Animal regeneration and microbial retention of nitrogen along coastal rocky shores

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Abstract. Animal contributions to ecosystem productivity via nutrient regeneration are increasingly recognized as significant for ecosystem productivity. The paradigm in coastal upwelling systems, in contrast, remains centered around the idea that nutrient supply is extrinsic and sourced from deep water. Here we tested for both animal contributions to nitrogen regeneration and microbial retention of nitrogen along a gradient of animal abundance in Washington State, USA. Using inferences from the concentration and isotopic composition of nitrogen forms, as well as the stable nitrogen isotopic composition of particulate organic matter, and plant and animal tissue, we found increased ammonium as well as greater stable nitrogen isotope values in these organisms in areas of increased animal abundance. We further tested the effect of the nearshore biota by comparing it with an immediately adjacent offshore area, and found an enriched $\delta^{15}N$ value for nitrate and particulate organic matter. Further, an analysis of the dual isotopes of $\delta^{15}N$ and the $\delta^{18}O$ of nitrate indicated increasing microbial processing in the nearshore. Isoscapes, or the spatial pattern of stable isotopes, revealed that animal and microbial processing of nitrogen leave a consistent signature and are thus a previously overlooked and essential component of nearshore productivity.

Key words: animal excretion; California Current Large Marine Ecosystem; California mussel, Mytilus californianus; *isoscapes; nitrification; nutrient retention; productivity; remineralization; rocky intertidal community; stable isotope analysis; Tatoosh Island, Washington, USA.*

Introduction

Nitrogen regeneration by animals has been shown to contribute to primary production and ecosystem function across a diversity of ecosystems, including lakes (Schindler et al. 2001, Vanni 2002), streams (McIntyre et al. 2008), African grasslands (McNaughton et al. 1997), and coral reefs (Meyer et al. 1983, Layman et al. 2011). In contrast, the dominant paradigm for marine ecosystems characterized by coastal upwelling and some of the highest primary productivity values in the ocean (Mann 1973), is that productivity is fueled by upwelled nitrogen in the form of nitrate. Although the importance of regenerated nitrogen from animal metabolism has been quantified in some open marine systems (Dugdale and Goering 1967, Eppley and Peterson 1979, Bronk et al. 1994), we know little about the importance of regenerated nitrogen to coastal productivity. The assumption that upwelling is the sole driving factor for productivity ignores the high animal abundance in nearshore areas that is likely to contribute to productivity via nutrient regeneration. Given recent and precipitous declines in animal abundance in coastal environments (Lindberg et

al. 1998, Worm et al. 2006), it is imperative to identify the non-trophic role that animals might play in these systems. We expect that animal abundance and metabolic rate are key factors determining their contribution to regenerated nitrogen.

There are several lines of evidence that nearshore environments have significant animal sources of nitrogen. Fish elevate nutrient concentrations (Meyer et al. 1983, McIntyre et al. 2008), and bird aggregations can create significant nutrient hotspots (Wootton 1991, Post et al. 1998). It follows that along rocky shores, abundant invertebrates may also be significant sources of nutrients. As evidence, increased ammonium concentrations have been observed over California mussel beds (Aquilino et al. 2009). Similarly, high densities of animals in tidepools, especially mussels, are associated with the accumulation of ammonium and enhancement of algal productivity (Bracken 2004, Pfister 2007) and algal diversity (Bracken and Nielsen 2004). Further, a shoreward peak in ammonium has been observed at Tatoosh Island, Washington, an area of extensive mussel-covered rocky benches, declining immediately away from the island (Pfister et al. 2007). Given that animals are a quantitatively significant source of nitrogen, there should be areas where animal-derived nutrients support primary production. Heavily exploited locales or those that do not favor animal survival might

be expected to show reduced nutrient cycling and retention, just as less exploited locales with increased animal densities might have enhanced local primary production.

Nitrogen regenerated as ammonium (NH_4^+) by animals can have multiple fates. First, NH_4^+ is the preferred source of nitrogen for primary producers and thereby can be taken up directly (Phillips and Hurd 2004, Bracken and Stachowicz 2006). Second, NH_4^+ is the substrate for microbial nitrification to nitrite (NO_2^-) and nitrate (NO_3^-), leading to the regeneration of these nitrogen forms (Ward 1990) that can also be utilized by diverse phototrophs and chemolithotrophs. Because these multiple nutrient gain and loss processes occur simultaneously in natural systems, quantifying them has been experimentally challenging. Although there are strong inferences from tracer additions in natural systems, e.g., streams (Peterson et al. 1997) and soils (Drake et al. 2006), evidence of large-scale patterns of nitrogen regeneration and retention is scarce (but see Epstein et al. 2012). In this study, we use inferences from stable isotopes of nitrogen and carbon over a gradient of animal abundance along a productive rocky shore and in both nearshore and offshore waters to assess how the nearshore biota contributes to nitrogen regeneration and retention.

