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LETTER

Diurnal variability in alkaline phosphatase activity and the potential role of zooplanktonC. Davis¹,^{1a*} M. C. Lohan,² R. Tuerena,³ E. Cerdan-Garcia,² E. M. S. Woodward,⁴ A. Tagliabue,¹ C. Mahaffey¹¹Department of Earth, Ocean and Ecological Sciences, University of Liverpool, Liverpool, UK; ²Ocean and Earth Science, National Oceanography Centre, University of Southampton, Southampton, UK; ³School of Geosciences, University of Edinburgh, Edinburgh, UK; ⁴Plymouth Marine Laboratory, Plymouth, UK**Scientific Significance Statement**

In phosphate deplete regions of the ocean, alkaline phosphatases (AP) are synthesized by microbes to enable access to the more abundant dissolved organic phosphorus (DOP) pool to support growth and productivity. However, the general assumption is that there is no diurnal variability in the activity of key enzymes involved in DOP acquisition. Our study provides evidence for significant diurnal variation in AP activity in the surface ocean. We identify diel vertical migration of zooplankton as a nocturnal source of AP in the surface ocean, with reduction or degradation of AP during the day. Our findings highlight a potentially important role of zooplankton in phosphorus regeneration in the surface ocean.

Abstract

Daily light–dark cycles drive the circadian rhythm of many ocean processes including photosynthesis, gene expression, and zooplankton diel vertical migration (DVM). In phosphate deplete surface ocean regions, microbes produce metalloenzymes, such as alkaline phosphatases (AP), to access dissolved organic phosphorus. Here, we provide novel evidence of diurnal variation in AP activity (APA) in the subtropical North Atlantic using two independent datasets, with APA being two- to three-fold higher at night. We demonstrate that zooplankton are a source of AP and postulate that zooplankton DVM is a source of enhanced AP in the surface waters at night, with reduction or degradation of AP during the day. Our results challenge the current assumption that APA is linear over a 24-h period. While future ocean scenarios predict intensification and expansion of oceanic phosphate limitation, our findings indicate a role for zooplankton in regenerating phosphate that is currently missing in conceptual and numerical models.

Diurnal variation in irradiance leads to well-known behavioral and physiological responses in organisms in the surface ocean, such as diel patterns in cellular processes including

metabolism, gene expression, and metal allocation (Lorenzen, 1963; Armbrust 2014; Saito et al. 2011). It also drives diel vertical migration (DVM) of zooplankton, whereby zooplankton

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Author Contribution Statement: CD wrote the manuscript with input from CM, MCL, and AT. CD designed and conducted all enzyme and zooplankton incubation experiments. RT and EMSW conducted phosphate analysis on JR15007 and JC150, respectively. ECG picked zooplankton on JC150.

Data Availability Statement: Data are available in the British Oceanographic Data Centre repository at https://www.bodc.ac.uk/data/published_data_library/catalogue/10.5285/80850823-28e1-3d69-e053-6c86abc083d5/

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migrate to the surface at night and to depth during the day (Longhurst 1991). The influence of DVM on biogeochemical nutrient cycles has been studied in relation to nitrogen, carbon, and iron (Steinberg et al. 2000, 2002; Schmidt et al., 2016) but is less well-studied in relation to phosphorus (P).

Throughout, much of the low-latitude Atlantic Ocean, surface phosphate concentrations are chronically low ($<10 \text{ nmol L}^{-1}$) and limit or colimit microbial growth, nitrogen fixation, and primary productivity (Moore et al. 2013). The prevalence and intensity of phosphate stress is predicted to increase because of enhanced stratification and nitrogen fertilization (Capotondi et al. 2012; Jickells et al. 2017). Under phosphate-stressed conditions, microbes may synthesize enzymes to access dissolved organic phosphorus (DOP) to meet their P requirements (Mahaffey et al. 2014). The DOP pool is composed of esters, anhydrides, and phosphonates and can be hydrolyzed by a range of enzymes including nucleotidases, phosphonates, adenosine-triphosphatases, and alkaline phosphatases (AP) (Duhamel et al. 2010).

