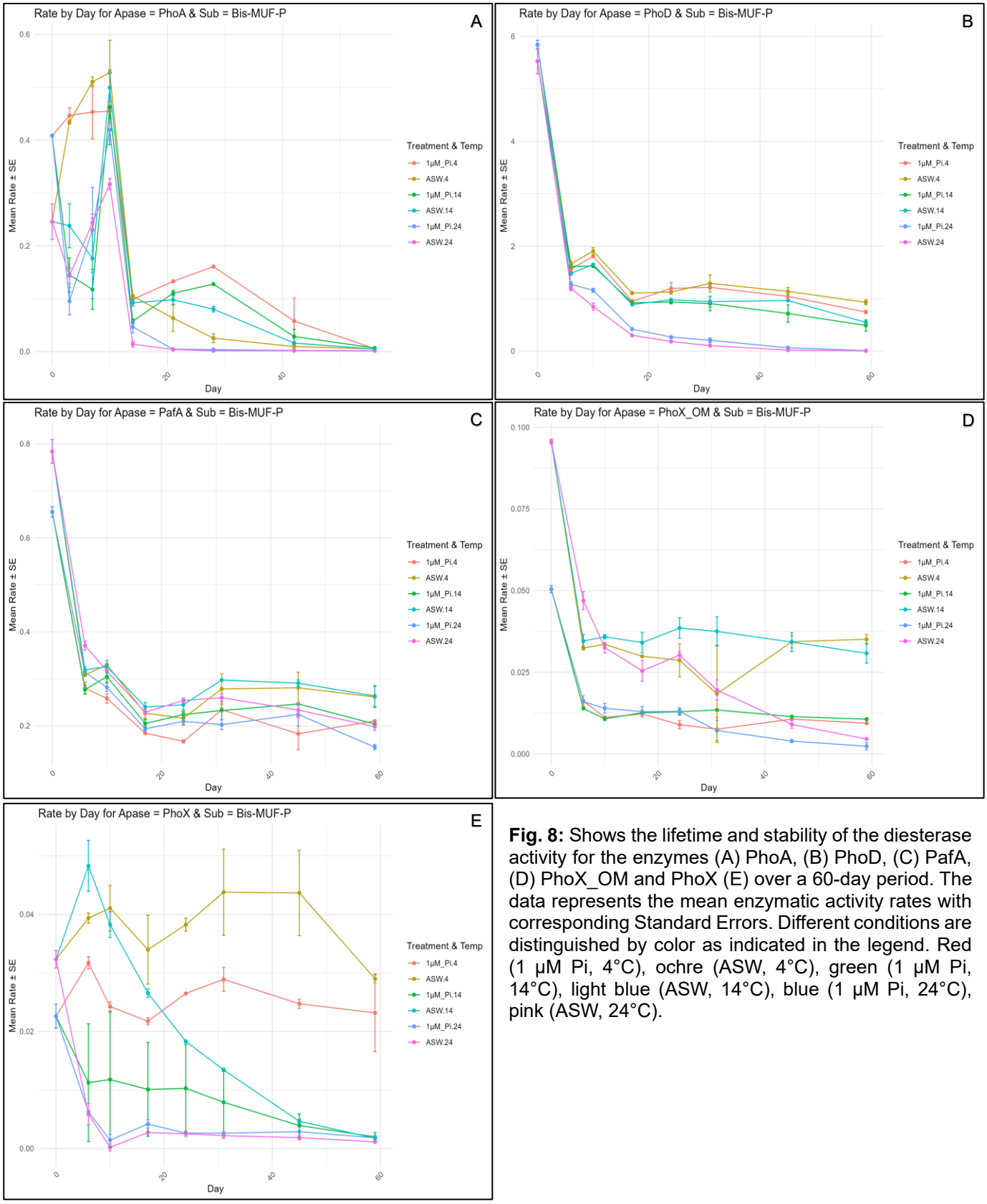

Fig. 8: Shows the lifetime and stability of the diesterase activity for the enzymes (A) PhoA, (B) PhoD, (C) PafA, (D) PhoX_OM and PhoX (E) over a 60-day period. The data represents the mean enzymatic activity rates with corresponding Standard Errors. Different conditions are distinguished by color as indicated in the legend. Red (1 µM Pi, 4°C), ochre (ASW, 4°C), green (1 µM Pi, 14°C), light blue (ASW, 14°C), blue (1 µM Pi, 24°C), pink (ASW, 24°C).

Influence of pH

The final set of experiments evaluated the enzymatic activity of PhoA, PhoD, PafA, PhoX_OM, PhoX under varying pH levels. Figures 9 and 10 illustrate the monoesterase and diesterase activity of these enzymes at three different pH levels: 7.6, 7.9, and 8.1. In addition, the impact of the presence of Pi at pH 8.1 was examined. For optimal enzyme stability, specific metal cofactors were included. Zinc was added for PhoA and PafA and iron for PhoD, PhoX and PhoX_OM.

The effects of pH on enzymatic activity are not consistent across all enzymes (Fig. 9). For monoesterase activity of PhoX and PafA, a decrease in pH corresponds to a notable decline in enzymatic activity. PhoX exhibits its highest activity with 0.095 nmol s^{-1} L^{-1} at pH 8.1. This enzyme demonstrates the most pronounced decline in activity with decreasing pH. The presence of Pi at pH 8.1 reduces PhoX activity approximately by half compared to its peak activity at pH 8.1. The monoesterase activity for PafA reaches its maximum at pH 8.1 with a value of 0.06 nmol s^{-1} L^{-1}. Both, the presence of Pi at pH 8.1 and a pH of 7.9 result in comparable activity levels, approximately 0.06 nmol s^{-1} L^{-1}. The lowest activity for PafA is observed at pH 7.6, although the difference to pH 7.9 is not pronounced. PhoA displays its highest monoesterase activity at pH 7.9 achieving 0.1 nmol s^{-1} L^{-1}. This is closely followed by the activity observed at pH 8.1 in the presence of Pi. At pH 7.6 PhoA is halved, reaching only 0.05 nmol s^{-1} L^{-1}, compared to its activity at pH 7.9. Among all tested enzymes PhoD generally exhibits the highest enzymatic activity overall. For PhoD the highest activity is observed at pH 8.1 and 7.9, both at 0.23 nmol s^{-1} L^{-1}. However, the presence of Pi at pH 8.1 seem to inhibit the enzymatic activity slightly. The lowest activity for PhoD can be investigated at pH 7.6, where activity decreases to one-fourth of the values observed at pH 8.1 and 7.9 (0.05 nmol s^{-1} L^{-1}). PhoX_OM shows consistent activity levels across all conditions, except for pH 7.6. At pH 8.1, 7.9 and in the presence of Pi at pH 8.1, the activity remains relatively stable at 0.055 nmol s^{-1} L^{-1}. The lowest activity for this enzyme is observed at pH 7.6.

A consistent trend in the diesterase activity under different pH conditions is observed across all enzymes (Fig. 10). Enzymatic activity decreases as pH declines. At pH 8.1

the presence of Pi results in a slight decrease in activity compared to pH 8.1. However, the inhibitory effect of Pi is less pronounced than the overall decline in activity caused by decreasing pH levels. The highest total enzymatic activity exhibits PhoD reaching 0.15 nmol s^{-1} L^{-1} at pH 8.1. However, its activity decreases below 0.05 nmol s^{-1} L^{-1} at pH 7.6. PhoA closely follows PhoD in terms of activity, showing its maximum activity at pH 8.1 with 0.12 nmol s^{-1} L^{-1} and its lowest enzymatic activity at pH 7.6 at with 0.049 nmol s^{-1} L^{-1}. Lowest activity in general can be investigated for PhoX_OM with its peak enzymatic activity with 0.04 nmol s^{-1} L^{-1}.at pH 8.1.