Natural variations in the stable isotopes of nitrogen and carbon have been used as powerful in situ tracers of biological processes through their capacity to alter isotopic ratios (Lajtha and Michener 1994, Fry 2006). First, nitrogen assimilation by phototrophs and ammonium oxidization by microbes typically discriminates against the heavier isotope, ^{15}N (e.g., Wada and Hattori 1991, Montoya and McCarthy 1995, Needoba et al. 2003). Second, subsequent NO_2^- oxidation to NO_3^- is known to result in an unusual inverse discrimination whereby the intermediate NO_2^- has a lower $\delta^{15}N$ value relative to both NH_4^+ and NO_3 (Casciotti 2009). Thus, a low $\delta^{15}N$ value for nitrite ($\delta^{15}N_{NO2}$) is indicative of active nitrification, even though nitrite is typically a small component of the total dissolved inorganic nitrogen pool. The net effect of animal excretion, microbial processing, and nitrate assimilation should result in an increase in the $\delta^{15}N$ value of water-column ammonium and nitrate. Hence, autotrophs in systems with strong nitrogen regeneration will have increased $\delta^{15}N$ values relative to organisms receiving their nitrogen from upwelling processes alone, because $\delta^{15}N_{NO3}$ values for upwelled water are relatively lower and set at the value from deep, upwelled water. Third, the dual isotopic composition of nitrate allows for the use of nitrogen ($\delta^{15}N_{NO3}$) and oxygen ($\delta^{18}O_{NO3}$) stable isotope analysis to diagnose the relative roles of photo-assimilation vs. regeneration. Last, stable isotopes of carbon also reveal information on biological processing. Deeper water has a relatively low $\delta^{13}C$ value for dissolved inorganic carbon ($\delta^{13}C_{DIC}$) (Emerson and Hedges 2008), and photosynthetic plankton reflect the $\delta^{13}C$ values of the dissolved inorganic carbon of their surrounding water (Freeman and Hayes 1992). Therefore, the $\delta^{13}C$ of phototrophs provides an additional indicator of exposure to newly upwelled water.

We tested whether geographic patterns in $\delta^{15}N$ values for the different chemical forms of inorganic nitrogen in seawater (NH_4^+, NO_2^-, NO_3^-) and major food web compartments (plankton, a marine angiosperm, and a filter-feeding mussel) support a hypothesis of increased nitrogen regeneration in areas with increased abundance of animals. We censused along a natural gradient of animal abundance, from the outer coast of Washington State at Tatoosh Island, hosting marine mammals, nesting seabirds, and benches of California mussels, eastward through the Strait of Juan de Fuca. We also contrasted locales immediately at the shore, and thus in proximity to diverse and abundant invertebrates and vertebrates, with those 2–3 km offshore, where animal abundance was reduced. We tested whether regeneration of nitrogen was greater in outer coast areas where animals were more abundant, and whether regeneration of nitrogen and retention via microbial processing was greater nearshore than offshore. If nitrogen regeneration and retention is greater at animal-rich outer coast sites, we expect (1) increased $\delta^{15}N$ values of plankton (particulate organic matter or POM), surfgrasses, and mussels toward the outer coast, (2) increased $\delta^{15}N$ values for nitrate and ammonium, and decreased $\delta^{15}N_{NO2}$ values, (3) deviations from the 1:1 relationship between $\delta^{18}O_{NO3}$ and $\delta^{15}N_{NO3}$ values, (4) deviations from a linear relationship between $\delta^{15}N_{NO3}$ and the amount of nitrate assimilation, and (5) surfgrass $\delta^{13}C$ values decoupled from upwelling patterns. Similarly, in our nearshore vs. offshore comparison, we expect greater $\delta^{15}N$ values for nearshore nitrate and POM. Overall, the spatial patterns of stable isotope values, or isoscapes, were consistent with these conditions and are evidence for processes that regenerate and retain nitrogen in areas of higher animal abundance and in the nearshore.

MATERIALS AND METHODS

We chose four shore-based sites that spanned a gradient of California mussel (*Mytilus californianus*) abundance from the outer coast of Washington State's Tatoosh Island (TI; 48.39° N, 124.74° W) eastward into the Strait of Juan de Fuca. Second Beach (2B; 48.23° N, 124.40° W) was 2 km east of Tatoosh, Slip Point (SP; 48.26° N, 124.25° W) 40 km eastward, and Observatory Point (OB; 48.15° N, 123.64° W) was farthest east at 85 km. The relative abundance of mussels was based on the prevalence of rocky shore within 5 km on either side of each of the four sites shown in aerial surveys of the shoreline (Washington Shore Zone Inventory, *available online*).[5] There was a strong gradient of rocky shore