AP is a metalloenzyme group with iron or zinc cofactors that can hydrolyze the labile monoester pool, which may account for as much as 75% of the DOP pool (Kolowitz et al. 2001; Hoppe 2003). AP activity (APA) rapidly increases below 30 nmol L^{-1} phosphate and thus is often used to infer phosphate stress or limitation across transects, over times series, and in bioassay experiments (Mahaffey et al. 2014). Such studies assume no diurnal variation in APA, despite there being a light dependence and diurnal variation in phosphate uptake (Duhamel et al. 2012).

Here, we use data from two field campaigns and a previously published dataset in the subtropical North Atlantic Ocean to address the following questions: (1) is there diurnal variability in APA, and (2) do zooplankton influence APA in the surface ocean? Quantifying the diurnal variability in APA and the role of zooplankton in surface ocean P cycling will allow more accurate assessment and detection of future changes in ocean P dynamics, which are inextricably linked to the nitrogen and carbon cycles.

Materials and methods

Near-surface APA

Seawater was collected on board the RRS *James Cook* during summer 2017 (JC150: 22 June to 12 August) between 30°W and 60°W and 22°N and 23°N in the subtropical North Atlantic (Fig. 1) using a trace metal clean “towed fish” at $\sim 2 \text{ m}$ depth. This sampling method allowed us to obtain a high spatial and temporal resolution of near-surface sampling. In total, 130 samples were collected at 2-h intervals at a spatial resolution of $0.23 \pm 0.07^\circ\text{W}$ ($26 \pm 8 \text{ km}$; Fig. 1).

APA rates were measured in unfiltered seawater using the fluorometric substrate 4-methylumbelliferyl-phosphate (MUF-P, Sigma Aldrich; Ammerman 1993). Samples were incubated with either 500 nmol L^{-1} (51°W – 30°W) or 2000 nmol L^{-1} (60°W – 51°W) MUF-P at in situ conditions. Fluorescence was

measured every 2 h for up to 12 h using a Turner 10AU field fluorometer (long wavelength oil lab filter kit 10-302R, 365 nm emission, 455 nm excitation). A calibration and daily single standard were produced using 4-methylumbelliferone (MUF, Sigma Aldrich, concentration range 0–2000 and 400 nmol L^{-1} , respectively) to convert the rate of change in fluorescence to MUF-P hydrolysis rate, considered synonymous with volumetric APA (APA_{vol} , $\text{nmol P L}^{-1} \text{ h}^{-1}$). Fluorescence measurements were corrected for abiotic hydrolysis or MUF-P degradation using blanks (boiled sample plus 500 nmol L^{-1} MUF-P).

To interrogate diurnal patterns in the data, APA_{vol} was normalized to local (within $\pm 1^\circ$) APA_{vol} measured at midnight ($\pm 1 \text{ h}$). This gave a value of the fractional change in volumetric APA_{vol} across a 24-h cycle, termed $\delta\text{APA}_{\text{vol}}$.

Zooplankton incubations and APA

To investigate the influence of zooplankton on APA (APA_{zoo}), replicate experiments were conducted on the RRS *James Clark Ross* (JR15007: June to July 2016, $n = 7$) and on the RRS *James Cook* (JC150, $n = 5$) (Fig. 1). Zooplankton were collected using WP2-size net rings (57 cm diameter) fitted with $200 \mu\text{m}$ mesh size and nonfiltering cod ends. During both cruises, plankton tows were performed at night from 200 m to the surface at 0.5 m s^{-1} (Giering et al. 2012). This depth was chosen to ensure sufficient biomass for replicate incubations and for intercruise consistency. Samples were immediately sorted after tows with healthy, active organisms being twice washed prior to incubation by gentle sequential transfer into filtered surface seawater ($0.7 \mu\text{m}$ GF/F during JR15007; $0.2 \mu\text{m}$ Durapore during JC150). Incubations consisted of taxa that were abundant at each station, namely calanoid copepods (JC150 and JR15007) and gelatinous organisms (JR15007 only, $\sim 15\%$).