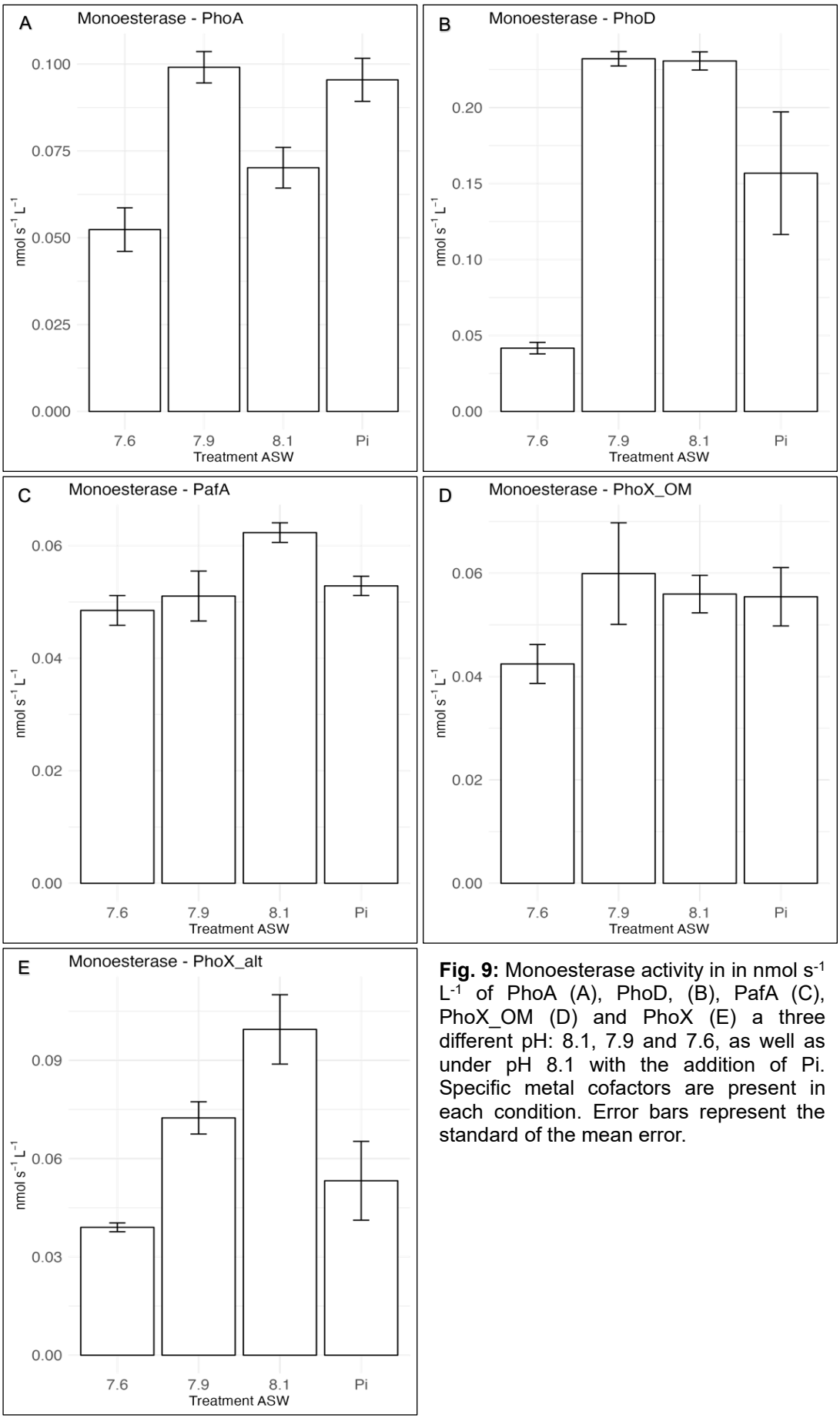

Fig. 9: Monoesterase activity in in nmol s^{-1} L^{-1} of PhoA (A), PhoD, (B), PafA (C), PhoX_OM (D) and PhoX (E) a three different pH: 8.1, 7.9 and 7.6, as well as under pH 8.1 with the addition of Pi. Specific metal cofactors are present in each condition. Error bars represent the standard of the mean error.

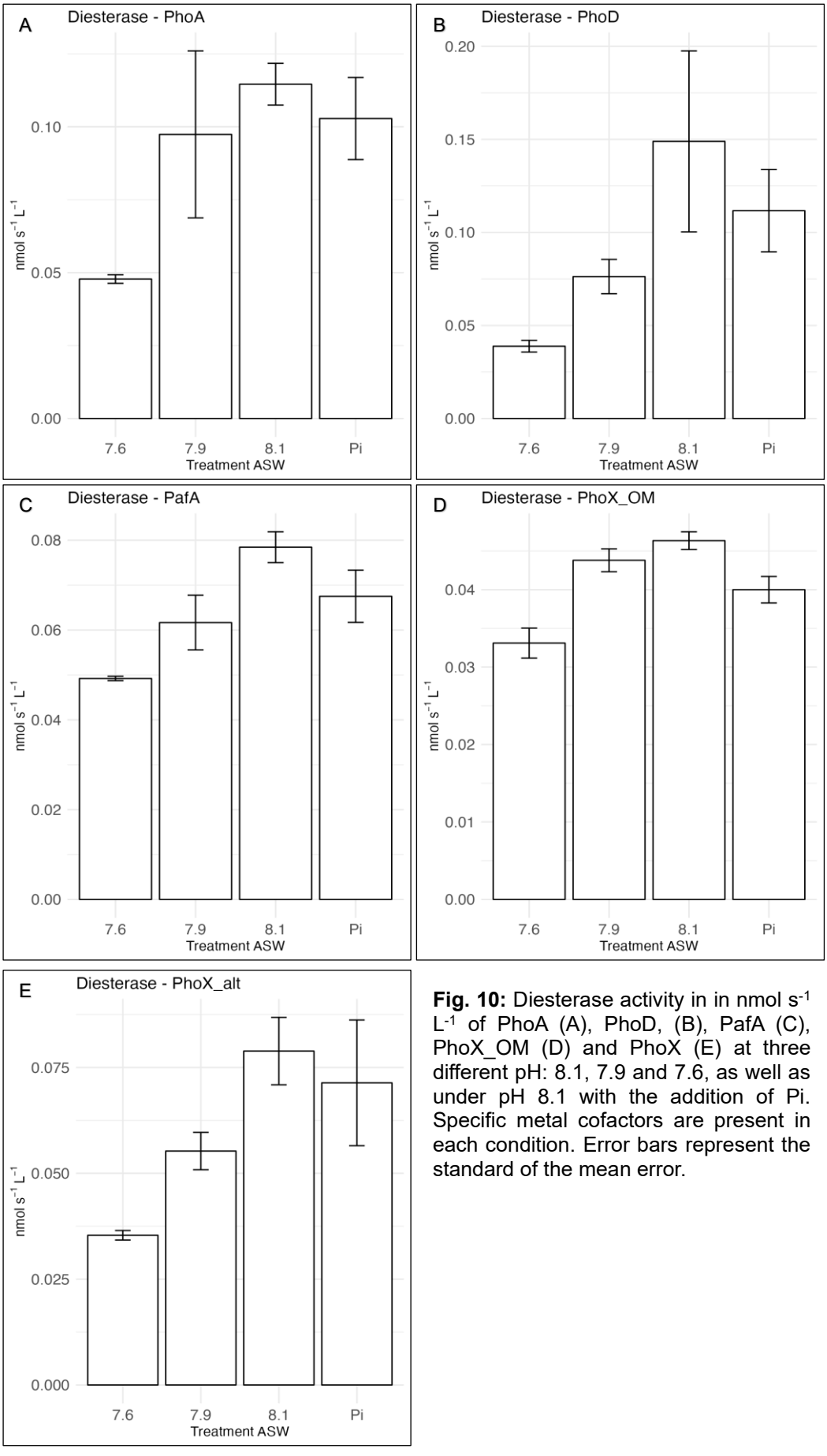

Fig. 10: Diesterase activity in in nmol s^{-1} L^{-1} of PhoA (A), PhoD, (B), PafA (C), PhoX_OM (D) and PhoX (E) at three different pH: 8.1, 7.9 and 7.6, as well as under pH 8.1 with the addition of Pi. Specific metal cofactors are present in each condition. Error bars represent the standard of the mean error.