[5] http://www.dnr.wa.gov/ResearchScience/Topics/AquaticHabitats/Pages/aqr_nrsh_inventory_projects.aspx
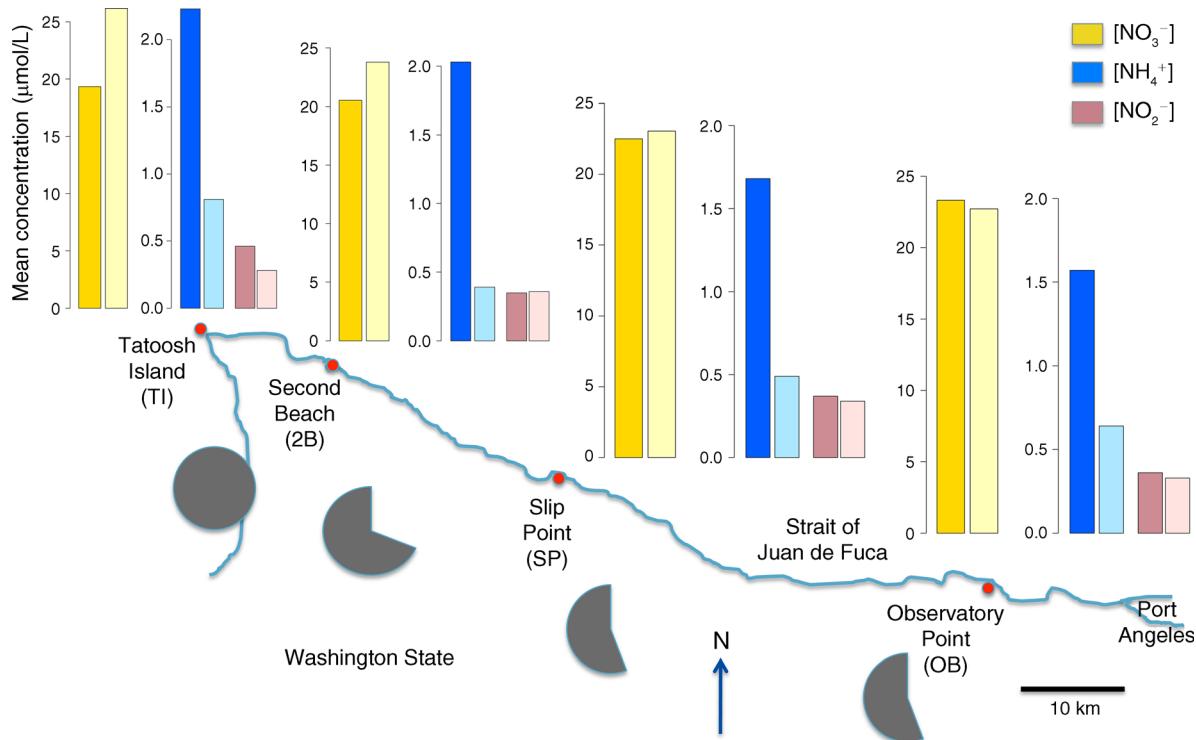
FIG. 1. Location of the four study sites along the Strait of Juan de Fuca in Washington State, USA; the distance scale bar is at the bottom right. The gray-shaded pie-circles represent the percentage of substrate within 5 km of each site that was suitable rocky bench habitat for mussels, based on aerial surveys by the Washington Department of Fish and Wildlife (see footnote 5). For each site, mean concentrations of nitrate $[NO_3^-]$ (left vertical axis) and ammonium $[NH_4^+]$ and nitrite $[NO_2^-]$ in seawater (right vertical axis) are pooled over all dates and years (finer resolution nutrient concentrations are shown in Appendix A; ANOVA analyses are in Appendix C). Darker hues are shore concentrations; lighter hues are offshore concentrations. Ammonium was greater at the shore than offshore at all sites; Tatoosh ammonium was greater than Observatory Point (two-way ANOVA and Tukey HSD). Nitrate concentrations were greater in the offshore compared with the nearshore only at Tatoosh (site × shore interaction, two-way ANOVA and Tukey HSD). Ammonium tended to be highest in August. Nitrite was greater at Tatoosh than other sites, and only here did the shore have significantly higher concentrations than offshore (site × shore interaction, two-way ANOVA and Tukey HSD).

from the west to the east. Within 5 km on either side of Tatoosh Island, 100% of habitat was rocky bench suitable for mussel populations. To the east, the quantity of suitable habitat was reduced to 70% of the total at Second Beach and 60% at Slip Point and Observatory Point (Fig. 1). Corresponding to each of these shore-based sites, we also sampled a site 1–3 km offshore at 60–70 km depth (for TI, 48.432° N, 124.73° W; for 2B, 48.37° N, 124.57° W; for SP, 48.27° N, 124.25° W; for OB, 48.17° N, 123.62° W). The offshore sites served as a comparison within locales for the effect of nearshore biota on nutrient dynamics.

