Vol. 84: 127–140, 2020
https://doi.org/10.3354/ame01930

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online April 30

Quantification of small-scale heterogeneity in aquatic aminopeptidase activity

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ABSTRACT: Leucine aminopeptidase (LAP) is one of the enzymes involved in the hydrolysis of peptides, and is sometimes used to indicate potential nitrogen limitation in microbes. Small-scale variability has the potential to confound interpretation of underlying patterns in LAP activity in time or space. An automated flow-injection analysis instrument was used to address the small-scale variability of LAP activity within contiguous regions of the Hudson River plume (New Jersey, USA). LAP activity had a coefficient of variation (CV) of ca. 0.5 with occasional values above 1.0. The mean CVs for other biological parameters — chlorophyll fluorescence and nitrate concentration — were similar, and were much lower for salinity. LAP activity changed by an average of 35 nmol l^{-1} h^{-1} at different salinities, and variations in LAP activity were higher crossing region boundaries than within a region. Differences in LAP activity were ±100 nmol l^{-1} h^{-1} between sequential samples spaced <10 m apart. Variogram analysis indicated an inherent spatial variability of 52 nmol l^{-1} h^{-1} throughout the study area. Large changes in LAP activity were often associated with small changes in salinity and chlorophyll fluorescence, and were sensitive to the sampling frequency. This study concludes that LAP measurements in a sample could realistically be expected to range from zero to twice the average, and changes between areas or times should be at least 2-fold to have some degree of confidence that apparent patterns (or lack thereof) in activity are real.

KEY WORDS: Aminopeptidase · Ectoenzyme activity · Variability · Coefficient of variation · Automated methods · River plume

1. INTRODUCTION

Bacteria, algae, and zooplankton use enzymes located on the cell surface, within the periplasmic space (for certain classes of bacteria), and/or released into the environment to convert polymeric, organic matter to smaller, readily utilizable forms (Allison et al. 2012 and references therein). Aminopeptidases catalyze the cleavage of amino acids from proteins, releasing amino acids that may alleviate nitrogen limitation (Cunha & Almeida 2009, Misic et al. 2006). Leucine aminopeptidase (LAP) is the aminopeptidase most often considered in environmental studies, though other amino-acid-containing substrates have been used (e.g. Pantoja et al. 1997, Tiquia 2011). However, LAP expression can also be an indicator of carbon limitation (Foreman et al. 1998, Sinsabaugh et al. 1997). Additionally, free (as opposed to cell-bound) enzyme production results in a potential decoupling between the source of enzyme production and the ultimate use of the enzyme (Vetter et al. 1998, Baltar 2018, Baltar et al. 2019).

The response of ectoenzyme activity to changing environmental conditions has been documented over

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hours to days (Martinez et al. 1996, Donachie et al. 2001, Williams & Jochem 2006, Cappello et al. 2007, Rochelle-Newall et al. 2008, Jaeger et al. 2009, Tiquia 2011), and manual measurements of LAP activity have been used to approximate the potential of organic matter processing over these temporal scales (Santos et al. 2009, Allison et al. 2012). Unfortunately, time-series measurements of ectoenzymes in marine systems do not specifically address local heterogeneity (Harbott et al. 2005). In addition, it is relatively rare for a mapping study to quantify the variability around each sampling location, despite the potential for activities to be uncorrelated with environmental parameters or spatial patterns (Bullock et al. 2017).

The requirement for rapid and consistent sampling and measurement of enzyme activities has been established (Obayashi et al. 2017). Automated shipboard (Ammerman & Glover 2000, Gaas & Ammerman 2007) and moored (Jaeger et al. 2009) ectoenzyme analysis systems can provide sampling methods that are ideal for characterizing variability in ectoenzyme activity, especially when combined with robust spatial data analysis techniques (e.g. Bulit et al. 2003, Askin & Kizilkaya 2006, Li & Heap 2014). The present study used a shipboard automated ectoenzyme analysis system to characterize the amount of small-scale spatial variability in homogeneous salinity regions. The spatial variability in ectoenzyme activity was also compared to the variability of analytes controlled by physical (salinity) and biophysical (chlorophyll *a* autofluorescence as a measure of phytoplankton biomass, and nitrate concentration for nutrient availability) processes. The results from this study may help explain the discrepancy between studies that alternatively do or do not find correlations among enzyme activities and other environmental parameters. It also provides information required to account for variability when designing experiments that rely on comparing enzyme activities between groups (e.g. making maps, contrasting surface water versus deep water).

2. MATERIALS AND METHODS

2.1. Study site

This work was performed as part of the Lagrangian Transport and Transformation Experiment (LaTTE), a multi-year, multi-platform study that looked at the physics and biogeochemistry of the Hudson River plume off the coast of New York and New Jersey (Chant et al. 2008a). In 2005, a 1-in-10-yr flood event combined with upwelling winds caused the formation of a freshwater bulge north of New Jersey (Chant et al. 2008a). Within the bulge, fresh estuarine waters mixed with aged and processed waters: estuarine water that had been retained and recirculated, and the site of high levels of biogeochemical processing by plankton. After the bulge collapsed, the processed waters created a downshelf coastal current. Nutrient uptake and primary production were very rapid in the bulge, resulting in nutrient-limited conditions within the coastal current (Moline et al. 2008). The dynamics of the bulge, including its formation, evolution, and eventual collapse, are detailed elsewhere (Chant et al. 2008a,b, Moline et al. 2008).

Four physical regions were defined based on contoured salinity values (Fig. 1). The river-affected region was identified from semi-circular contours with salinities between 17 and 23, and corresponds to where freshwater entered the shelf. The bulge region also formed rounded salinity contours near the coast and is where water of salinity 23–25 was entrained by upwelling-favorable southerly winds. Bulge formation started on 9 April and its main growth started on 13 April (Chant et al. 2008b). Primarily, vertical contours (seen after bulge collapse) with salinities >27 represent the offshore region. The offshore region was separated from the bulge by a sharp physical divide, as indicated by the tight contours around salinity 26. While the bulge was present, freshwater extended further off the shelf and reduced the area of the offshore region. The coastal current region had wide, vertical contours along the New Jersey coast with salinities >27. The return of the coastal current after bulge collapse manifested as a southward stretching of the salinity 25 through 27 contours.