Next, during JR15007, healthy active organisms were gently pipetted into 10 liter unfiltered surface seawater and incubated in the dark in triplicate at an incubation density of $14 \pm 5 \text{ mg L}^{-1}$. Samples were sacrificed at each time point (every 30 to 60 min) during the 9 h incubations. Prescreened ($63 \mu\text{m}$ mesh) subsamples were taken for determination of APA ($10 \mu\text{mol L}^{-1}$ MUF-P; see section Near-Surface APA), and the remaining sample was filtered onto preweighed $0.7 \mu\text{m}$ GF/F and stored at -20°C for later determination of dry weight biomass.

During JC150, calanoid copepods were gently pipetted into 1 liter surface seawater and incubated in the dark in triplicate with an incubation density of $7 \pm 5 \text{ animals L}^{-1}$ in 1 liter polycarbonate bottles. Duplicate experiments were conducted using filtered ($0.2 \mu\text{m}$ Durapore) and unfiltered surface seawater, to investigate whether APA_{zoo} was influenced by excretion or grazing processes. Incubation bottles were spiked with MUF-P (final concentration $2 \mu\text{mol L}^{-1}$) and fluorescence measurements were taken every 30 to 60 min for 6 to 8 h. This duration was sufficiently long for animal adaptation and grazing (Nöges 1992) and sufficiently short that the change in fluorescence remained linear, consistent with the previous APA incubations.