Seawater was filtered through a 47-mm GF/F filter (Whatman; Sigma-Aldrich, St. Louis, Missouri, USA) and immediately frozen for later analysis of nutrient concentrations (at University of Washington Chemical Oceanography Lab) and stable nitrogen isotopes of nitrate and ammonium. For shore-based sampling, a 2.5-cm hose was placed into the water and hand-pumped through a Pall 47-mm filter holder (Pall Corporation, Port Washington, New York, USA). Nitrogen stable

isotopes of ammonium were measured according to a modified version of the NH_4^+ oxidation method detailed in Zhang et al. (2007). Briefly, NH_4^+ is oxidized to nitrite using a hypobromite solution and then reduced to N_2O using a sodium azide-acetic acid reagent before analysis on an IRMS (isotope ratio mass spectrometer). The published method was modified with respect to removal of preexisting NO_2^- prior to hypobromite addition. Instead of sulfanilic acid addition followed by boiling, we added 340 μL 20 mmol/L sulfamic acid together with 10 μL 10% HCl to a 20-mL sample volume, which was allowed to react for 12 h at room temperature. A second modification was the addition of 6 mol/L HCl to reduce the pH of the sample below 7 prior to the addition of the azide-acetic acid reagent. This latter reagent was made up using 20% acetic acid as opposed to 100% acetic acid. All other procedures were as detailed in Zhang et al. (2007). The stable isotope ratios of nitrogen ($\delta^{15}N_{NO3}$) and oxygen ($\delta^{18}O_{NO3}$) in nitrate were measured by cadmium reduction to NO_2^-, followed by reaction with azide to N_2O (McIlvin and Altabet 2005), as modified

by Ryabenko et al. (2009). Isotope determinations were made at the University of Massachusetts Dartmouth using a GV IsoPrime IRMS (Manchester, UK), a custom purge-trap sample preparation system, and a CTC PAL autosampler (Leap Technologies, Carrboro, North Carolina, USA). External precision for isotope analysis, including both sample preparation and IRMS analysis, was better than ±0.5‰.

Stable nitrogen isotope values for water column plankton were analyzed by retaining the GF/F Whatman filter (0.7-μm pore size) used in water sampling. The volume pumped ranged from 1.0 to 9.7 L, depending upon how rapidly the filter clogged. Filters were dried and one-fourth of the area of the filter was packed into a 5×9 mm tin capsule prior to isotopic analysis. Samples were analyzed using a Costech 4010 Elemental Analyzer combustion system (Costech, Valencia, California, USA) coupled to a Thermo DeltaV Plus IRMS (Thermo Fisher Scientific, Waltham, Massachusetts, USA) via a Thermo Conflo IV interface (at the University of Chicago). The reproducibility was 0.11‰ for ^{13}C and 0.17‰ for ^{15}N. Some samples were run on another Elemental Analyzer coupled to a Thermo DeltaXP Advantage IRMS via a Thermo Conflo III interface (at Yale University); reproducibility between the two laboratories on identical samples was 0.6‰.

The surfgrass *Phyllospadix scouleri* (henceforth surfgrass) was collected in the low intertidal at each site ($n = 6$–10). Tissue was collected from plants at a tide level from −0.3 m to 0.0 m; several blades 3 cm long were cut from the basal meristematic region just distal to the sheath and placed in aluminum foil envelopes, dried at 50°C for 48 h, and then ground to a fine powder with a 3-mm stainless steel ball in a Genogrinder (Spex Sample Prep., Metuchen, New Jersey, USA) and packed into a 3.5×5 mm tin capsule prior to isotopic analysis.

Mussel tissue was sampled by excising the adductor muscle only and processing it in the identical way as for surfgrass. We chose the adductor muscle because it provided a tissue type that we could sample consistently and without contamination, and because the muscle tissue has been demonstrated to provide a low-variance, time-integrated signal (Cabanellas-Reboredo et al. 2009).

All shore-based sampling was done approximately monthly in April or May and then June, July, and August of 2009, 2010, and 2011 when low tides allowed access to sites and organisms. We sampled 1–2 km offshore (depth of 60–70 m) of each site in June, July, and August of 2010 and 2011 from the R/V *Clifford Barnes*. For each site, a 12-sample CTD array (Seabird Electronics, Bellevue, Washington, USA) with 10-L Niskin bottles (General Oceanics, Miami, Florida, USA) was used. Casts were made to depths of ~60 m with seawater collected via Niskin bottles at four depths (surface, 10, 20, and 30 or 40 m) in triplicate; seawater was collected and filtered to obtain dissolved nutrients, natural abundance of nitrogen isotopes in nitrate, and POM using the same methodology used at the shore-based sites, except that each Niskin bottle was simply gravity filtered. We were unable to estimate the $\delta^{15}N$ values for ammonium in offshore waters because the ammonium concentrations were too low.

In total, we had multiple determinations of nutrient concentrations and stable nitrogen isotope values spanning four sites, multiple time periods, and both nearshore and offshore habitats. We used multifactor ANOVA to test our expectations, including whether sites differed, whether there was seasonal change, and whether onshore values differed from offshore. An ANOVA framework further allowed us to test for statistical interactions, such as whether differences among sites depended upon sampling date. All statistic analyses were done using R v. 2.15 (R Development Core Team 2013).