The 2005 LaTTE sampling program was conducted using 2 research vessels deployed simultaneously from 9 to 20 April 2005. The following analytes were measured between the 2 vessels: LAP activity, salinity, chlorophyll *a* autofluorescence (referred to as chlorophyll fluorescence), and nitrate concentration. The R/V Oceanus was the platform used to measure LAP activity, salinity, and chlorophyll fluorescence. Samples were taken from an uncontaminated seawater flow centered at a depth of 3 m. LAP activity was measured using the automated Enzyme Activity Analysis System (EAAS, described briefly below). Salinity was acquired from a Sea-Bird Electronics SBE 45, and chlorophyll fluorescence from a flow-through WETLabs WetStar fluorometer (460 nm excitation/695 nm emission wavelengths). Nitrate concentrations and salinity were measured in water from

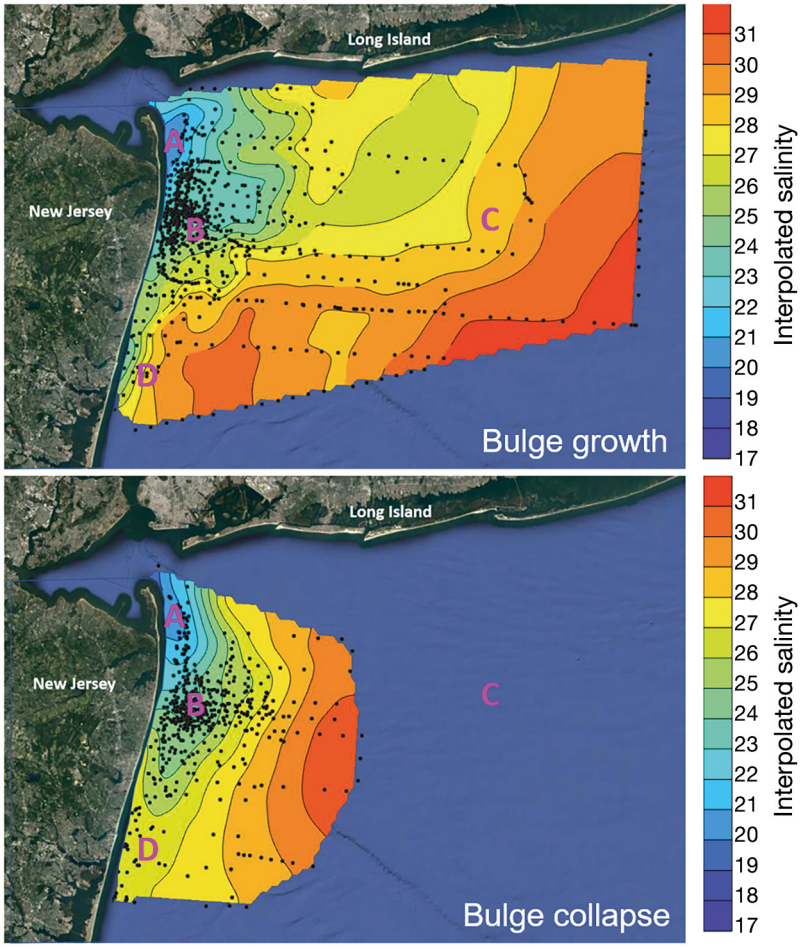

Fig. 1. Regions defined by salinity contours. Contours are the ordinary point kriging results from the observed salinity data from both ships. The bulge growth data (top panel) included salinity measurements between 9 and 16 April. The bulge collapse data (bottom panel) were from 17 to 20 April. Black dots are the locations of salinity measurements. The letters identify the 4 physical regions: (A) river-affected, (B) bulge, (C) offshore, and (D) coastal current

the 2 m depth intake of the R/V Cape Hatteras. Nitrate concentrations were measured with an Autolab automated nutrient analyzer. Salinity aboard the R/V Cape Hatteras was measured using a Sea-Bird SBE 21. The 2 ships, while surveying the same general areas, did not follow the same cruise tracks and did not necessarily sample the same locations at the same times (Fig. 2).

2.2. Enzyme Activity Analysis System (EAAS)

The EAAS is based on automated, stopped-flow, sequential flow-injection analysis technology. The instrumental details of the EAAS are described elsewhere (Gaas & Ammerman 2007). The EAAS used a mixture of seawater and the leucine-bound fluorophore substrate L-leucine-7-amido-4-methylcoumarin (Leu-AMC) to measure ectoenzyme activity every 14 min. Ectoenzyme activity was measured indirectly as the rate of fluorescence increase that occurs through hydrolysis of Leu-AMC. The substrate concentration used in the LaTTE program was 200 µM, the observed saturating concentration based on LAP kinetics run at the beginning of the cruise (Gaas & Ammerman 2007). However, mixing and dispersion within the EAAS reduced the substrate concentration to 20 µM, closer to the half-saturation value (K_m; Taylor et al. 2003). Ectoenzymes generally operate at natural substrate concentrations near the K_m, as this provides the most effective response to changing substrate concentrations (Sinsabaugh et al. 2014). Although the substrate concentrations in this project were not saturating, they may more closely reflect the natural substrate concentrations and variability of the environment. Fresh substrate was created from frozen stock solutions every 2 d and fresh standards every 3 d.

LAP activity was calculated as the ratio of the sample slope to an internal standard slope (units: nmol l^{-1} h^{-1}). The sample slope was the slope of the linear least squares regression of Leu-AMC fluorescence versus time observed in a solution of seawater plus substrate (units: AMC fluorescence s^{-1}). Internal standard slopes were calculated by subtracting the fluorescence of the blank (0 nM standard) from the fluorescence of an internal standard run periodically during each transect, and dividing by 99 nM (concentration of the internal standard in the flow cell) (units: nmol l^{-1} AMC fluorescence^{-1}). The internal standard slope corrects for changing environmental effects on AMC fluorescence due to salinity, particle scattering, and absorption of excitation light or fluorescence by chlorophyll and colored dissolved organic matter.

2.3. Quantifying variability

Since true replicate samples were not available for this project, a different approach was required to

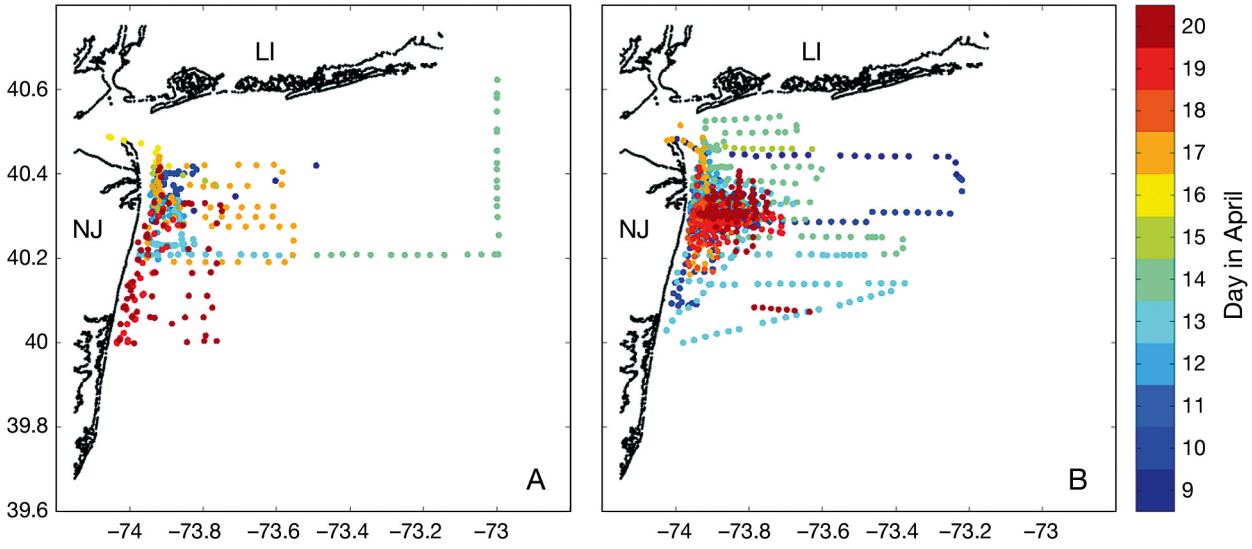

Fig. 2. Ship tracks for (A) R/V Oceanus and (B) R/V Cape Hatteras off the coasts of New Jersey (NJ) and Long Island (LI). The day of the cruise (in April 2005) follows the color scale. Salinity, leucine aminopeptidase (LAP) activity, and chlorophyll fluorescence were measured on the R/V Oceanus, while salinity and nitrate concentration were measured on the R/V Cape Hatteras