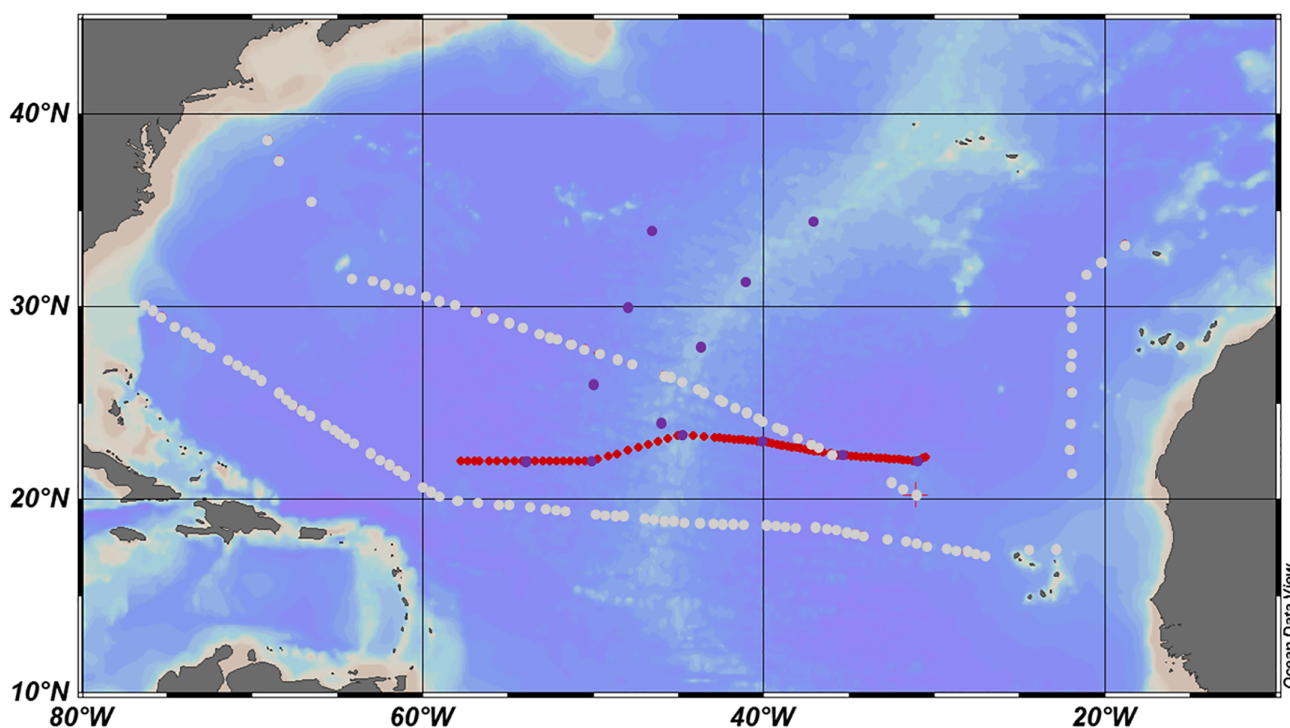


Fig. 1. Sampling locations for data collected during June–August 2017 on JC150 (red); for Wurl et al. (2013) during U.S. GEOTRACES GA03 subtropical North Atlantic cruise in November 2010 and December 2011 (gray); and for zooplankton incubation experiments during both JR15007 (June–July 2016) and JC150 (July–August 2017) (purple).

APA was determined from the linear change in fluorescence over time. All APA_{zoo} measurements were blank corrected (boiled seawater control to monitor abiotic changes and surface seawater only control to measure background APA).

Dissolved phosphate

During JR15007, samples for phosphate analysis were collected using 20-liter Niskin bottles on a stainless-steel rosette frame supporting a Seabird 911 conductivity, temperature, depth (CTD) instrument and fluorometer. During JC150, samples were collected using the “towed fish” at ~ 2 m depth and the CTD. Samples were collected directly into aged, acid-washed, Milli-Q rinsed, and sample rinsed 60 mL HDPE Nalgene® bottles. Nutrients were analyzed on board using a Bran and Luebbe segmented flow colorimetric auto-analyzer (A4111) using techniques described in Woodward and Rees (2001). Nutrient reference materials (KANSO Technos) were analyzed daily. On JR15007 and JC150, precision was between 2% and 3%. Limits of detection were $0.02 \mu\text{mol L}^{-1}$ for phosphate during JR15007 but lower during JC150 (0.5 nmol L^{-1}) due to the use of a 2 m liquid waveguide (Zhang and Chi 2002).

Flow cytometry

During JC150, samples for picophytoplankton and heterotrophic bacterial analysis were collected from the “towed fish” sampling system using 125 mL polycarbonate bottles (acid washed, Nalgene®). Immediately after sampling, 1.9 mL of

sample was pipetted into 2 mL cryovials containing $20 \mu\text{L}$ of 50% glutaraldehyde solution (Fisher Scientific), inverted 10 times, and stored at 4°C for 1 h. Samples were then flash frozen with liquid nitrogen and stored at -80°C .

Samples were analyzed using a flow cytometer (FACSort™ B0043) at Plymouth Marine Laboratory using techniques described by Tarran et al. (2001). Four groups were identified and enumerated: *Prochlorococcus*, *Synechococcus*, picoeukaryotes, and total heterotrophic bacteria (high and low nucleic acid groups), which collectively were used as total cell abundance.

Results

Diurnal variation in APA

There was significant diel periodicity in δAPA_{vol} ($R^2 = 0.52$, $p = 0.017$; Fig. 2a) and APA normalized to total cell abundance (δAPA_{cell} , $R^2 = 0.41$, $p = 0.011$; Fig. 2b), which more than doubled between the minimum rates in early afternoon when irradiance was highest ($\sim 0.6 \text{ kW m}^{-2}$) and maximum rates at night (Fig. 2a). Applying the same derivation to the data of Wurl et al. (2013), collected in the subtropical North Atlantic (U.S. GEOTRACES GA03, 15 October to 27 November 2010, 06 November to 11 December 2011, RV *Knorr*; Fig. 1), revealed a remarkably similar diurnal variation in δAPA_{vol} and with rates doubling between day and night ($R^2 = 0.55$, $p = 0.07$; Fig. 2c).