If water column factors are determinants for the stable isotope values of nearshore biota, then we expect correlates among sea surface temperature (SST), upwelling, and nitrate concentrations, and the $\delta^{15}N$ values from plankton, surfgrass, and mussels, and the $\delta^{13}C$ values from dissolved inorganic carbon. Temperature data were collected at each site during the time of collection with a Hach DS5 (Hach, Loveland, Colorado, USA) for Tatoosh Island (e.g., Wootton and Pfister 2012), or with a Hach MS5 or a HOBO data logger (Onset, Wareham, Massachusetts, USA) at the other three sites. Upwelling data (daily, monthly) for 48° N, 125° W is an index based on wind stress measurements (*available online*).[6] We tested for relationships among environmental variables and our stable isotope values using linear mixed-effect models where date was treated as a random effect.

RESULTS

The mean concentration of ammonium was approximately three times greater onshore than offshore at all sites (Fig. 1), with the highest concentrations tending to be at the shore at Tatoosh (Appendices A–C). Ammonium concentrations showed strong seasonal patterns, with August concentrations greater than June concentrations overall (two-way ANOVA and Tukey test). At Tatoosh, nitrate concentrations were reduced by 26.2% at the shore and showed a disparity with offshore concentrations (based on two-way ANOVA and Tukey test); also note the site × shore interaction in Appendix C. Nitrite was always twice the concentration at the shore vs. offshore (Fig. 1 and Appendix A), but showed the highest concentrations onshore at Tatoosh (Appendices A–C).

Isoscapes along a shore-based gradient of mussel abundance

Surfgrass $\delta^{15}N$ values showed an overall pattern of greater $\delta^{15}N$ values westward toward the outer coast (Fig. 2a; see Appendix D), as well as an overall seasonal

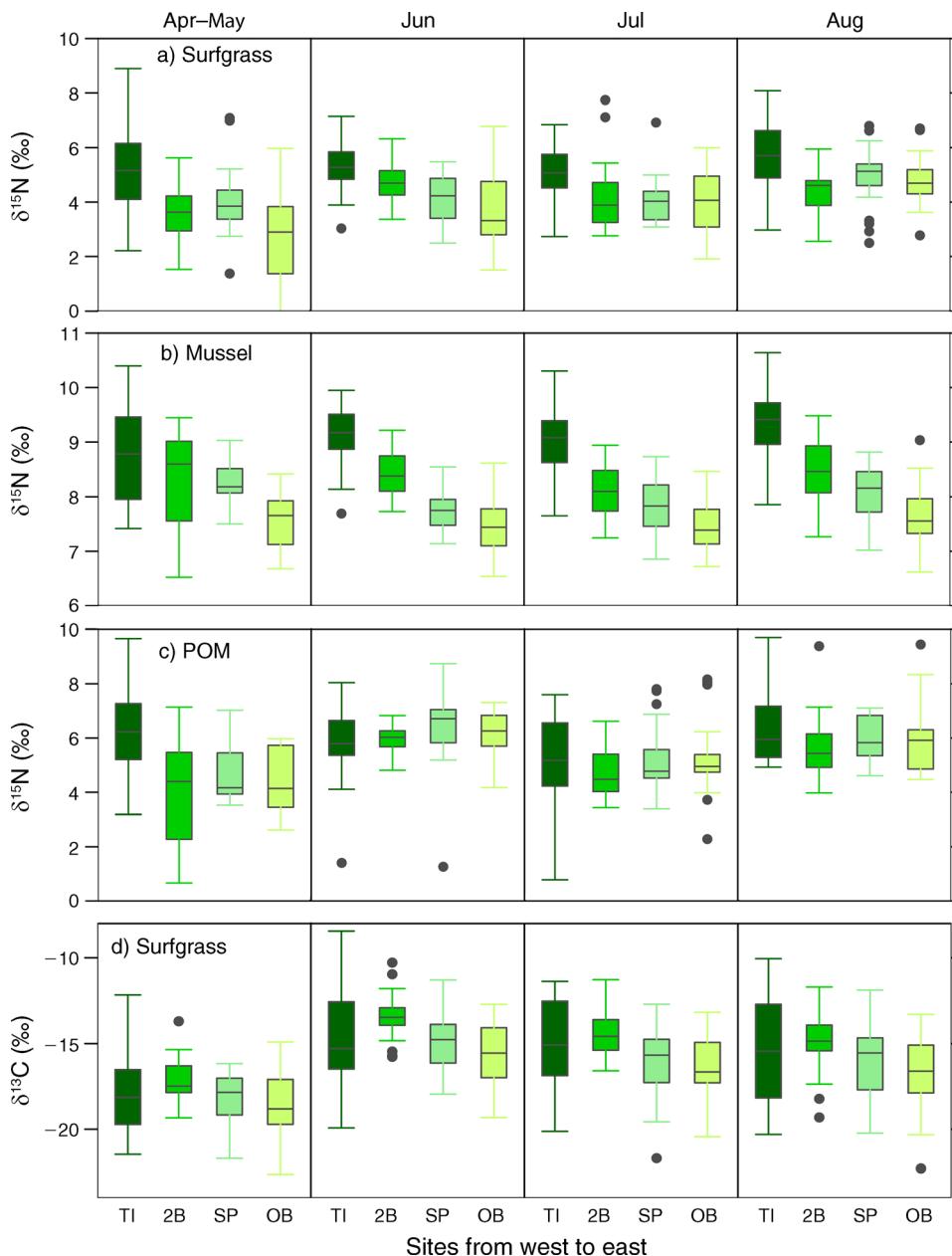[6] http://www.pfeg.noaa.gov/products/PFEL/modeled/indices/upwelling/upwelling.html