group observations for statistical summary. Salinity was used to define regions where summary statistics (e.g. mean, coefficient of variation) were calculated for salinity, LAP activity, chlorophyll fluorescence, and nitrate. Salinity was chosen because it can be measured accurately, it was continually measured on both ships, and the processes controlling salinity (e.g. river discharge) resulted in contiguous, physically meaningful areas. Observed salinity values, rounded down to the nearest integer, were used to define regions with the same (unit width) salinity each day; measurements of salinity, LAP activity, chlorophyll fluorescence, and nitrate concentration were grouped into these salinity bins. The residence time of drifters inside the bulge was about 1 wk (Chant et al. 2008a), during which salinity remained relatively constant (Moline et al. 2008), so a daily time step appeared reasonable. A shorter time step would also have made data interpretation more difficult by increasing the number of time–space combinations that needed to be considered.

The coefficient of variation (CV; standard deviation divided by the mean) for each environmental parameter (salinity, LAP activity, chlorophyll fluorescence, nitrate concentration) was calculated daily within each salinity bin. This approach provides a summary statistic characterizing the relative variability of each parameter without inflating variability by comparing dissimilar water masses (e.g. nutrient-laden river outflow to nutrient-depleted offshore water).

The activity slope, defined here as the absolute change in LAP activity per unit change in distance, salinity, or chlorophyll fluorescence between sequential samples, was calculated from the time-series of LAP activity created along the cruise track. The activity slope should not be confused with the sample slope or internal standard slope used in LAP activity calculations. It is also not a regression slope. Data from 17 April were used to calculate activity slopes because samples on that day were collected from a wide range of salinities, and had the highest sample count of LAP activity measurements. Activity slopes were also calculated on the same 17 April data set, but where every second sample (or second and third samples) was removed. These downsampled data sets approximated the observation frequency of a fluorescent microplate reader (one-half original EAAS rate) or manual fluorometer (one-third original EAAS rate). Activity slopes were not calculated across gaps created when the instrument was not sampling (e.g. running quality control samples or cleaning cycles). Activity slopes were not calculated for nitrate, since nitrate concentrations were not measured at the same locations as LAP activity.

Variogram analysis was also applied to the LAP activity data. Semi-variograms are statistical models that describe the relationship between the physical distance among data points and the dissimilarity of the observations (their semi-variance). Data are

placed into bins by distance from each other, and the semi-variance γ is calculated in each bin as:

$$\gamma = \frac{1}{2N}\Sigma\left|z_i - z_j\right| \quad (1)$$

where z_i and z_j are pairs of data within a specific distance (lag distance), and N is the total number of data pairs within the lag distance. In general, observations are more similar the closer they are to each other in space and, conversely, more dissimilar the further the locations are from each other. A non-zero semi-variance where the samples are practically at the same location (zero lag distance) indicates inter-sample variability that is not explained by distance (the 'nugget' effect). Semi-variograms were created in R (R Core Team 2008) using the default parameters under the gstat package (Pebesma 2004). A different semi-variogram was created for each of the 4 physical regions (river affected, bulge, offshore, and coastal current) for each day. Models were fit to each function using linear and non-linear least squares regression, and the value of the nugget semi-variance (also equal to the y-intercept of the model fit) was recorded. The standard deviation of the nugget semi-variance will be referred to as the nugget deviation, and is a measure of the fundamental variability of LAP activity within the sampled area.

3. RESULTS

3.1. Mean values within salinity bins

LAP activity, chlorophyll fluorescence, and nitrate concentration each had distinct spatial patterns (Fig. 3, Table S1 in the supplement at www.int-res.com/articles/suppl/a084p127_supp.pdf). Mean LAP activities were >150 nmol l^{-1} h^{-1} in the bulge region (salinity bins 23–25) during the fastest portion of bulge growth (13–17 April). The highest activities (>200 nmol l^{-1} h^{-1}) were observed in the coastal current after the bulge had collapsed. LAP activities out-