Discussion

APases are arguably the most important enzyme in the marine P-cycle. A huge part is the supply of bioavailable P, through the conversion of DOP. As APases are found throughout the global ocean (Su et al., 2023), we further investigated in the specific resonance of this enzymes as they are presented to different environmental stressors. Different settings allowed us, to further investigate the enzymatic activity, stability, and flexibility of this important enzyme.

The anthropogenic impact on marine ecosystems represents major ecological challenges. Our findings corroborate existing research that demonstrates the detrimental effects of human activities on oceanic systems. Rising atmospheric CO_2 levels have led to enhanced CO_2 uptake by the ocean (Feely et al., 2004), resulting in a consequential decrease in pH at the sea surface but progressively affecting deeper waters through mixing and biological processes (Caldeira & Wickett, 2003). This phenomenon of ocean acidification fundamentally disrupts the biogeochemical processes in the sea and threatens the stability of ecosystems.

Because APase activity with MUF-P and bis-MUF-P is a ubiquitous process documented in marine environments (Su et al., 2023), these were the substrates of choice for our experiments. With this we also emphasized the critical importance of examining the promiscuous activity potential of APases, as highlighted by Srivastava et al. (2021).

Kinetic performance of mono- and diesterase activity in presence of zinc and iron

Metals are essential in biological systems, often serving as cofactors or structural components of proteins and enzymes. Enzymatic activity is dependent on the availability of metal cofactors, as they can further stabilize the enzyme and catalyze the enzymatic reaction. Not only the biogeochemical cycling of Carbon, Nitrogen and P, but also the speciation, and bioavailability of trace metals in seawater is expected to be influenced by the changing ocean chemistry (Buck et al., 2018). Understanding

the impact of metal availability on APase function is crucial for assessing the potential consequences of oceanic trace metal fluctuations on the marine P-cycle.

Previous studies have identified dependencies on native metals for APases. PhoD and PhoX require iron and calcium as metal cofactors in their catalytic centers (Wu et al., 2007; Yong et al., 2014; Coleman, 1992; Ali & Hamaza, 2012), whereas PhoA and PafA are dependent on zinc (Wu et al., 2007). The availability of some metals in the marine environment can be highly variable, with significant limitations in certain regions. Iron deficiency is particularly pronounced in areas such as the equatorial and subarctic Pacific, as well as the Southern Ocean, and coastal upwelling zones, affecting phytoplankton growth and primary production (Martin & Fitzwater, 1988; Boye et al., 2005; Boyd et al., 2007). Iron scarcity in oceanic waters is primarily caused by its low solubility and rapid uptake by phytoplankton from the surface ocean (Liu & Millero, 2002).

The availability of zinc is also limited, especially in surface waters, where its concentration remains low across the global ocean. Only in greater depths does its concentration increase again due to remineralization of sinking detritus (de Souza et al., 2018). Given the essential role of iron and zinc as cofactors for APases, this study investigated whether the substitution of these metals affects catalytic efficiency. By assessing key kinetic parameters, including substrate affinity and reaction velocity, we aimed to determine how metal availability influences enzymatic performance. The Michaelis–Menten constant (K_M) provides insights in to enzyme-substrate affinity, with lower K_M values indicating a stronger binding affinity. The maximum reaction velocity (V_{max}) represents the upper limit of enzymatic activity under substrate saturation (Segel, 1975; Duman & Tekin, 2020).

The results Indicate that the addition of metal cofactors effected the enzymatic kinetics, though the extend and direction of this effect varied dependent on enzyme and specific cofactor. The catalytic rate remained relatively stable among the enzymes exhibiting the monoesterase activity. The only notable exception was PhoX, where the presence of zinc strongly enhanced kinetic performance, increasing K_{cat} by a factor of 1.4. In contrast, the enzyme substrate affinity showed the most pronounced variation for PafA, displaying a 4.7-fold increase for monoesterase activity in the presence of iron. This shift in affinity was further reflected in the catalytic efficiency, as the K_{cat}/K_M

ratio was notably elevated in the presence of iron. However, for PhoD, the presence of both metals resulted in a decreased catalytic efficiency. PhoX_OM even showed in the presence of iron a reduced catalytic efficiency by a factor of 3.5.

For diesterase activity, both kinetic parameters remained relatively stable across individual enzymes in the presence of their respective metal cofactors. Similarly, the catalytic efficiency showed only low differences between enzymes and their associated metal cofactors, suggesting that metal substitution is feasible and does not negatively impact diesterase activity. Interestingly, despite this general stability, the catalytic efficiency of PafA was slightly reduced in the presence of zinc, a trend that was also observed for PhoD, indicating a subtle influence of zinc on enzymatic performance. These findings suggest that, unlike monoesterase activity, diesterase activity appears to be less dependent on specific metal cofactors, allowing for functional flexibility under varying metal availability conditions.

Despite numerous studies on APase production by marine microorganisms, kinetic analyses of this process are still limited (Ma et al., 2007; Maruthiah et al., 2017; Cheng et al., 2015). Additionally, the comparison of K_M and V_{max} values among marine microbial APases is challenging due to the heterogeneity in experimental conditions. Our observation show that the choice of substrates impacts the determination of kinetic parameters. This introduces systematic biases when attempting to compare reported enzymatic activity in other studies (Van Loo et al., 2010).

Further our findings indicate limited flexibility in metal cofactor utilization across the APases. Enzymatic activity consistently showed lower activity in the presence of non-native cofactors compared to the native metal, suggesting a high degree of metal specificity. However, our experimental design was limited to observe whether the residual activity resulted from the incorporation of non-native metals or from the native metal which is already incorporated in the enzymes from the beginning. This suggests that if the non-native metals support enzymatic function, they do so with lower efficiency. This underscores the role of specific metal cofactors in optimizing enzymatic performance and highlight potential functional adaptations in response to metal availability in the marine environment and that metal availability plays a crucial role in modulating enzyme functionality within the marine P-cycle.

Enzymatic activity in presence of different metal cofactors

Previous studies on the role of trace metals in microbial dynamics emphasized their focus on the response of autotrophic phytoplankton to iron fertilization, while the influence of other biogeochemically relevant metals remains an area requiring further investigation (Baltar et al., 2018b). The availability of trace metals in marine environments is largely constrained by their low solubility, with concentrations decreasing rapidly with distance from the coast (Johnson et al., 1997).

The impact of trace metal availability on heterotrophic bacteria has been demonstrated in iron, cobalt, and iron+cobalt amendments (Wisniewski et al., 2008). Revealing an increase in bacterial abundance in response to iron, suggesting that the availability of these metals limits the heterotrophic bacterial growth (Jain et al., 2015; Browning et al., 2017). Such findings align with evidence that APases are able to incorporate different metal ions as cofactors (Lin et al., 2019. In enzyme models, the presence of a single metal ion can enhance the second-order rate acceleration even up to six orders of magnitude (Ma et al., 2007; Baweja et al., 2017).