Fig. 2. Isotope $\delta^{15}N$ values of (a) surfgrass (*Phyllospadix scouleri*), (b) mussels (*Mytilus californianus*), and (c) POM (particulate organic matter), and (d) $\delta^{13}C$ values of surfgrass, all measured at each of the four shore-based sites in all months of collection. Years (2008–2011) have been pooled and showed highly concordant patterns. For panel (a) surfgrass, $\delta^{15}N$ differed by site and month. An interaction between site and month indicated that the seasonal change diminished into the summer (Appendix D; $n = 24$ samples) and was supported by one-way ANOVAs and Tukey tests for each site showing that only TI had greater $\delta^{15}N$ in July and August. In panel (b) mussels showed a consistent pattern of increased $\delta^{15}N$ value in the west during all months. The significant interaction term between site and month was the result of an increasing mussel $\delta^{15}N$ at TI only (one-way ANOVAs and Tukey tests). Each bar represents $n = 30$–36 mussel samples. For filtered POM in panel (c), the sites and months differed, with ^{15}N enrichment greatest for TI, SP, and OB and the month of August, but there was no interaction. Each bar represents $n = 8$–16 samples. In panel (d), the $\delta^{13}C$ of surfgrass was greater at TI and 2B compared with SP and OB, and these values increased as the season progressed (one-way ANOVAs and Tukey test). Horizontal lines in the boxplots are medians, the box denotes the 25th–75th percentiles; the vertical dashed lines represent 2 SD, and single points are outlier data that fall beyond 2 SD. All means and standard errors are in Appendix B; two-way ANOVA results are in Appendix D.
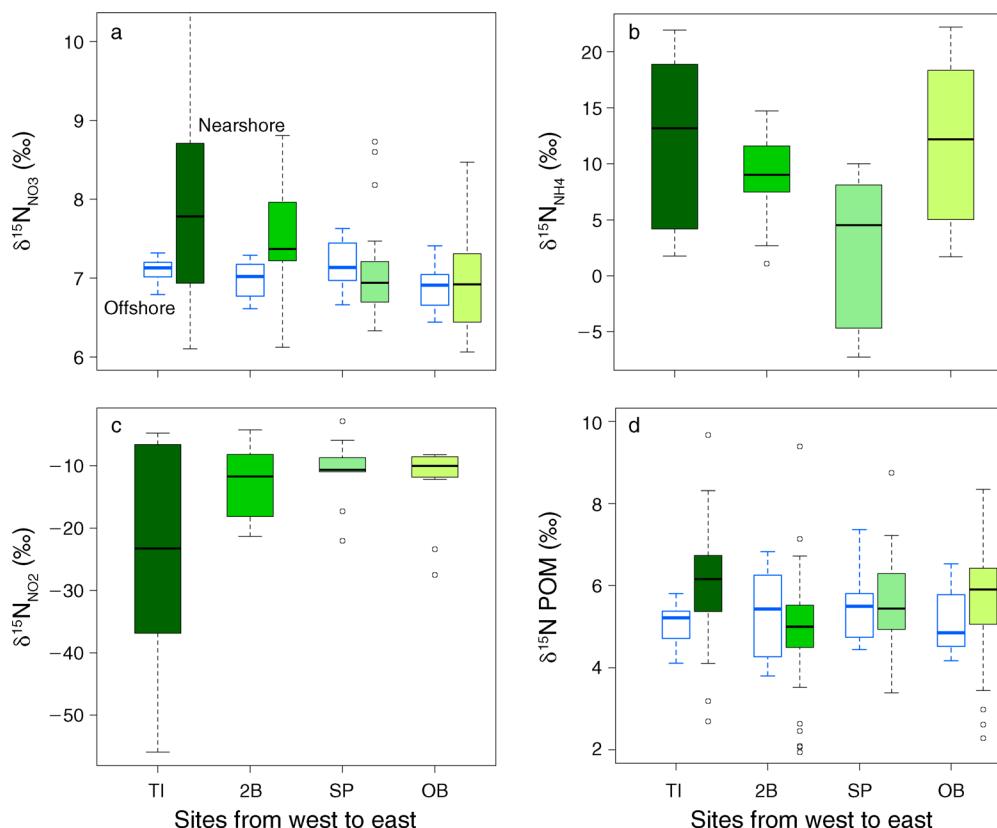
FIG. 3. Nitrogen isotopes of dissolved N forms and suspended POM. (a) The $\delta^{15}N_{NO3}$ of seawater collected at each of the four sites, both onshore and offshore. The offshore collections (open boxplots) did not differ, but the shore-based collections (shaded boxplots) showed significantly greater $\delta^{15}N_{NO3}$ values at Tatoosh Island and Second Beach ($F_{3,97} = 8.025$, $P < 0.001$ and Tukey test). (b) The nitrogen isotopes of ammonium ($\delta^{15}N_{NH4}$), although highly variable, showed that site SP had a lower $\delta^{15}N_{NH4}$ than the other sites ($F_{3,68} = 8.127$, $P < 0.001$ and Tukey test). (c) The nitrogen isotopes of nitrite ($\delta^{15}N_{NO2}$) were significantly lower at Tatoosh Island compared with other sites ($F_{3,43} = 4.130$, $P = 0.012$ and Tukey test). (d) The $\delta^{15}N$ of POM was greater at Tatoosh Island ($F_{3,220} = 3.720$, $P = 0.012$), and showed a nearly statistically significant interaction between the four sites and onshore or offshore location ($F_{3,220} = 2.466$, $P = 0.063$). Nearshore sites are in green, with the darkest hue at TI and lightening to the east. All means and standard errors are given in Appendix B. Boxplot components are as in Fig. 2.