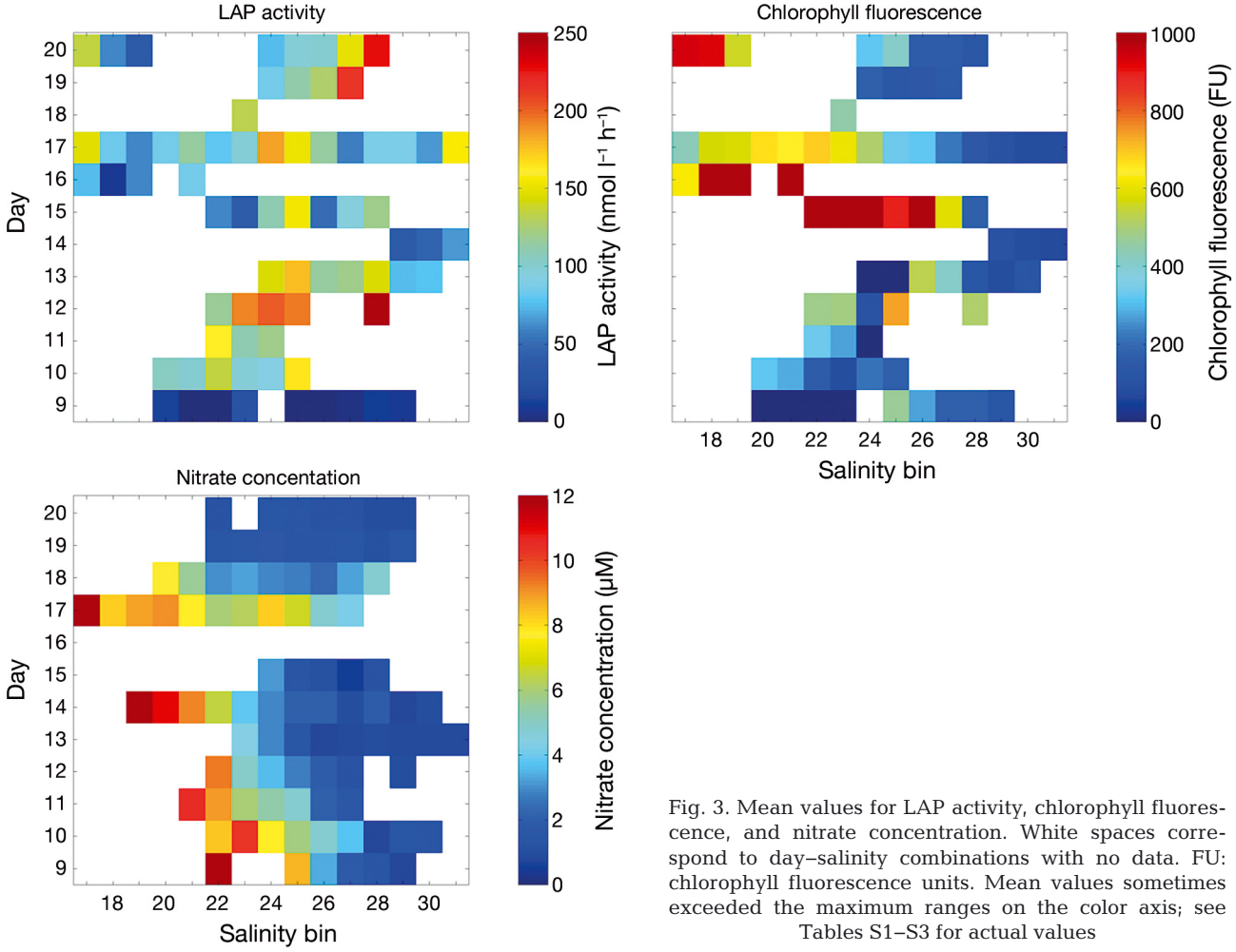

Fig. 3. Mean values for LAP activity, chlorophyll fluorescence, and nitrate concentration. White spaces correspond to day–salinity combinations with no data. FU: chlorophyll fluorescence units. Mean values sometimes exceeded the maximum ranges on the color axis; see Tables S1–S3 for actual values

side of these 2 regions were generally between 50 and 100 nmol l^{-1} h^{-1}. A maximum mean activity of 380 nmol l^{-1} h^{-1} occurred on 12 April at salinity 28, and could represent a microbial response to the shutdown of normal southern flow (down the coast) during bulge formation. The highest activity measurement was 1.2 µmol l^{-1} h^{-1}.

Chlorophyll fluorescence increased during the growth of the bulge from 9 to 15 April (Fig. 3, Table S2). Chlorophyll fluorescence peaked on 15 and 16 April and extended from salinity 18 to 26, which included some of the river-affected region. Within 2 d, chlorophyll fluorescence within the bulge decreased to half of the maximum; this occurred at the same time the bulge was collapsing. In addition, peak chlorophyll fluorescence became more localized and occurred in fresher water. Unlike LAP activity, high chlorophyll measurements were not observed in the coastal current region.

The highest mean nitrate concentration was 14.4 µM (Fig. 3, Table S3), which is lower than the maximum nitrate measurement of 15.9 µM from within the river plume. Offshore nitrate concentrations were around 1.2 µM. Nitrate concentrations decreased from low salinity to high salinity (left to right in Fig. 3) as riverine input was diluted. Nitrate concentrations also decreased during bulge formation at salinities 23–26 from 9 to 15 April (bottom to top in Fig. 3) due to nutrient uptake. Bulge formation did not appear to affect nitrate concentrations in offshore waters. During the bulge collapse, mean nitrate concentrations in the bulge increased from 2.41 µM on 15 April to 7.7 µM on 17 April. Concentrations again decreased and reached a background of 1.5 µM on 19 April. The increase in nitrate concentration on 17 April was not reflected in changes in either LAP activity or chlorophyll fluorescence.

3.2. Variability within salinity bins

The CVs for measured salinity were between <0.01 and 0.02 and were consistently the lowest of all the parameters (Fig. 4, Table S4). Although salinity had the largest number of measurements of the 4 parameters, the number of samples should only affect the precision of the estimates (i.e. mean and standard deviation) and not the actual values of the estimates. The variabilities of LAP activity, chlorophyll fluorescence, and nitrate concentrations were substantially higher, with CVs occasionally exceeding 1.0. The standard deviation was positively correlated to the mean for these 3 parameters (Spearman $r_s > 0.5$, $p < 0.01$). The explanatory power of the relationship (r_s^2) between the standard deviation and mean increased by 11% and 19% for chlorophyll fluorescence and nitrate concentration, respectively, if data from the river-affected region were excluded; the r_s^2 stayed the same for LAP activity. A linear regression of the standard deviation on the mean for LAP activity returned a slope of 0.51 ($r^2 = 0.32$, df = 36, $p < 0.01$) which equates to an estimated CV of ca. 0.5. The slopes for chlorophyll fluorescence and nitrate concentration were 0.21 and 0.37 respectively, but the residuals of the linear model showed the regression was a poor fit for these data. The mean CVs over all 12 d and salinity bins were 0.01 for salinity, 0.48 for LAP activity, 0.54 for chlorophyll fluorescence, and 0.40 for nitrate concentration. There was no direct correlation among the CVs of the parameters: Spearman correlation coefficients ranged from −0.14 to +0.21 (Table S8). The lack of correlation between the CVs of salinity against the other parameters suggests that variations in LAP activity, chlorophyll fluorescence, and nitrate concentrations were influenced by a different set of biogeochemical processes than that influencing salinity.

The CV for LAP activity exceeded 1.0 four times: 9 April in salinity bin 27, 10 April in salinity bin 23, 16 April in salinity bin 21, and 20 April in salinity bin 28 (Table S5). High variability in LAP activity was present in multiple areas and was not restricted to the dynamic bulge region. There was a general tendency for low CVs to be present in the bulge region, where mean LAP activities were higher. The CVs for chlorophyll fluorescence and nitrate concentration exceed 1.0 in 9 and 4 instances, respectively (Tables S6 & S7). The CVs for chlorophyll fluorescence were especially high between 10 and 13 April during the growth of the bulge, and also increased starting on 17 April during the collapse of the bulge. The CVs for chlorophyll fluorescence of ≈0.4 on 17 April coincided with the localization of the chlorophyll fluorescence maximum on 17 April. Although chlorophyll fluorescence decreased after the bulge collapsed, the variability remained about the same. The highest variability in nitrate concentration occurred in offshore waters, indicating that nitrate CVs may be inflated by the low mean concentrations further away from the coast. A pocket of relatively high CVs (≈0.5) for nitrate occurred in the bulge between 11 and 13 April. Nitrate concentrations were decreasing at this time, so the higher variability could be related to when the concentrations were measured during the day.