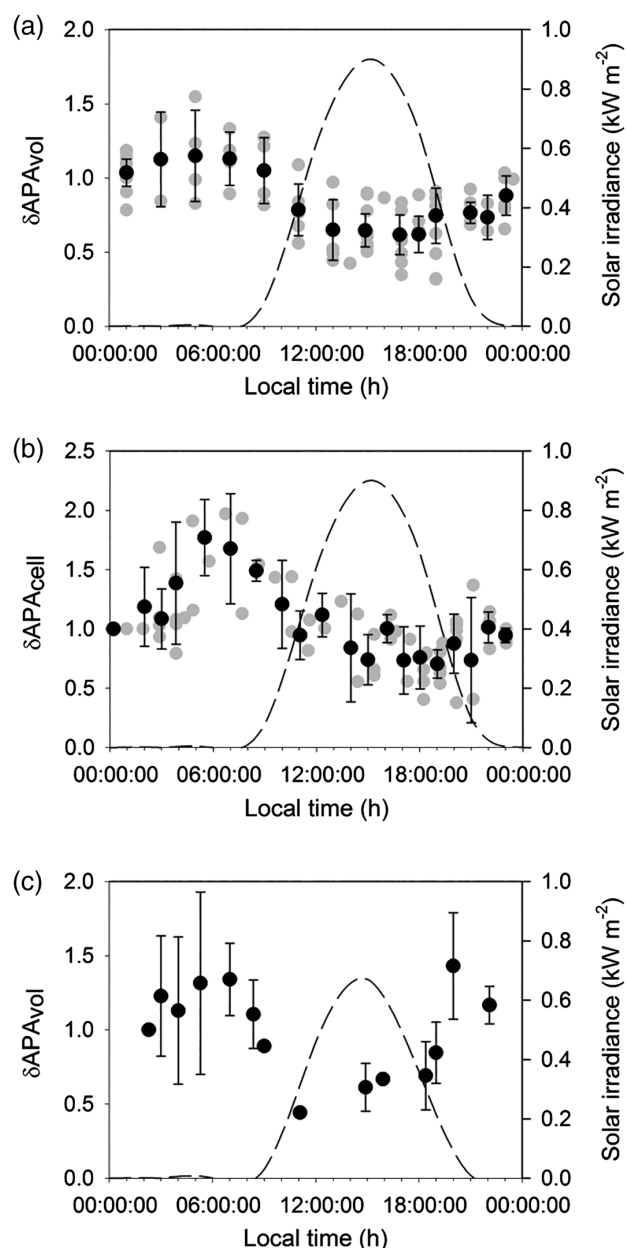


Fig. 2. Hourly mean volumetric APA normalized to local volumetric midnight activity ($\delta\text{APA}_{\text{vol}}$, closed circles) and discrete values (gray circles) vs. time of day (local) and solar irradiance (kW m^{-2} , dashed line) for (a) all APA_{vol} data collected from the zonal transect at 22°N during 03 July 2017 to 08 August 2017 on JC150, modified sine regression: $R^2 = 0.52$, $p = 0.017$, (b) APA_{vol} normalized to cell abundance (APA_{cell} $\text{nmol P } 10^6 \text{ cell}^{-1} \text{ h}^{-1}$) prior to normalization to local midnight values ($\delta\text{APA}_{\text{cell}}$), modified sine regression: $R^2 = 0.41$, $p = 0.011$, and (c) APA_{vol} data reported in Wurl et al. (2013) U.S. GEOTRACES GA03 tropical North Atlantic cruises in November 2010 and December 2011, downloaded from www.bco-dmo.org, polynomial (order 3) overall regression: $R^2 = 0.55$, $p = 0.07$. Error bars represent 95% confidence intervals.

Zooplankton and APA

APA_{vol} was significantly higher in incubations with zooplankton than without zooplankton, such that APA_{zoo} was

positive throughout (Table 1). During JC150, APA_{zoo} was up to 17-fold higher in filtered seawater and up to 6-fold higher in unfiltered seawater compared to controls. Once corrected for blank and background activity and normalized to the number of animals in each incubation, APA_{zoo} was similar in filtered and unfiltered seawater experiments ($9\text{--}61 \text{ nmol P animal}^{-1} \text{ d}^{-1}$ and $7\text{--}71 \text{ nmol P animal}^{-1} \text{ d}^{-1}$, respectively). APA_{zoo} in filtered seawater incubations showed no correlation to $\text{APA}_{\text{control}}$, whereas in unfiltered seawater incubations APA_{zoo} was highest when $\text{APA}_{\text{control}}$ was lowest (Table 1).