pattern in which $\delta^{15}N$ values increased through the summer at all sites. The strength of the geographic pattern changed seasonally, however, as indicated by the significant interaction between the two factors (Appendix D). By July and August, only TI surfgrass had greater $\delta^{15}N$ than the three sites to the east (one-way ANOVA and Tukey tests). Mussel $\delta^{15}N$ values consistently showed higher values to the west in all months, although a seasonal increase was only significant at Tatoosh (one-way ANOVA and Tukey tests). POM did not show these strong differences found for benthic phototrophs and filter feeders, although POM also showed higher ^{15}N enrichment at TI, SP, and OB as compared to 2B, and during the month of August as compared to the other months (Fig. 2c).

The $\delta^{15}N$ of dissolved inorganic nitrogen showed spatial patterns in which the $\delta^{15}N_{NO3}$ value increased westward to the outer coast and was almost 1.0‰ higher in the nearshore relative to offshore at Tatoosh Island (Fig. 3a). The isotopic signature of ammonium

($\delta^{15}N_{NH4}$) in the nearshore was lowest at SP (Fig. 3b). Indeed, the variability in the $\delta^{15}N_{NO3}$ and $\delta^{15}N_{NH4}$ values increased from east to west, with Tatoosh Island showing the greatest variability. In contrast, the $\delta^{15}N_{NO2}$ values were significantly lower at this location (Fig. 3c).

A 1:1 relationship between the $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ values is expected if photoassimilation is the only process that affects nitrate (Granger et al. 2004, Wankel et al. 2006). For our study, we can set the intercept for the expected relationship at $\delta^{15}N_{NO3} = 6.7$‰ and $\delta^{18}O_{NO3} = 2.9$‰, based on our measurements at 40 m depth at the offshore Tatoosh locale, and thus the value for deep, upwelled water in this region. Many of our data fall above this expected relationship by 0.8‰ to 1.0‰ at all nearshore sites and have a slope of 1.24 (SE = 0.05; Fig. 4 inset), suggesting that $\delta^{15}N_{NO3}$ values are consistently lower than expected (Fig. 4).

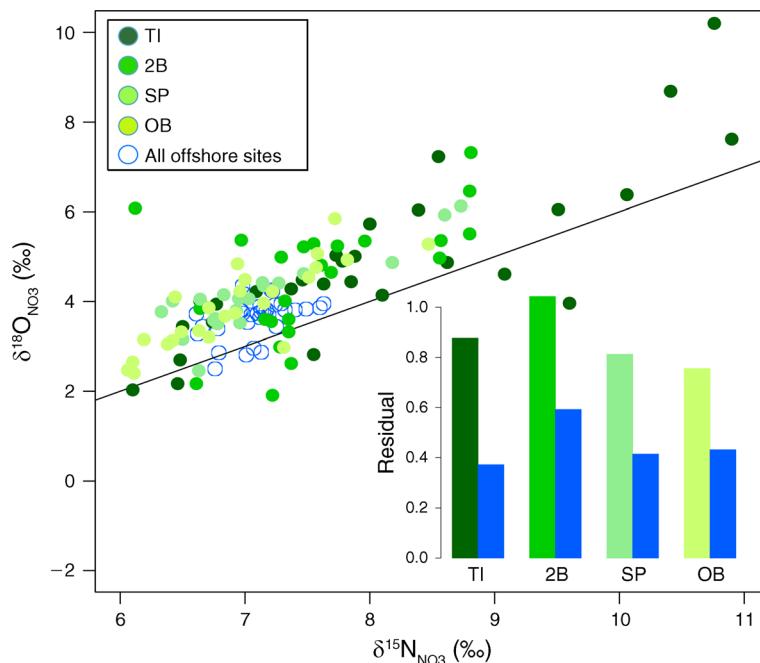A linear relationship between $\delta^{15}N_{NO3}$ and the relative use of nitrate (as the $\ln([NO_3]_t/[NO_3]_{max})$ is also