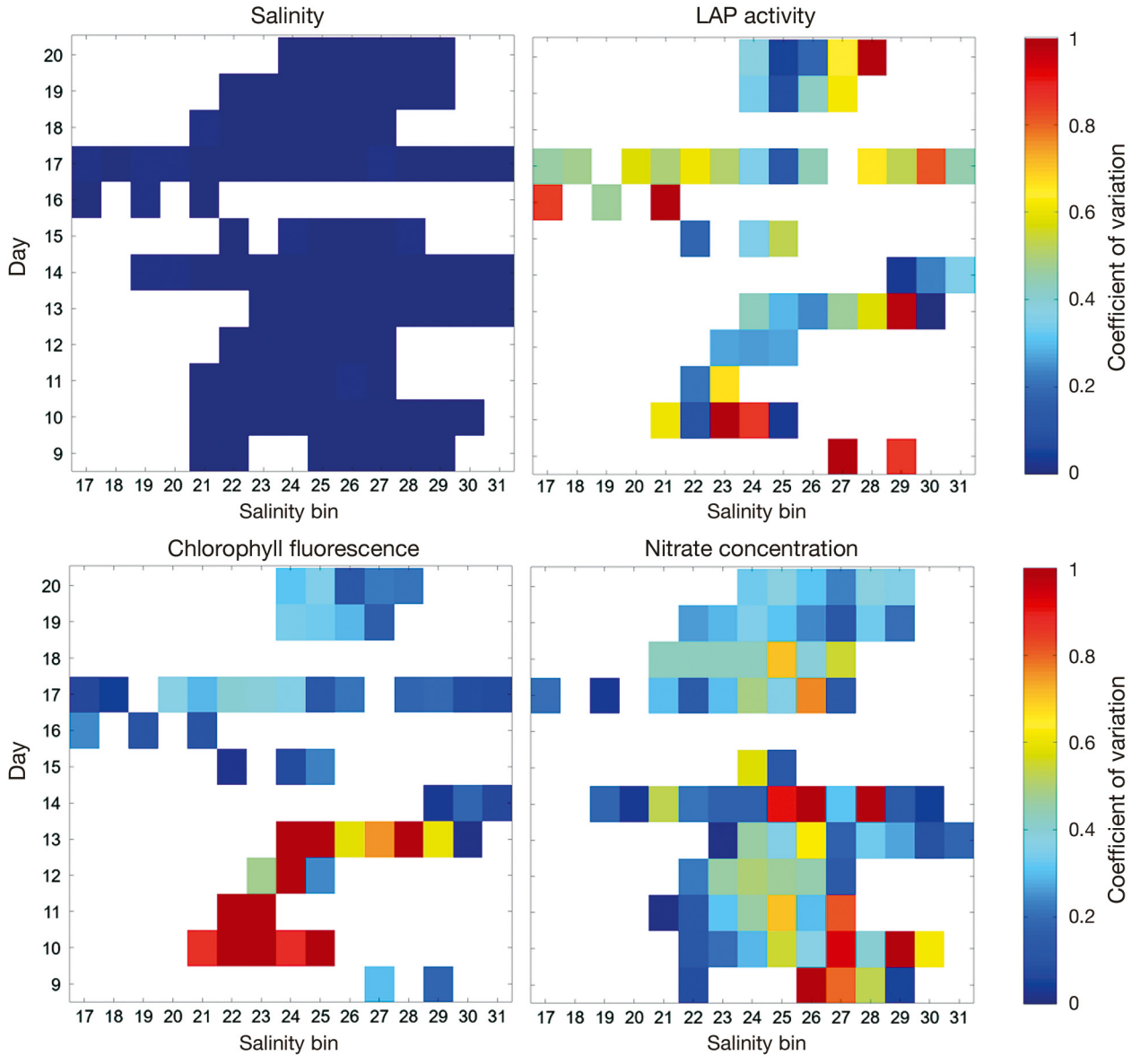

Fig. 4. Coefficients of variation (CVs) for salinity, LAP activity, chlorophyll fluorescence, and nitrate concentration. White spaces correspond to day–salinity combinations with no data. Calculated CVs sometimes exceeded 1.0; see Tables S4–S7 for actual values

3.3. Spatial variability in LAP activity

The change in LAP activity between adjacent salinity bins was highest when the salinity bins were between salinity 22 and 28, though this value depended on the day of sampling (Fig. 5). On average, LAP activity changed by 35 nmol l^{-1} h^{-1} when changing salinity bins (derived from Table S2). The relatively large changes in mean LAP activity across salinity bins overlapped the salinity range that defined the bulge (salinity 23–25), reinforcing the idea that the bulge was an area of high biogeochemical activity.

Since LAP activity was measured at the same locations as salinity and chlorophyll fluorescence, rates of change in LAP activity could be calculated for each sequential pair of measurements relative to distance traveled, or relative to the change in salinity or chlorophyll fluorescence. Changes in LAP activity between sequential samples were around ±100 nmol l^{-1} h^{-1} at distances less than 10 m, and were also high when changes in salinity or chlorophyll fluorescence were small, especially within the bulge region (Fig. 6). Changes in LAP activity decreased to less than ±40 nmol l^{-1} h^{-1} at a spacing of 10 km between sample pairs. The large distances between samples occurred

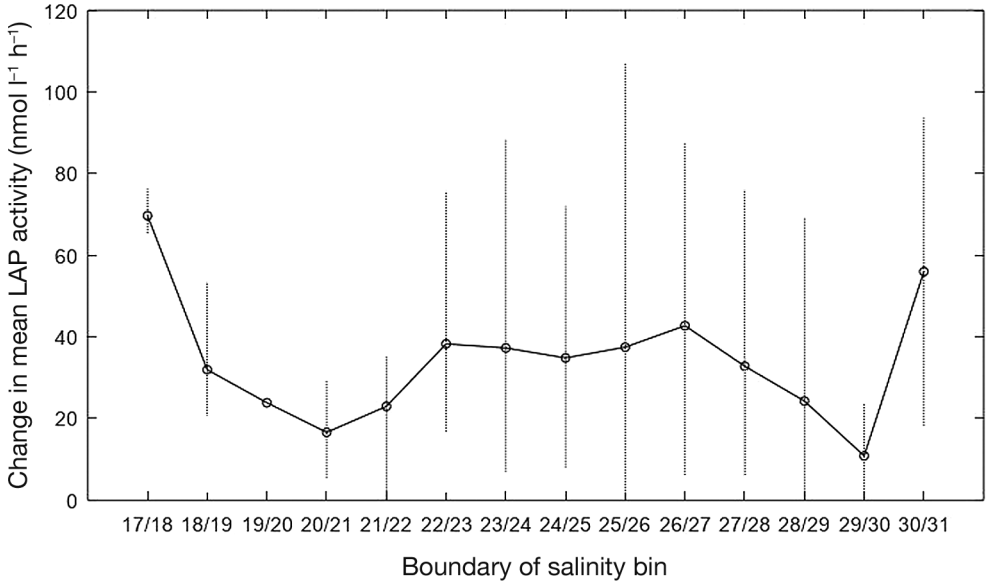

Fig. 5. Change in mean LAP activity across salinity bins. The *x*-axis indicates the boundary between salinity bins (e.g. 17/18 is the boundary between salinity bins 17 and 18). Change in mean LAP activity (*y*-axis) across a salinity boundary was calculated as: (LAP activity on Day 9/Sal 18 bin–Day 9/Sal 17 bin), (Day 10/Sal 18–Day 10/Sal 17), etc. Circles indicate the mean of the differences for each salinity bin boundary across all days. Dotted lines indicate the range of differences for each salinity bin boundary across all days