Further supporting the role of metal availability in regulating APase function, Mahaffey et al. (2014) observed an enhancement of APase-mediated DOP hydrolysis in model marine microbes upon metal addition in axenic cultures. This suggests that APase activity in natural environments may be limited by trace metal availability. While iron and zinc are well-established cofactors for marine APases, there is evidence that these enzymes may also interact with other trace metals. For instance, dissolved cobalt concentrations are significantly lower in the North Atlantic compared to the South Atlantic, potentially due to its incorporation into PhoA enzymes, fulfilling the natural Co requirements of marine microbes (Wojciechowski et al., 2002), or due to Co substitution for zinc (Jakuba et al., 2008; Saito et al., 2017). Additionally, in the Southern Ocean, the seasonal depletion of Mn, iron, and zinc has been shown to enhance microbial cadmium uptake, further emphasizing the complex interactions between trace metal availability and microbial enzymatic function (Baars et al., 2014).

Building on these considerations, our findings indicate that the presence of metal cofactors did not massively enhance monoesterase activity, suggesting that these

enzymes may exhibit a degree of functional independence from specific metal cofactors under the tested conditions. In contrast, for diesterase activity, the most pronounced positive effect of metal cofactors was observed for PhoA, where enzymatic activity was at least twice as high compared to the control, highlighting a stronger dependency on metal availability.

However, these results should be interpreted with caution, as the potential for metal contamination in experimental setups remains a considerable concern. Given the high risk of trace metal contamination, even small unintended metal inputs could have influenced enzymatic activity. One possible explanation for the lack of more enhanced metal effects on enzymatic activity is the sterilization process of ASW via autoclaving, which may have introduced trace amounts of metals, thereby masking the impact of the intentionally added cofactors. This suggests that only minimal concentrations of metal cofactors may be required to achieve high enzymatic functionality, potentially explaining why the differences between various metals were less pronounced than initially expected.

These findings emphasize the importance of carefully controlling metal concentrations in enzymatic assays and suggest that even trace amounts of metal cofactors could be sufficient to sustain phosphatase activity in marine environments.

Half-lifetime & lifetime

Extracellular enzymatic activity manifests in two primary forms: cell-associated enzymes, which are either bound to the cell wall or found in the periplasm, and cell-free ectoenzymes suspended in the water column. Previous research has established that these cell-free enzymes can remain active for extended periods, ranging from days to several weeks (Hoppe et al., 2003; Baltar, 2018a; Steen and Arnosti, 2011; Baltar et al., 2013). Moreover, these dissolved enzymes constitute a substantial portion of the total extracellular enzyme pool, as documented in multiple studies (Kamer and Rassoulzadegan, 1995; Li et al., 1998; Baltar et al., 2010; Duhamel et al., 2010; Baltar et al., 2019).

PhoA emerged as the most stable enzyme at 4°C and in the presence of Pi, with an half-lifetime of 25 days. Similarly, PhoX demonstrated long-lasting stability at 14°C, with a half-lifetime of 24 days for diesterase activity, both with and without Pi. For monoesterase activity, the control conditions without the presence of Pi generally resulted in a longer half-lifetime, but not at a temperature of 24°C.

P plays a key role, as it is the main limiting nutrient for primary production in the open oceans (Björkman & Karl, 2003). Despite relatively high ambient Pi concentrations, P-stress and limitation have been documented in both freshwater and seawater (Trommer et al., 2013; Wang et al., 2021). Surface mixed-layer ocean waters exhibit significant variation in phosphate concentrations, ranging from less than 1 nM in regions like the Sargasso and Mediterranean Seas to 1–3 µM in coastal upwelling zones (Duhamel et al., 2021). DOP concentrations frequently exceed those of phosphate, particularly in oligotrophic marine environments. APase activity can serve as an indicator of microbial phosphate stress, as an indicator of microbial DOP utilization (Su et al., 2023), and eutrophication (Singh & Pandey, 2023). This enzyme is typically induced at extremely low phosphate concentrations, resulting in elevated APase activity in phosphorus-limited oceanic regions (Su et al., 2023).

Our analysis revealed intriguing patterns in enzyme stability under different temperature conditions and in the presence or absence of Pi. Since APase activity is upregulated at low Pi concentrations, we hypothesized that elevated Pi levels might have a negative impact APase stability. However, our results revealed a more complex relationship between the presence of Pi and the stability of the enzymes. The presence of Pi did not uniformly reduce enzyme half-lifetimes compared to Pi-free conditions in ASW. Notably, PhoA exhibited enhanced stability in the presence of Pi. The effects of temperature on enzyme half-lifetimes on the enzymes also varied. While higher temperatures were generally associated with accelerated decay rates, some enzymes, particularly the diesterase activity of PhoA and PhoD, deviated from this pattern.

In our experimental observations regarding the activity of the enzymes during a 60-day observation period, most enzymes followed a similar activity pattern: high initial activity followed by a sharp decline and subsequent stabilization at a lower activity plateau. However, distinct variations emerged among the different APases. PhoA

monoesterase activity exhibited unique behavior, showing delayed activity decline compared to other enzymes and, increased activity in the presence of Pi.

Temperature emerged as a significant factor influencing lifetime enzyme stability. Both PhoA monoesterase and PhoX demonstrated more pronounced decay at higher temperatures, following congruent patterns. However, the influence of Pi varied among enzymes. While Pi presence appeared to have a substantial impact on PhoA activity, its effect on PhoX was less pronounced compared to temperature effects.

The diesterase activity patterns showed similarities to monoesterase activity, although the relative influences of Pi and temperature were less distinct. PhoD showed particular sensitivity to elevated temperatures, with notably reduced activity at 24°C regardless of Pi presence. In contrast, PhoX_OM demonstrated greater sensitivity to Pi presence than temperature, maintaining higher enzymatic activity in Pi-free conditions throughout the experimental period.

The key findings of this experiment demonstrate variation in the overall lifetime between the APase families. PhoA showed a deviating peak in activity after several days of incubation, distinguishing it from other APases. Notably, Pi exerted a positive effect on the longevity of the enzymes, which is most pronounced for PhoA. These variations suggest distinct evolutionary adaptations among phosphatases, potentially reflecting their roles in different ecological niches or environmental conditions.

These findings contribute to our understanding of extracellular enzyme dynamics in marine systems and highlight the complexity of phosphatase regulation in response to environmental variables. Future research should focus on elucidating the mechanisms underlying these differential responses and their ecological implications.

Influence of pH

The pH of marine ecosystems is a fundamental parameter that influences APase activity and thereby modulating the dynamic utilization of DOP within the marine P-cycle (Weiss et al., 2018; Guo et al., 2023). The regulatory relationship between

APase activity and phosphate availability, serves as a key indicator of microbial phosphate stress**,** DOP utilization (Su et al., 2023), and eutrophication processes (Singh & Pandey, 2023) in oceanic systems. We aimed to further assess the inhibition of APase activity with Pi and APase activity under decreasing pH conditions.

The progressive acidification of seawater has been demonstrated to affect the dynamics of metal-binding, with a particular effect in iron complexation mechanisms. Avendaño et al. (2016) demonstrated that reduced pH conditions enhance ligand binding strength relative to inorganic complexation processes, primarily attributed to a decrease in hydroxide ion concentration. This pH-dependent shift in metal binding dynamics suggests that ongoing ocean acidification, disrupts the balance of bioavailable iron affecting enzymatic processes central to marine biogeochemical cycles.