During JR15007, APA doubled over a 9-h period and biomass-normalized APA (APA_{wt}) increased 2.6-fold (Fig. 3). The linear increase in APA_{wt} over incubation time ($R^2 = 0.96$, $p < 0.001$) suggests that AP release was relatively constant when normalized to zooplankton biomass. We suggest this was not a stress response because AP and other Pho regulon genes are not upregulated in zooplankton as a stress response (Lauritano et al. 2012) and there was no mortality during our short incubations ($<10 \text{ h}$), which were designed to avoid animal mortality following Nöges (1992).

During JC150, there were too few zooplankton to accurately measure biomass in each incubation. Instead, body size to mass equations (Fernandez Aroz 1991) were used to estimate mean zooplankton mass ($1.6 \pm 0.3 \text{ mg}$), and APA_{vol} was normalized to zooplankton biomass based on the number of animals in each incubation. Combined APA_{wt} data from both cruises showed a significant logarithmic relationship with phosphate integrated across the surface 200 m (i.e., tow depth), with the highest APA_{wt} at the lowest integrated phosphate values (Fig. 4).

Discussion

Using two independent datasets, we have shown that there is a significant two-fold diurnal variation in APA_{vol} that is zonally, seasonally, and annually persistent, suggesting that it is a consistent feature in the subtropical North Atlantic. Phosphate concentrations are known to be chronically low and limit productivity in our study region, where APA is commonly used as an indicator of phosphate limitation (Mahaffey et al. 2014). Yet, the degree of phosphate stress in the microbial community did not have an apparent effect on $\delta\text{APA}_{\text{vol}}$ or $\delta\text{APA}_{\text{cell}}$ (Fig. 2). Such a diurnal variation in APA_{vol} may be driven by circadian rhythms in microbial gene expression (Cohen and Golden 2015; Frischkorn et al. 2018). For example, changes in cellular enzymes can be a physiological response to the light–dark cycle, to oxidative stress, or to modulate cellular resource demands (Saito et al. 2011; Morris et al. 2016). The marine diazotroph *Crocospaera watsonii* alternates its enzyme inventory across a diel cycle to economize cell quotas by transferring iron between N_2 fixation and photosynthetic processes (Saito et al. 2011). Such a process could also apply to AP, which has zinc or iron metal cofactors (Coleman 1992; Yong et al. 2014). An indirect circadian control may occur as a result of certain symbiotic unicellular cyanobacteria, such as

Table 1. Volumetric ($\text{nmol L}^{-1} \text{d}^{-1}$) and normalized ($\text{nmol animal}^{-1} \text{d}^{-1}$) rates of zooplankton associated APA (APA_{zoo}) during JC150 (03 July 2017 to 08 August 2017) in unfiltered and 0.2 μm filtered surface seawater and associated control activity ($\text{APA}_{\text{control}}$, $\text{nmol L}^{-1} \text{d}^{-1}$). SD is the standard deviation of three replicate incubations. Normalized APA_{zoo} values have been corrected for blank associated activity.

Longitude (°W)	Density (animals L^{-1})	$\text{APA}_{\text{control}}$ ($\text{nmol L}^{-1} \text{d}^{-1}$)				APA_{zoo} ($\text{nmol L}^{-1} \text{d}^{-1}$)				APA_{zoo} ($\text{nmol animal}^{-1} \text{d}^{-1}$)			
		Filtered water		Unfiltered water		Filtered water		Unfiltered water		Filtered water		Unfiltered water	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
58	8	42.7	4.4	191.0	32.4	144.3	5.7	312.9	5.7	12.7	1.6	15.6	0.4
54	3	11.8	2.6	43.2	3.8	195.3	123.1	255.6	197.2	61.2	41.0	70.8	65.7
44°47'	5	17.5	4.7	361.1	10.0	94.7	35.0	396.2	29.0	15.4	3.7	7.3	3.2
40	3	5.0	1.6	137.6	4.7	71.0	33.7	198.4	24.7	22.0	5.6	20.3	4.2
31	15	14.3	1.3	114.6	15.3	154.7	21.3	344.1	25.8	9.4	0.7	15.3	1.0