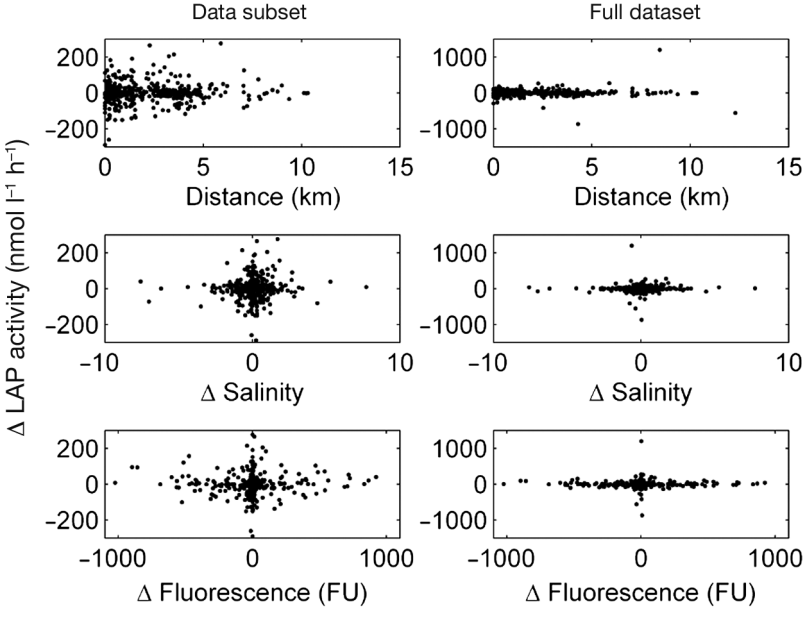

Fig. 6. Input for activity slope calculations. The activity slope is the ratio of the change in LAP activity between sequential measurements (*y*-axis values) to distance between stations, change in salinity, or change in chlorophyll fluorescence between sequential measurements (*x*-axis values). Activity slopes were calculated as the ratio of each (*x*,*y*) pair and summarized in Table 1. The data subset column is a zoomed-in version of the full data set column with the zoom centered on the location of the highest data density. Changes were measured between sequential measurements only, rather than for every possible data pairing. Δ: difference between sequential data values. Linear regression slopes based on the 3 full data set graphs (right column) were not significant ($p > 0.45$)

in the offshore region, where physical conditions were more uniform and LAP activities were lower. The changes in LAP activity were symmetrical around zero, indicating that the direction of change in LAP activity was random between samples. There was no relationship between the difference in sequential measurements of LAP activity and distance between stations, change in salinity, or change in chlorophyll fluorescence (linear model, n = 442 pairs, $p > 0.45$ for all tests).

Activity slope statistics varied with sampling frequency (Table 1). Maximum activity slopes based on distance suggest that LAP activities could vary on the order of 1 µmol l^{-1} h^{-1} km^{-1}, which matches the maximum observed difference in LAP activity between subsequent measurements of 1.2 µmol l^{-1} h^{-1}. The maximum activity slopes using salinity and chlorophyll fluorescence were around 1000 times higher than the median activity slopes. In contrast, the maximum activity slope using distance was closer to 100 times higher than the median.

Table 1. Leucine aminopeptidase (LAP) activity slopes from 17 April 2005. Activity slopes were calculated as the absolute change in LAP activity per unit distance, or unit change in salinity or chlorophyll (chl) fluorescence. Distance slope units = nmol l^{-1} h^{-1} km^{-1}. Salinity slope units = nmol l^{-1} h^{-1} (salinity unit)$^{-1}$. Chlorophyll fluorescence slope units = nmol l^{-1} h^{-1} (chlorophyll fluorescence unit)$^{-1}$. Downsampling factor is the ratio of the number of observations in the full data set to the number of observations in the subsampled data set (e.g. downsampling factor = 2 means every other data point was excluded). Slopes corresponding to changes in nitrate concentration were not available as LAP activity and nitrate concentration were not sampled at the same locations

Activity slope parameter	Downsampling factor = 1			Downsampling factor = 2			Downsampling factor = 3		
	Minimum slope	Median slope	Maximum slope	Minimum slope	Median slope	Maximum slope	Minimum slope	Median slope	Maximum slope
Distance	0.03	14	1277	0.01	9.3	1070	0.02	8.5	1860
Salinity	0.04	65	151 170	0.02	52	60 707	0.16	45	35 686
Chl fluorescence	0.0009	0.56	777	0.001	0.64	474	0.003	0.39	433

The lower median slopes indicate that smaller spatial changes in LAP activity were common. Minimum activity slopes were close to zero regardless of whether LAP activity was compared to distance, salinity, or chlorophyll fluorescence. This is because the activity slopes have a mathematical limit of zero since the slopes could not be negative. There was a significant relationship between the activity slopes based on distance with those based on salinity, likely due to riverine outflow driving the distribution of salinity (linear model, n = 430 pairs, $p < 0.08$).

Downsampling (ignoring every second or second and third sample to mimic a lower sampling rate) did not have a consistent effect on the activity slopes (Table 1, Fig. S1). The magnitude of the statistics among activity slopes was similar, with the exception of the maximum activity slope based on salinity, which was an order of magnitude higher in the 2-fold downsampled data set compared to the original and 3-fold downsampled data sets. The similarity of the summary statistics despite the change in sampling frequency indicates that although the sampling frequency can affect the value of the statistics, the results are not biased.

Most (85%) of the semi-variograms for LAP activity resulted in non-zero small-scale variance. The nugget semi-variances (equivalent to a variance for measurements within the smallest lag distance) were on the order of hundreds to thousands of nmol^2 l^{-2} h^{-2} (Table S9). The mean nugget deviation (equivalent to a standard deviation for measurements within the smallest lag distance) was 52 nmol l^{-1} h^{-1}, and ranged from 0 to 165 nmol l^{-1} h^{-1}. This result is consistent with observed CVs (Figs. 3 & 4), again indicating the variability in LAP activity is expected to be similar in magnitude to the activities themselves. The distribution of nugget deviations did not differ across regions (Kruskal-Wallis $\chi^2 = 1.26$, df = 3, $p = 0.74$), date range (before bulge formation: 9–12 April; bulge formation and aging: 13–17 April; bulge collapse: 17–20 April) ($\chi^2 = 1.04$, df = 2, $p = 0.59$), or a combination of the two ($\chi^2 = 7.82$, df = 10, $p = 0.65$). The insignificant differences among date–region combinations persisted even if different date ranges were used.

4. DISCUSSION

The majority of recent work on enzyme activities tends to either report activities in a specific environment or correlate activities with other environmental parameters such as nutrient concentration. The present study is unique within the ectoenzyme literature for its focus on the variability of enzyme activity. It specifically quantifies the level of small-scale variability: differences in LAP activity that are unrelated to regional patterns. Knowledge about the variability in enzyme activity measurements is critical to correctly interpreting activity data, as high variability has the potential to mask or distort trends in space, time, or correlations between variables (e.g. enzyme activity and biomass). It is also useful for designing studies where activities will be compared between 2 or more groups, as highly variable measurements will require more samples to distinguish between groups.

Of the 4 environmental parameters examined (salinity, LAP activity, chlorophyll fluorescence, and nitrate concentration), salinity had the least amount of variability (Fig. 4, Table S4). The main influence on salinity in the study area was the river discharge, which formed contiguous regions of different salinities. The low variability in salinity was due to the large areas influenced by river discharge, relative to the spacing of salinity measurements. If the variabilities of LAP activity, chlorophyll fluorescence, and

nitrate concentration were similar in magnitude to that of salinity, then this would suggest that spatial variations in these other parameters were also dominated by regional, transport-driven processes. The much smaller variability in salinity indicates that the source of variability in LAP activity, chlorophyll fluorescence, and nitrate concentrations is not regional but more localized.