A pronounced pH-dependent decline in monoesterase activity across all tested APases was observed. Both PafA and PhoX demonstrated diminished activity under lower pH conditions, with the inhibitory effects being strikingly pronounced in the presence of Pi. Particularly**,** PhoX activity decreased by 50% in the presence of Pi, suggesting a complex interplay between pH and phosphate-mediated inhibition. PhoD maintained stable activity at pH 8.1 and 7.9 but showed a drastic decline at pH 7.6. Similarly**,** PhoX_OM showed stable activity between pH 8.1 and 7.9 but exhibited a decrease in enzymatic activity at pH 7.6**.** Overall, all five enzymes of the APase exhibited their lowest activity at pH 7.6, indicating a general negative effect of acidification on APase activity.

Analysis of diesterase activity revealed a clear pH-dependent trend, characterized by with activity reduction with increasing acidity. Notably, PhoX_OM was an exception, where the presence of Pi showed less inhibition at pH 7.9 compared to other pH levels. Which highlights the complex interactions between enzyme activity and environmental parameters.

Structural analyses of related phosphatase enzymes further support the observed pH dependency. BcPMH, while phylogenetically distinct from APases (Jonas et al., 2008; Hanson et al., 2004), demonstrates pH-dependent catalytic activity, with an optimal range between pH 6 and 10 (Bert van Loo et al., 2010). This broad pH tolerance

contrasts with the more restricted range for APase activity, which typically peaks under alkaline conditions between pH 8 and 10.5. This has been demonstrated for various marine bacterial and fungal species, including *Pseudoalteromonas sp.* 129–1 **(**Ji et al., 2012; Wu et al., 2015). This indicated that APases exhibit pH dependent activity patterns which depend in their environment. However, our experiments show that certain APase families are more sensitive to pH fluctuations. The monoesterase activity of PhoD and remains stable at pH 8.1 and 7.9, but decreases sharply at pH 7.6, a trend that is also observed for PhoX_OM, albeit to a lesser extent. The remaining APases show a more gradual decrease in activity as pH decreases, suggesting varying degrees of pH tolerance among different APase families.

The demonstrated sensitivity of APase activity to pH variations, carries strong implications for marine biogeochemistry under future climate scenario. Therefore, it is crucial to further investigate the potential consequences of ocean acidification on the marine phosphorus cycle. A deeper understanding of how pH fluctuations influence APase functionality will be essential for predicting future disruptions in microbial phosphorus acquisition and assessing potential threats to phosphorus cycling in marine ecosystems.

Conclusion

In this study we comprehensively studied the enzymatic activity of five different APases PhoA, PhoD, PafA, PhoX and PhoX_OM in several different conditions, including metal cofactors availability, Pi concentration, temperature, and pH. Although no clear trend in enzymatic activity with the substitution of different cofactors could be made, our findings provide novel insights into enzyme-specific metal dependencies and stability patterns.

The half-lifetime of most APases, were not strongly influenced by the presence of Pi, compared to the P-free controls. However, PhoA exhibited enhanced stability in the presence of Pi, suggesting an adaptation of PhoA at elevated Pi concentrations. Temperature was a critical factor influencing enzymatic stability, with most APases exhibiting a faster decay at higher temperatures. The overall lifetime between the APase families show variation. Notably the presence of Pi exerted a positive effect on

the longevity of the APases, which was most pronounced for PhoA. Finally, the change towards more acidic pH shows a clear influence on enzymatic activity. Here a clear trend of reduced enzymatic activity can be seen, as the pH in decreased to 7.6. This implicates the huge impact ocean acidification could have on the functionality of APases and therefore disrupting the P-cycle in the marine ecosystems.

These findings underscore the importance of considering multiple environmental stress factors together, as enzymatic activity is highly complex. Future research should explore the interactive effects of pH, temperature, Pi availability, and especially metal cofactors. A deeper mechanistic understanding will be crucial for predicting the impact of oceanic shifts on enzymatic functions and phosphorus cycling.

Sources

Ali, A. F., & Hamza, B. N. (2012). Purification and characterization of alkaline phosphatase enzyme from the periplasmic space of *Escherichia coli* C90 using different methods. *African Crop Science Journal, 20*(2), 125–135.

Altieri, A. H., & Gedan, K. B. (2015). Climate change and dead zones. *Global Change Biology, 21*(4), 1395–1406. https://doi.org/10.1111/gcb.12754

Avendaño, L., Gledhill, M., Achterberg, E. P., Rérolle, V. M. C., & Schlosser, C. (2016). Influence of ocean acidification on the organic complexation of iron and copper in Northwest European shelf seas: A combined observational and model study. *Frontiers in Marine Science, 3*, 58. https://doi.org/10.3389/fmars.2016.00058

Azam, F., & Malfatti, F. (2007). Microbial structuring of marine ecosystems. *Nature Reviews Microbiology, 5*, 782–791. https://doi.org/10.1038/nrmicro1747

Baars, O., Abouchami, W., Galer, S. J. G., Boye, M., & Croot, P. L. (2014). Dissolved cadmium in the Southern Ocean: Distribution, speciation, and relation to phosphate. *Limnology and Oceanography, 59*, 385–399. https://doi.org/10.4319/lo.2014.59.2.0385

Baltar, F. (2018a). Watch out for the “living dead”: Cell-free enzymes and their fate. *Frontiers in Microbiology, 8*, 2438. https://doi.org/10.3389/fmicb.2017.02438

Baltar, F., Arístegui, J., Gasol, J. M., Sintes, E., van Aken, H. M., & Herndl, G. J. (2010). High dissolved extracellular enzymatic activity in the deep Central Atlantic Ocean. *Aquatic Microbial Ecology, 58*(3), 287–302. https://doi.org/10.3354/ame01377

Baltar, F., Arístegui, J., Gasol, J. M., Yokokawa, T., & Herndl, G. J. (2013). Bacterial versus archaeal origin of extracellular enzymatic activity in the Northeast Atlantic deep waters. *Microbial Ecology, 65*(2), 277–288. https://doi.org/10.1007/s00248-012-0113-2

Baltar, F., De Corte, D., Thomson, B., & Yokokawa, T. (2019). Teasing apart the different size pools of extracellular enzymatic activity in the ocean. *The Science of the Total Environment*, *660*, 690–696. https://doi.org/10.1016/j.scitotenv.2019.01.083

Baltar, F., Gutiérrez-Rodríguez, A., Meyer, M., Skudelny, I., Sander, S., Thomson, B., Nodder, S., Middag, R., & Morales, S. E. (2018). Specific effect of trace metals on marine heterotrophic microbial activity and diversity: Key role of iron and zinc and hydrocarbon-degrading bacteria. *Frontiers in Microbiology, 9*, 3190. https://doi.org/10.3389/fmicb.2018.03190

Baweja, M., Singh, P. K., Sadaf, A., Tiwari, R., Nain, L., Khare, S. K., & Shukla, P. (2017). Cost-effective characterization process and molecular dynamic simulation of detergent compatible alkaline protease from *Bacillus pumilus* strain MP27. *Process Biochemistry, 58*, 199–203. https://doi.org/10.1016/j.procbio.2017.05.019

Bell, D. W., Pellechia, P. J., Ingall, E. D., & Benitez-Nelson, C. R. (2020). Resolving marine dissolved organic phosphorus (DOP) composition in a coastal estuary. *Limnology and Oceanography, 65*, 2787–2799. https://doi.org/10.1002/lno.11487

Berthold, M., Kleeberg, A., Löffler, J., & Hupfer, M. (2019). Magnitude and influence of atmospheric phosphorus deposition on the southern Baltic Sea coast over 23 years: Implications for coastal waters. *Environmental Sciences Europe, 31*, 27. https://doi.org/10.1186/s12302-019-0206-8

Björkman, K. M., & Karl, D. M. (2003). Bioavailability of dissolved organic phosphorus in the euphotic zone at station ALOHA, North Pacific Subtropical Gyre. *Limnology and Oceanography, 48*(3), 1049–1057. https://doi.org/10.4319/lo.2003.48.3.1049