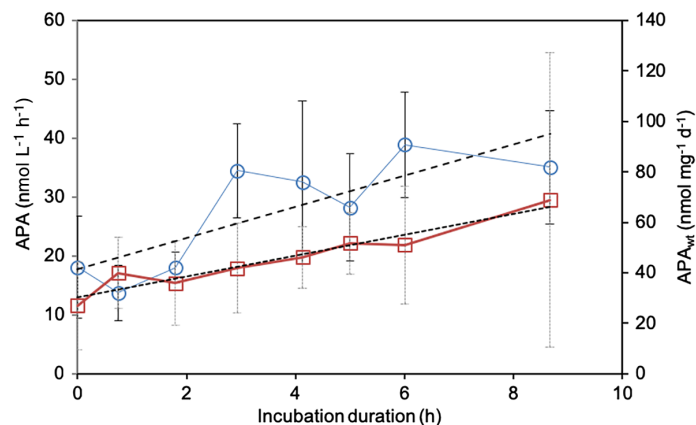


Fig. 3. Mean volumetric APA ($\text{nmol L}^{-1} \text{h}^{-1}$, open blue circles, linear regression R^2 0.80, $p = 0.015$) and biomass-normalized APA (APA_{wt} , $\text{nmol mg}^{-1} \text{d}^{-1}$, open red squares, linear regression R^2 0.96, $p < 0.001$) against time of replicate sacrifice (incubation duration, h) for zooplankton incubation experiments conducted on JR15007 (June–July 2016). Error bars represent 95% confidence intervals.

UCYN-A and UCYN-B, inducing diurnal P stress in their photosynthetic hosts as a result of diel variation in N_2 fixation (Tripp et al. 2010; Zehr et al. 2016).

Alternatively, our study shows that the presence of zooplankton significantly enhanced APA in our incubations and so we hypothesize that zooplankton DVM can significantly contribute to the diurnal variation in APA_{vol} in the surface ocean. In our study, $\delta\text{APA}_{\text{vol}}$ was highest at night coinciding with zooplankton DVM to the surface waters (Fig. 2). Zooplankton phosphatase activity has been previously studied in freshwater (Wynne and Gophen 1981) and coastal regions (Jamet and Boge 1998) but to our knowledge has not been directly studied in the open ocean. There are several mechanisms by which zooplankton may enhance APA, including release from their prey during grazing, release of digestive enzymes to the particulate or dissolved pool through excretion

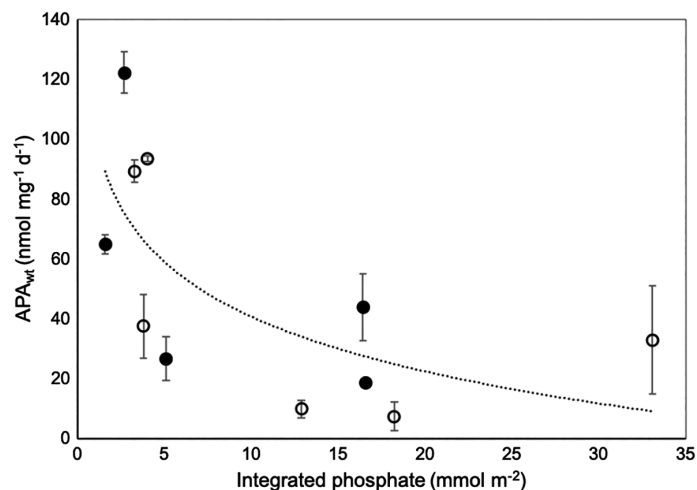


Fig. 4. Dry weight zooplankton biomass-normalized APA (APA_{wt} , $\text{nmol mg}^{-1} \text{d}^{-1}$) against integrated phosphate in the surface 200 m (net haul depth) for JR15007 (June–July 2016, open circles) and JC150 (July–August 2017, closed circles; exponential decay regression R^2 0.48, $p = 0.018$). Each data point represents a single incubation experiment, error bars represent 1 SD.

or mortality, or release via their bacterial epibiont community (Båmstedt 1988; Davis and Mahaffey 2017; De Corte et al. 2018).