Salinity may only loosely correspond to the biological processes affecting LAP activity, chlorophyll fluorescence, or nitrate (Salerno & Stoecker 2009, Allison et al. 2012). For instance, nitrate concentrations were consistently high closest to the river discharge and low offshore, but were reduced over time within the bulge while maintaining a relatively consistent salinity (Moline et al. 2008). The change in light penetration in the coastal current during the LaTTE program occurred without concomitant changes in salinity (Moline et al. 2008), signaling a decoupling from the physical movement of the water (as indicated by salinity) and biologically important processes. LAP activity could be related to the outflow-driven distribution of metal cofactors (Rasmussen & Olapade 2016). Concentrations of dissolved metals in the study area decreased with increasing salinity due to dilution with shelf waters (Moline et al. 2008), and bulge, offshore, and coastal current regions were also differentiable using metal concentrations (Wright et al. 2010). Salinity was likely related to LAP activity via the amount of organic nitrogen (LAP substrate) and/or inorganic nitrogen (LAP hydrolysis product) to the study area (Chrost 1991). Unfortunately, data were not available to directly relate the variability of organic or inorganic nitrogen concentrations to that of LAP activity. While salinity may be useful in indicating large-scale regions of interest and can correlate with community composition and metabolic analysis (Monticelli et al. 2014, Wear et al. 2014), high within-region variability of enzyme activity, chlorophyll fluorescence, and nutrient concentrations may still occur (as it did in the present study).

There was a general tendency for low CVs and large changes across salinity boundaries to be present in the salinity range 23–25 corresponding to the bulge region (Figs. 4 & 5). This combination indicates the bulge region had relatively sharp biogeochemical boundaries. Peaks in LAP activity were associated with rapid changes in salinity on a cruise track along the New Jersey coast that made excursions out to sea and back to shore (Gaas & Ammerman 2007). The bulge was both an area of high biological activity as well as a transitional zone from the river-affected region to the offshore region. Transitional zones are known to have fluctuating responses to biogeochemical drivers (Caruso et al. 2005, 2013), so it is not surprising that the activity slopes were highest in this region.

The presence of sharp physical boundaries could contribute to the high variabilities identified in this study. Data from 2 distinct biogeochemical regions (on either side of a physical boundary) but with similar salinities could have been binned together, inflating the variability. Similarly, semi-variances are calculated by binning data by the distance separating them. Higher semi-variances and nugget deviations are expected if the boundaries between higher and lower LAP activity regions are small enough to fall within a single distance bin within the semi-variogram. Activity slopes, which do not rely on binning, would also contain high values if a measurement was taken on one side of a boundary between high- and low-LAP-activity regions and the subsequent value taken on the other side. In these cases, the small-scale variability still exists but would be related to the physical structure of the environment. In fact, observing highly variable LAP activities could indicate the existence of physical discontinuities that cannot be identified using salinity. It is worth noting that sharp boundaries were not expected in the offshore region of this study, yet high CVs and nugget deviations were still observed (Fig. 4, Table S9). Although crossing physical boundaries can contribute to the overall variability in LAP activity, substantial variability also existed within discrete biogeochemical regions.

The relative variability in biological parameters — LAP activity, chlorophyll fluorescence, and nitrate — were around half of the magnitude of the measured activities (CV ca. 0.5). Similarly, the standard deviation of LAP activity, chlorophyll fluorescence, and nitrate concentration increased with increasing mean values, and LAP activity followed a slope of 0.5 (standard deviation was half of the mean). This means that biological measurements could realistically be expected to range from zero to twice their average, and changes between areas or times should be at least 2-fold to have some degree of confidence that the apparent pattern (or lack thereof) is real. Similar variability has been noted in studies from other environments (see Table S10) and is not limited to the present study, or coastal environments in general. This work also used variogram analysis to quantify an absolute measure of small-scale variability for LAP activity. The nugget deviation (standard deviation of the semi-variogram at zero lag distance) was on the order of tens to 100 nmol l^{-1} h^{-1} (Table S9).

These values were similar to the activity slopes observed for locations away from the plume with relatively stable conditions and can be interpreted as a detection limit for changes in LAP activity. The nugget deviations indicate that changes in LAP activities up to 100 nmol l^{-1} h^{-1} between samples constituted background variability unrelated to smooth spatial patterns. At least in this environment, analyses involving LAP activities less than 100 nmol l^{-1} h^{-1} run the risk of underlying patterns being masked or creating spurious correlations. Nugget deviations were calculated for river-affected, biogeochemically active, and offshore waters. There was no difference in the nugget deviation for LAP activity among these physical regions, suggesting that similarly high background variabilities could be applicable to other environments.

The data were binned by day and salinity in order to calculate summary statistics. Binning the data differently could impact the summary statistics, in particular the CVs. For instance, the spatial grouping could have been done using distance from the river mouth instead of using salinity. Using a different spatial grouping would not affect the variogram or activity slope results, as they do not depend on assigning data to a specific group. However, the data were also binned by time (1 d), and some of the variability observed in a single salinity bin could be due to changes occurring on time scales less than 1 d. Ectoenzyme activities can change over the course of hours (e.g. Kamer & Rassoulzadegan 1995), so including the higher and lower ends of a daily range in the same group could inflate the CVs. Since the variogram analysis grouped points by lag distance within an entire day, the nugget deviation could reflect both small-scale spatial variations as well as differences in activity that occurred over the course of a day. The activity slope results are the least likely to be affected by changes in LAP activity occurring over the course of day. The activity slopes were calculated between subsequent measurements, so most of the slopes only differ by 14 min (the sampling rate of the EAAS instrument). The median activity slopes with distance were ca. 10 nmol l^{-1} h^{-1} (Table 1). This is lower than the estimate of the nugget deviation (52 nmol l^{-1} h^{-1}), which suggests that changes in activity over the course of the day could have contributed to the overall variability.

Community changes are often invoked to explain variability in ectoenzyme activity that has no direct relationship with other observed parameters (e.g. Allison et al. 2012). There was a change in community structure at the boundary between the bulge and the coastal current from diatoms to dinoflagellates, cryptophytes, and other flagellates (Moline et al. 2008). This community change could alter the role of bacterioplankton in nutrient cycling, which is dependent on the stoichiometry of organic to inorganic nutrient sources (Cunha & Almeida 2009), as well as the source, composition, and quantity of dissolved organic matter (Wear et al. 2014). Community structure can have a direct impact on bulk LAP activities (e.g. Martinez et al. 1996). However, not all changes in enzyme activities are linked to changes in community composition (Arnosti et al. 2014). Bacterioplankton community structure may have a limited role in controlling the processing of the dissolved organic carbon pool compared to trophic factors and physical conditions (Dinasquet et al. 2013). The similar CVs among LAP activity, chlorophyll fluorescence, and nitrate concentration suggest that the variabilities in these parameters are caused by a similar biophysical process(es). Three alternative explanations to community changes that could contribute to the variability of LAP activity include: hydrolysis rate changes with temperature, hydrolysis kinetics related to substrate concentration, and the random sampling of particles.