Boyd, P. W., Jickells, T., Law, C. S., Blain, S., Boyle, E. A., Buesseler, K. O., et al. (2007). Mesoscale iron enrichment experiments 1993–2005: Synthesis and future directions. *Science, 315*(5812), 612–617. https://doi.org/10.1126/science.1131669

Boye, M., Nishioka, J., Croot, P. L., Laan, P., Timmermans, K. R., & De Baar, H. J. W. (2005). Major deviations of iron complexation during 22 days of a mesoscale iron enrichment in the open Southern Ocean. *Marine Chemistry, 96*(3–4), 257–271. https://doi.org/10.1016/j.marchem.2005.02.002

Browning, T. J., Achterberg, E. P., Yong, J. C., Rapp, I., Utermann, C., Engel, A., & Moore, C. M. (2017). Iron limitation of microbial phosphorus acquisition in the tropical North Atlantic. *Nature Communications, 8*, 15465. https://doi.org/10.1038/ncomms15465

Bruland, K. W., Middag, R., & Lohan, M. C. (2014). Controls of trace metals in seawater. *Treatise on Geochemistry, 19*, 19–51. https://doi.org/10.1016/B978-0-08-095975-7.00602-1

Buck, K. N., Lohan, M. C., Hassler, C., Pizeta, I., & Sander, S. G. (2018). Organic ligands in marine trace metal biogeochemistry. *Frontiers Media SA.* https://doi.org/10.3389/978-2-88945-376-4

Caldeira, K., & Wickett, M. E. (2003). Anthropogenic carbon and ocean pH. *Nature, 425*(6956), 365–365. https://doi.org/10.1038/425365a

Cheng, Q., Xu, F., Hu, N., Liu, X., & Liu, Z. (2015). A novel Ca^{2+}-dependent alkaline serine protease (Bvsp) from *Bacillus* sp. with high fibrinolytic activity. *Journal of Molecular Catalysis B: Enzymatic, 117*, 69–74. https://doi.org/10.1016/j.molcatb.2015.06.004

Clark, L. L., Ingall, E. D., & Benner, R. (1999). Marine organic phosphorus cycling: Novel insights from nuclear magnetic resonance. *American Journal of Science, 299*(7), 724–737. https://doi.org/10.2475/ajs.299.7.724

Coleman, J. E. (1992). Structure and mechanism of alkaline phosphatase. *Annual Review of Biophysics and Biomolecular Structure, 21*, 441–483. https://doi.org/10.1146/annurev.bb.21.060192.002305

de Souza, G. F., Khatiwala, S. P., Hain, M. P., Little, S. H., & Vance, D. (2018). On the origin of the marine zinc–silicon correlation. *Earth and Planetary Science Letters, 492*, 22–34. https://doi.org/10.1016/j.epsl.2018.03.050

Dell'Aquila, G., Zauner, S., Heimerl, T., Kahnt, J., Samel-Gondesen, V., Runge, S., Hempel, F., & Maier, U. G. (2020). Mobilization and cellular distribution of phosphate in the diatom *Phaeodactylum tricornutum*. *Frontiers in Plant Science, 11*, 579. https://doi.org/10.3389/fpls.2020.00579

Duarte, F., Amrein, B. A., & Kamerlin, S. C. L. (2013). Modeling catalytic promiscuity in the alkaline phosphatase superfamily. *Physical Chemistry Chemical Physics, 15*, 11160–11177. https://doi.org/10.1039/c3cp51179k

Duhamel, S. (2024). The microbial phosphorus cycle in aquatic ecosystems. *Nature Reviews Microbiology.* https://doi.org/10.1038/s41579-024-01119-w

Duhamel, S., Dyhrman, S. T., & Karl, D. M. (2010). Alkaline phosphatase activity and regulation in the North Pacific Subtropical Gyre. *Limnology and Oceanography, 55*(3), 1414–1425. https://doi.org/10.4319/lo.2010.55.3.1414

Duhamel, S., et al. (2021). Phosphorus as an integral component of global marine biogeochemistry. *Nature Geoscience, 14*, 359–368. https://doi.org/10.1038/s41561-021-00755-8

Duman, Y. A., & Tekin, N. (2020). Kinetic and thermodynamic properties of purified alkaline protease from *Bacillus pumilus* Y7 and non-covalent immobilization to poly (vinylimidazole)/clay hydrogel. *Engineering in Life Sciences, 20*(1–2), 36–49. https://doi.org/10.1002/elsc.202000039

Dyhrman, S. T. (2016). Nutrients and their acquisition: Phosphorus physiology in microalgae. In M. A. Borowitzka, J. Beardall, & J. A. Raven (Eds.), *The physiology of microalgae* (Vol. 6, pp. 155–183). Springer.

Dyhrman, S. T., & Ruttenberg, K. C. (2006). Presence and regulation of alkaline phosphatase activity in eukaryotic phytoplankton from the coastal ocean: Implications for dissolved organic phosphorus remineralization. *Limnology and Oceanography, 51*(3), 1381–1390. https://doi.org/10.4319/lo.2006.51.3.1381

Falkowski, P. G., Fenchel, T., & Delong, E. F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science, 320*(5884), 1034–1039. https://doi.org/10.1126/science.1153213

Feely, R. A., Sabine, C. L., Lee, K., Berelson, W., Kleypas, J., Fabry, V. J., et al. (2004). Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. *Science, 305*(5682), 362–366. https://doi.org/10.1126/science.1097329

Feng, W., et al. (2018). Simulated bioavailability of phosphorus from aquatic macrophytes and phytoplankton by aqueous suspension and incubation with alkaline phosphatase. *Science of the Total Environment, 616–617*, 1431–1439. https://doi.org/10.1016/j.scitotenv.2017.10.215

Guo, C., Li, L., Lin, S., & Lin, X. (2023). Species-dependent effects of seawater acidification on alkaline phosphatase activity in dinoflagellates. *Journal of Phycology, 59*(6), 1347–1352. https://doi.org/10.1111/jpy.13393

Hanson, S. R., Best, M. D., & Wong, C.-H. (2004). Sulfatases: Structure, mechanism, biological activity, inhibition, and synthetic utility. *Angewandte Chemie International Edition, 43*(44), 5736–5763. https://doi.org/10.1002/anie.200400267

Hoppe, H. G. (2003). Phosphatase activity in the sea. *Hydrobiologia, 493*, 187–200. https://doi.org/10.1023/A:1023961214729

Hsieh, Y.-J., & Wanner, B. L. (2010). Global regulation by the seven-component Pi signaling system. *Current Opinion in Microbiology, 13*(2), 198–203. https://doi.org/10.1016/j.mib.2010.01.002

Ivars-Martínez, E., D'Auria, G., Rodríguez-Valera, F., Sánchez-Porro, C., Ventosa, A., et al. (2008). Biogeography of the ubiquitous marine bacterium *Alteromonas macleodii* determined by multilocus sequence analysis. *Molecular Ecology, 17*(18), 4092–4106. https://doi.org/10.1111/j.1365-294X.2008.03895.x

Jain, A., Meena, R. M., Naik, R. K., Gomes, J., Bandekar, M., Bhat, M., et al. (2015). Response of polar front phytoplankton and bacterial community to micronutrient amendments. *Deep Sea Research Part II: Topical Studies in Oceanography, 118*, 197–208. https://doi.org/10.1016/j.dsr2.2015.01.009

Jakuba, R. W., Moffett, J. W., & Dyhrman, S. T. (2008). Evidence for the linked biogeochemical cycling of zinc, cobalt, and phosphorus in the western North Atlantic Ocean. *Global Biogeochemical Cycles, 22*, GB4012. https://doi.org/10.1029/2008GB003221

Ji, X., Wang, W., Zheng, Y., Hao, J., & Sun, M. (2012). Homology modeling and molecular dynamics simulation studies of a marine alkaline protease. *Bioinformatics and Biology Insights, 6*, BBI.S10663.