Focusing on the western basin (JC150), the diurnal variation in APA_{vol} ranged from 31 $\text{nmol P L}^{-1} \text{d}^{-1}$ in the day to 74 $\text{nmol P L}^{-1} \text{d}^{-1}$ at night (data not shown). Applying values of APA_{zoo} from Table 1 to a grazing density of 1.5 animals L^{-1} (Hays et al. 1996) over a 12-h grazing period (Bianchi and Mislán 2015), we estimate that APA_{zoo} could contribute between 9 and 53 $\text{nmol P L}^{-1} \text{d}^{-1}$ (21% to 124%) of the diurnal variation in APA_{vol} . Meanwhile, the relationship between APA_{zoo} and phosphate (Fig. 4) is similar to that of APA and phosphate reported by Mahaffey et al. (2014). Therefore, this reveals a potential link between APA_{zoo} and phosphate stress of

the ambient microbial community, suggesting a contribution from the bacterial epibiont to APA_{zoo} (De Corte et al. 2018).

If zooplankton are a nocturnal source of AP in the surface ocean, then to resolve the diurnal variation in APA_{vol} there must be a net sink of AP during the day. Factors driving AP loss in the surface could include microbial degradation of the enzyme to access protein as an N source, loss associated with sinking particles and fecal material, competitive binding and inhibition at the active site, or daytime photodegradation and oxidative inhibition of enzyme activity (Boavida and Wetzel 1998; Scully et al. 2003; Baltar et al. 2013; Davis and Mahaffey 2017). Future work into the turnover time of AP under different conditions in seawater is needed to improve our understanding of potential AP sink terms.

Phosphate-limited regions are predicted to expand and intensify due to enhanced anthropogenic and natural nitrogen addition in excess of phosphorus (Kim et al. 2014; Jickells et al. 2017). Future warming will enhance stratification at low latitudes, reducing marine productivity because of the restricted vertical nutrient supply (Bopp et al. 2013; Capotondi et al. 2012). The absolute contribution of APA_{zoo} in the future is unclear, with changes in prey abundance, refuge depth and predation all being influential factors (Hays 2003; Cohen and Forward Jr. 2009; Chust et al. 2014). Future changes in the relative importance of APA_{zoo} to surface APA are also unclear, as evidence of a similar relationship between phosphate concentration and APA_{zoo} (Fig. 4) and APA_{vol} associated with the microbial community (Mahaffey et al. 2014) suggests that both may increase under phosphate limitation. Clearly, more work is required to unravel the complex interplay among zooplankton biomass, autotrophic biomass, and phosphorus recycling that underpins the accuracy of future climate predictions.

The major implications of our findings are: (1) there is significant diurnal variation in APA_{vol} in the surface ocean, which is an important consideration when interpreting hydrographical survey work or time series data investigating phosphorus dynamics; and (2) zooplankton provide a significant source of AP to the surface ocean during DVM, encouraging non-Redfieldian DOM remineralization and significantly contributing to ambient dissolved APA_{vol}. Our study demonstrates that changes in DVM because of enhanced stratification, water transparency, temperature gradients, and oxygen depletion will not only influence nitrogen regeneration and carbon export but also surface ocean P regeneration via reduction of zooplankton-derived AP. Given the predicted increase in phosphate stress in subtropical oceanic regions, any changes in zooplankton biomass or behavior will have significant ramifications for phytoplankton community structure and biomass.

Conclusions

We have shown that there is a seasonally and spatially persistent doubling of APA_{vol} between day and night in the surface subtropical North Atlantic using two independent datasets.

Furthermore, we have provided evidence that APA is enhanced by the presence of zooplankton and that the degree of enhancement has a robust relationship with surface layer phosphate. Thus, we conclude that zooplankton are a potentially important source of AP in the surface ocean and that this signal combined with degradation or removal of the enzyme during the day dominates the diel variability in surface APA_{vol}. These findings reveal a critical role of zooplankton in the surface cycling of phosphorus that may be vital to improve our understanding of surface ocean biogeochemistry in the contemporary ocean and accurate prediction of future ocean scenarios.

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