Enzyme activity is directly linked to water temperature (e.g. Manna et al. 2019, Celussi et al. 2019), and microbial activity is limited in general by temperature–substrate interaction effects (Pomeroy & Wiebe 2001). LAP activities observed in temperate seawater (10–21°C) by Ayo et al. (2017) had Q_{10} values between 1.6 and 2.3. This range of Q_{10} values is much lower than the Q_{10} above 10 (i.e. an order of magnitude change in LAP activity with a 10°C temperature change) measured in an Arctic system (Piontek et al. 2014). The bulge region in our study area was almost twice as warm as temperatures off the coastal shelf (11°C versus 6°C, respectively; Moline et al. 2008), which would correspond to a 40% increase in LAP activities when moving to a warmer physical region. A similar enhancement in growth/metabolism rate could contribute to changes in chlorophyll fluorescence and nitrate concentration. However, no correlation was seen between LAP activity and temperature in the Hudson River or Long Island Sound unless it was coupled with nutrients and other biotic properties (Taylor et al. 2003). Also, the variability of temperature within a given salinity bin is expected to be similar to that of salinity (CV of 0.01–0.04), and does not explain the higher within-bin CVs observed for LAP activity (0.28–1.87).

High CVs could indicate inconsistent availability of enzyme substrate (Caruso et al. 2005). Under the

non-saturating conditions of the present study and the hyperbolic relationship between substrate concentration and LAP activity observed on the cruise, a small change in natural substrate concentration could result in a large change in LAP activity. Around 60 nM changes in the K_m of alkaline phosphatase were observed within the same water mass in the Mediterranean Sea (Van Wambeke et al. 2009), similar to the differences in median K_m values measured for LAP activity by Williams & Jochem (2006) in different locations within Florida Bay. If that value is applicable to LAP activities near the New Jersey coast, the change in K_m would be around 1% (60 nM variability/10 µM K_m), and the kinetic effect would make negligible changes in hydrolysis rates. However, Santos et al. (2009) observed a 40% change in LAP kinetic parameters during 6 h tidal cycles in a Portuguese estuary, and the K_m in the northwest Mediterranean Sea also varied (±2 SEM) by ≈48% (Unanue et al. 1999). Kinetic effects of this magnitude could alter hydrolysis rates in the study area by around 40 nmol l^{-1} h^{-1} and explain much of the variability observed between sequential samples. In effect, this mechanism replaces the small-scale variability in LAP activity with equally small-scale variability in substrate concentration.

The previous 2 mechanisms each rely on an underlying secondary factor — substrate concentration and temperature — to create variability in enzyme activities. In contrast, the distribution of LAP enzymes (or enzyme-producing cells) itself may be patchy. For instance, LAP changed by ±100 nmol l^{-1} h^{-1} between samples that were only meters apart. The reduction in scale between how salinity changes (kilometers) and how other parameters change (meters or less) is due to the small distances at which biological processes (e.g. enzyme production and hydrolysis, cell growth, nutrient uptake and release) operate. LAP is produced at the level of a single cell, and differences in LAP activity may occur at distances of micrometers (Arnosti et al. 2014). Substantial proportions of total enzyme activity can occur away from cells entirely as long as the population is close enough to access the hydrolyzed substrate (Baltar 2018, Baltar et al. 2019). LAP activity is enhanced on particles (Smith et al. 1992, Martinez et al. 1996, Misic & Fabino 2006, Kiersztyn et al. 2012, Orcutt et al. 2013), and samples with higher proportions of particles could lead to hot spots of activity. Bacteria associated with gels can have 3 orders of magnitude higher counts than surrounding waters, and could also create nutrient-enriched zones within the micrometer range (Azam 1998, Baltar et al. 2010).

Enzyme-producing cells may create and release enough hydrolysis product to support a population of non-enzyme producing cells (Vetter et al. 1998, Allison 2005, Arnosti 2011). The fraction of cell-free (often referred to as 'dissolved') LAP activity can comprise a large proportion of the total (Kamer & Rassoulzadegan 1995, Obayashi & Suzuki 2008, Baltar 2018, Baltar et al. 2019). An optimal, free enzyme foraging distance of 10 µm was calculated by Vetter et al. (1998), which could support a 'producer/non-producer' dichotomy. A bacterial biomass of 10^9–10^{10} cells per liter in the Hudson River (Taylor et al. 2003) implies that only 1 in 10 or 1 in 100 cells might produce extracellular LAP within the EAAS cuvette (100 µl). If cells actively producing LAP were this rare, it would only take a small increase in the number of LAP-producing cells captured in the EAAS cuvette to elevate the LAP activity in the sample. This variability is not contingent on composition differences in the microbial community, but rather is a result of randomly sampling a discrete population.

Presumably, similar results could arise by randomly sampling particles exhibiting LAP activity versus no activity, different rates of activity, or different peptidases that can hydrolyze Leu-AMC (Steen et al. 2015). Particles and gels offer the potential to change substrate concentrations on the micrometer scale, which could be sufficient to explain the observed variability in LAP activity. Variability in chlorophyll fluorescence was presumably also related to particle distribution (i.e. variability in chlorophyll fluorescence is primarily driven by differing numbers of phytoplankton). The magnitude of the variability in chlorophyll fluorescence was similar to that of LAP activity, reinforcing the idea that the spatial variability in LAP activity is related to particles. Micrometer-scale differences between samples could contribute to the poor correlations observed in studies between enzyme activity and explanatory variables such as nutrient concentrations or biomass. Further work is required to determine whether the variability in LAP activity, chlorophyll fluorescence, and nitrate concentration observable at distances of meters can be derived from processes occurring on the micrometer scale.

Large changes in LAP activity occurred over short spatial scales. Although the present study was restricted to LAP activity, the effects considered here — kinetic changes due to substrate concentration; micrometer-scale production of enzymes — are not unique to LAP. As a result, other ectoenzymes could be expected to exhibit small-scale variability of around the same magnitude. The downsampling manipulation showed that the amount of data cap-

tured can have a major (up to a 4-fold change in activity slope; Table 1) and unpredictable effect on how LAP activities are characterized. Furthermore, high spatial heterogeneity at small scales makes interpolating between observed LAP activities prone to error, even in relatively static environments. High sampling rates and very consistent methods may be required for accurately characterizing enzyme activity. This conclusion highlights the need to account for small-scale variability in aquatic enzyme activity to avoid distorting underlying patterns.

Acknowledgements. We thank IMCS researchers R. Chant, J. Reinfelder, and O. Schofield for the opportunity to apply EAAS to the LaTTE program and for technical discussions. Additional thanks are owed to J. Reinfelder for providing the nitrate concentration data, to the late Garth Klein for help developing the EAAS, and to the crew of the R/V Oceanus and R/V Cape Hatteras for operating the ships that let us collect the data in the first place. The input of three anonymous reviewers greatly helped the quality of this paper. Supported by NSF Biocomplexity IDEA (DBI 0216154) to J.W.A., G. P. Klinkhammer, and R. J. Chant.

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Editorial responsibility: Jed Fuhrman, Los Angeles, California, USA

Submitted: July 30, 2019; Accepted: March 3, 2020
Proofs received from author(s): April 27, 2020