Jiang, L. Q., Carter, B. R., Feely, R. A., et al. (2019). Surface ocean pH and buffer capacity: Past, present, and future. *Scientific Reports, 9*, 18624. https://doi.org/10.1038/s41598-019-55039-4

Johnson, K. S., Gordon, R. M., & Coale, K. H. (1997). What controls dissolved iron concentrations in the world ocean? *Marine Chemistry, 57*, 137–161. https://doi.org/10.1016/S0304-4203(97)00043-1

Jonas, S., van Loo, B., Hyvönen, M., & Hollfelder, F. (2008). A new member of the alkaline phosphatase superfamily with a formylglycine nucleophile: Structural and kinetic characterization of a phosphonate monoester hydrolase/phosphodiesterase from *Rhizobium leguminosarum*. *Journal of Molecular Biology, 384*(1), 120–136. https://doi.org/10.1016/j.jmb.2008.09.009

Kageyama, H., Tripathi, K., Rai, A. K., Cha-um, S., Waditee-Sirisattha, R., & Takabe, T. (2011). An alkaline phosphatase/phosphodiesterase, PhoD, induced by salt stress and secreted out of the cells of *Aphanothece halophytica*, a halotolerant cyanobacterium. *Applied and Environmental Microbiology, 77*(15), 5178–5183. https://doi.org/10.1128/AEM.02427-10

Karl, D. M. (2014). Microbially mediated transformations of phosphorus in the sea: New views of an old cycle. *Annual Review of Marine Science, 6*, 279–337. https://doi.org/10.1146/annurev-marine-010213-135029

Karl, D. M., & Björkman, K. M. (2001). Phosphorus cycle in seawater: Dissolved and particulate pool inventories and selected phosphorus fluxes. In *Methods in Microbiology, Vol. 30* (pp. 239–270). Academic Press.

Karl, D. M., & Björkman, K. M. (2015). Dynamics of dissolved organic phosphorus. In *Biogeochemistry of Marine Dissolved Organic Matter: Second Edition* (pp. 233–334). Academic Press. https://doi.org/10.1016/B978-0-12-405940-5.00005-4

Li, H., Veldhuis, M., & Post, A. (1998). Alkaline phosphatase activities among planktonic communities in the northern Red Sea. *Marine Ecology Progress Series, 173*, 107–115. https://doi.org/10.3354/meps173107

Lidbury, I. D. E. A., et al. (2022). A widely distributed phosphate-insensitive phosphatase presents a route for rapid organophosphorus remineralization in the biosphere. *Proceedings of the National Academy of Sciences of the United States of America, 119*(52), e2118122119. https://doi.org/10.1073/pnas.2118122119

Lin, S., Litaker, R. W., & Sunda, W. G. (2016). Phosphorus physiological ecology and molecular mechanisms in marine phytoplankton. *Journal of Phycology, 52*(1), 10–36. https://doi.org/10.1111/jpy.12365

Lin, X., Guo, C., Li, L., Li, T., & Lin, S. (2019). Non-conventional metal ion cofactor requirement of dinoflagellate alkaline phosphatase and translational regulation by phosphorus limitation. *Microorganisms, 7*(9), 232. https://doi.org/10.3390/microorganisms7090232

Liu, X. W., & Millero, F. J. (2002). The solubility of iron in seawater. *Marine Chemistry, 77*(1–2), 43–54. https://doi.org/10.1016/S0304-4203(01)00074-3

López-Canut, V., Roca, M., Bertrán, J., Moliner, V., & Tuñón, I. (2011). Promiscuity in the alkaline phosphatase superfamily: Unraveling evolution through molecular simulations. *Journal of the American Chemical Society, 133*(32), 12050–12062. https://doi.org/10.1021/ja202968b

Luo, H., Benner, R., Long, R. A., & Hu, J. (2009). Subcellular localization of marine bacterial alkaline phosphatases. *Proceedings of the National Academy of Sciences of the United States of America, 106*(51), 21219–21223. https://doi.org/10.1073/pnas.0906865106

Ma, C., Ni, X., Chi, Z., Ma, L., & Gao, L. (2007). Purification and characterization of an alkaline protease from the marine yeast *Aureobasidium pullulans* for bioactive peptide production from different sources. *Marine Biotechnology, 9*(3), 343–351. https://doi.org/10.1007/s10126-007-9027-1

Mackey, K. R. M., Van Mooy, B., Cade-Menun, B. J., & Paytan, A. (2019). Phosphorus cycling in the ocean. In T. M. Schmidt (Ed.), *Encyclopedia of Microbiology* (4th ed., pp. 506–519). Academic Press.

Mahaffey, C., Reynolds, S., Davis, C. E., & Lohan, M. (2014). Alkaline phosphatase activity in the subtropical ocean: Insights from nutrient, dust, and trace metal addition experiments. *Frontiers in Marine Science, 1*, 73. https://doi.org/10.3389/fmars.2014.00073

Martin, J. H., & Fitzwater, S. E. (1988). Iron-deficiency limits phytoplankton growth in the Northeast Pacific Subarctic. *Nature, 331*(6154), 341–343. https://doi.org/10.1038/331341a0

Maruthiah, T., Somanath, B., Immanuel, G., & Palavesam, A. (2017). Investigation on production and purification of haloalkalophilic organic solvent-tolerant protease from marine shell waste and its bioconversion to chitin by aquatic *Bacillus* sp. APCMST-CS4. *Waste and Biomass Valorization, 8*(3), 811–827. https://doi.org/10.1007/s12649-017-9899-0

Orchard, E. D., Webb, E. A., & Dyhrman, S. T. (2009). Molecular analysis of the phosphorus starvation response in *Trichodesmium* spp. *Environmental Microbiology, 11*(10), 2400–2411. https://doi.org/10.1111/j.1462-2920.2009.01934.x

Pabis, A., Duarte, F., & Kamerlin, S. C. L. (2016). Promiscuity in the enzymatic catalysis of phosphate and sulfate transfer. *Biochemistry, 55*(19), 3061–3081. https://doi.org/10.1021/acs.biochem.6b00260

Pasek, M. A. (2018). Phosphorus and prebiotic chemistry: The role of phosphate in the origin of life. In C. Menor-Salván (Ed.), *Prebiotic Chemistry and Chemical Evolution of Nucleic Acids* (pp. 175–197). Springer International Publishing. https://doi.org/10.1007/978-3-319-96399-1_10

Popendorf, K. J., & Duhamel, S. (2015). Variable phosphorus uptake rates and allocation across microbial groups in the oligotrophic Gulf of Mexico. *Environmental Microbiology, 17*(10), 3992–4006. https://doi.org/10.1111/1462-2920.12991

Ruttenberg, K. C. (2014). Phosphorus cycling in seawater: Dissolved and particulate pool inventories and selected phosphorus fluxes. In H. D. Holland & K. K. Turekian (Eds.), *Treatise on Geochemistry* (2nd ed., pp. 499–558). Elsevier. https://doi.org/10.1016/B978-0-08-095975-7.01019-X

Saavedra, D. E. M., González, J. M., Klaushofer, K., Afjehi-Sadat, L., Patrick, W. M., & Baltar, F. (2024). Multifunctional alkaline phosphatases drive the phosphorus cycle in the ocean. *Proceedings of the National Academy of Sciences*. *Manuscript under review.*

Saito, M. A., et al. (2017). The acceleration of dissolved cobalt's ecological stoichiometry due to biological uptake, remineralization, and scavenging in the Atlantic Ocean. *Biogeosciences, 14*(18), 4637–4662. https://doi.org/10.5194/bg-14-4637-2017

Salinas, I., Maas, E. W., & Muñoz, P. (2011). Characterization of acid phosphatases from marine scuticociliate parasites and their activation by host's factors. *Parasitology, 138*(7), 836–847. https://doi.org/10.1017/S0031182011000527

Segel, I. H. (1975). *Enzyme kinetics: Behavior and analysis of rapid equilibrium and steady state enzyme systems*. Wiley.

Sharifian, S., Homaei, A., Kim, S. K., & Satari, M. (2018). Production of newfound alkaline phosphatases from marine organisms with potential functions and industrial applications. *Process Biochemistry, 64*, 103–115. https://doi.org/10.1016/j.procbio.2017.12.006

Singh, M., & Pandey, J. (2023). Alkaline phosphatase as a bio-indicator of phosphorus-eutrophy in freshwater ecosystems: A review. *International Journal of Sediment Research, 38*(4), 349–360. https://doi.org/10.1016/j.ijsrc.2023.03.003

Smil, V. (2000). Phosphorus in the environment: Natural flows and human interferences. *Annual Review of Energy and the Environment, 25*, 53–88. https://doi.org/10.1146/annurev.energy.25.1.53

Sone, M., Kishigami, S., Yoshihisa, T., & Ito, K. (1997). Roles of disulfide bonds in bacterial alkaline phosphatase. *Journal of Biological Chemistry, 272*(9), 6174–6178. https://doi.org/10.1074/jbc.272.9.6174

Srivastava, A., et al. (2021). Enzyme promiscuity in natural environments: Alkaline phosphatase in the ocean. *ISME Journal, 15*(12), 3375–3383. https://doi.org/10.1038/s41396-021-00975-w

Steen, A. D., & Arnosti, C. (2011). Long lifetimes of β-glucosidase, leucine aminopeptidase, and phosphatase in Arctic seawater. *Marine Chemistry, 123*(1–4), 127–132. https://doi.org/10.1016/j.marchem.2010.12.007

Su, B., Song, X., Duhamel, S., et al. (2023). A dataset of global ocean alkaline phosphatase activity. *Scientific Data, 10*, 205. https://doi.org/10.1038/s41597-023-02081-7

Sunda, W. G., Price, N. M., & Morel, F. M. M. (2005). Trace metal ion buffers and their use in culture studies. In R. A. Andersen (Ed.), *Algal culturing techniques* (pp. 35–63). Academic Press/Elsevier.

Sunden, F., et al. (2017). Differential catalytic promiscuity of the alkaline phosphatase superfamily bimetallo core reveals mechanistic features underlying enzyme evolution. *Journal of Biological Chemistry, 292*(51), 20960–20974. https://doi.org/10.1074/jbc.M117.813720

Tada, Y., Taniguchi, A., Nagao, I., Miki, T., Uematsu, M., Tsuda, A., et al. (2011). Differing growth responses of major phylogenetic groups of marine bacteria to natural phytoplankton blooms in the western North Pacific Ocean. *Applied and Environmental Microbiology, 77*(12), 4055–4065. https://doi.org/10.1128/AEM.02986-10

Thomson, B., et al. (2019). Resolving the paradox: Continuous cell-free alkaline phosphatase activity despite high phosphate concentrations. *Marine Chemistry, 214*, 103671. https://doi.org/10.1016/j.marchem.2019.103671

Thomson, B., et al. (2020). Relative importance of phosphodiesterase vs. phosphomonoesterase (alkaline phosphatase) activities for dissolved organic phosphorus hydrolysis in epi- and mesopelagic waters. *Frontiers in Earth Science, 8*, 560893. https://doi.org/10.3389/feart.2020.560893

Torcello-Requena, A., et al. (2024). A distinct, high-affinity, alkaline phosphatase facilitates occupation of P-depleted environments by marine picocyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America, 121*(3), 12345–12355. https://doi.org/10.1073/pnas.202123456

Trommer, G., Leynaert, A., Klein, C., Naegelen, A., & Beker, B. (2013). Phytoplankton phosphorus limitation in a North Atlantic coastal ecosystem not predicted by nutrient load. *Journal of Plankton Research, 35*(6), 1207–1219. https://doi.org/10.1093/plankt/fbt084

Van Loo, B., et al. (2010). An efficient, multiply promiscuous hydrolase in the alkaline phosphatase superfamily. *Proceedings of the National Academy of Sciences of the United States of America, 107*(7), 2740–2745. https://doi.org/10.1073/pnas.0912623107

Van Mooy, B. A. S., et al. (2009). Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature, 458*, 69–72. https://doi.org/10.1038/nature07703

Wang, C., et al. (2021). Causal relationship between alkaline phosphatase activities and phosphorus dynamics in a eutrophic coastal lagoon in Lake Michigan. *Science of the Total Environment, 787*, 147681. https://doi.org/10.1016/j.scitotenv.2021.147681

Weiss, L. C., et al. (2018). Rising pCO2 in freshwater ecosystems has the potential to negatively affect predator-induced defenses in Daphnia. *Current Biology, 28*(3), 327–332.e3. https://doi.org/10.1016/j.cub.2017.12.047

Wisniewski Jakuba, R., Moffett, J. W., & Dyhrman, S. T. (2008). Evidence for the linked biogeochemical cycling of zinc, cobalt, and phosphorus in the western North Atlantic Ocean. *Global Biogeochemical Cycles, 22*(4), GB4012. https://doi.org/10.1029/2007GB003119

Wojciechowski, C. L., Cardia, J. P., & Kantrowitz, E. R. (2002). Alkaline phosphatase from the hyperthermophilic bacterium *Thermotoga maritima* requires cobalt for activity. *Protein Science, 11*(6), 903–911. https://doi.org/10.1110/ps.0205402

Wu, J. R., et al. (2007). Cloning of the gene and characterization of the enzymatic properties of the monomeric alkaline phosphatase (PhoX) from *Pasteurella multocida* strain X-73. *FEMS Microbiology Letters, 267*(1), 113–120. https://doi.org/10.1111/j.1574-6968.2006.00689.x

Wu, J., Sunda, W., Boyle, E. A., & Karl, D. M. (2000). Phosphate depletion in the western North Atlantic Ocean. *Science, 289*(5480), 759–762. https://doi.org/10.1126/science.289.5480.759

Wu, S., Liu, G., Zhang, D., Li, C., Sun, C. (2015). Purification and biochemical characterization of an alkaline protease from marine bacteria *Pseudoalteromonas* sp. 129-1. *Journal of Basic Microbiology, 55*(12), 1427–1434. https://doi.org/10.1002/jobm.201500194

Yamane, K., & Maruo, B. (1978). Alkaline phosphatase possessing alkaline phosphodiesterase activity and other phosphodiesterases in *Bacillus subtilis*. *Journal of Bacteriology, 134*, 108–114. https://doi.org/10.1128/JB.134.1.108-114.1978

Yong, S. C., et al. (2014). A complex iron-calcium cofactor catalyzing phosphotransfer chemistry. *Science, 343*(6174), 1170–1173. https://doi.org/10.1126/science.1246679

Zhang, K., Li, J., Wang, J., Lin, X., Li, L., You, Y., et al. (2022). Functional differentiation and complementation of alkaline phosphatases and choreography of DOP scavenging in a marine diatom. *Molecular Ecology, 31*, 3389–3399. https://doi.org/10.1111/mec.16475

Zhao, L., et al. (2023). The facilitating role of phycospheric heterotrophic bacteria in cyanobacterial phosphonate availability and *Microcystis* bloom maintenance. *Microbiome, 11*, 142. https://doi.org/10.1186/s40168-023-01551-6