FIG. 4. Dual isotopes of nitrate ($\delta^{18}O_{NO3}$ and $\delta^{15}N_{NO3}$), showing the relationship between the oxygen and nitrogen isotopes of nitrate greater than 1:1 (line shown) expected when assimilation is the only process that affects nitrate (slope = 1.24, SE = 0.05); green circles are the four nearshore sites; open blue-outlined circles are for all offshore sites pooled. Accordingly, $\delta^{15}N_{NO3}$ was consistently lighter than expected, e.g., a positive residual from the expected relationship. Inset: The mean absolute deviation from the 1:1 line varied by locale and whether the sample was offshore (blue) or nearshore (green, different hues by site). Offshore samples were significantly closer to a 1:1 relationship (ANOVA, $P = 0.009$), while locales did not differ ($P = 0.697$), and there was no significant interaction ($P = 0.934$). The color key is as in Fig. 3; means and standard errors are in Appendix B.

expected if uptake is the sole determinant of isotopic composition, with the slope of this Rayleigh plot reflecting the magnitude of isotope fractionation (Altabet 2005). The data are described by a mean slope of −0.59 (SE = 0.08), indicating fairly low fractionation but with high variance around the relationship ($r^2 = 0.10$). Nitrate samples unaffected by assimilation (e.g., near the $[NO_3]_{max}$ of 30.09 μmol/L sampled from offshore Tatoosh deep waters at 40 m), had $\delta^{15}N_{NO3}$ values near the expected 6.7‰ (Fig. 5). The estimated relationship has a shallow slope due to a large cluster of data points in the lower right quadrant. Deviations around that estimated linear relationship differed with locale, with the greatest deviations at Tatoosh Island (ANOVA, $P < 0.001$; Fig. 5 inset).

Stable isotopes of carbon in surfgrass, an indication of the source of dissolved inorganic carbon (DIC) that these marine angiosperms utilize, showed patterns similar to that of the $\delta^{15}N$ of surfgrass, with greater $\delta^{13}C$ values westward at TI and 2B compared with SP and OB. The $\delta^{13}C$ values of surfgrass also increased ~1.5‰ seasonally through the summer months (Fig. 2d; Appendix B, Tukey test).

Isoscapes in the nearshore vs. offshore

Suspended POM in the nearshore was 1.3‰ higher compared with offshore at Tatoosh Island, whereas the POM $\delta^{15}N$ values nearshore and offshore were statistically indistinguishable at the three other sites (Fig. 3d). Stable nitrogen isotopes of nitrate ($\delta^{15}N_{NO3}$) resulted in mean values of 7.5‰ at our shore sites and 6.9‰ at the surface at offshore sites with strong enrichment patterns in the nearshore sites of Tatoosh Island and Second Beach (Fig. 3a).

Offshore patterns of dual fractionation for $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ were tightly clustered and showed small deviations from the 1:1 expected relationship, indicating assimilation as a dominant process, whereas the nearshore samples (Fig. 4) deviated as much as 4‰ from the 1:1 expected line. Similarly, the $\delta^{15}N_{NO3}$ values as a function of nitrate depletion for the offshore samples showed relatively little deviation from a fitted line and most clustered relatively tightly near the region of little nitrate assimilation, whereas shore-based Tatoosh samples were highly divergent from the fitted relationship (Fig. 5).

Environmental correlates of isoscapes

SST, upwelling, and nitrate concentrations were correlated with stable isotope values, although the sign of the relationship could differ depending on the variable analyzed. The POM $\delta^{15}N$ values from filtering nearshore water were negatively related to SST, but mussel, surfgrass, and $\delta^{15}N_{NO3}$ values were positively

related to SST using linear mixed-effects models with date as a random effect (Table 1). POM $\delta^{15}N$, $\delta^{15}N_{NO2}$, and $\delta^{15}N_{NH4}$ values were positively related to nitrate concentrations through time, whereas $\delta^{15}N_{NO3}$ values had the expected negative relationship with nitrate (e.g., Fig. 5). Mussel $\delta^{15}N$ values also correlated negatively with nitrate concentrations (Table 1). Surfgrass $\delta^{13}C$ values were related positively to SST and upwelling (Table 1) and positively with ammonium concentration.

DISCUSSION

Inferences from isoscapes

Evidence for biological processing and reprocessing of nitrogen, particularly in the nearshore environment, was indicated by: (1) higher ammonium concentrations nearshore and at the westerly sites (Appendix A); (2) increased $\delta^{15}N$ values of mussels, surfgrass, and plankton at the most westerly sites (Fig. 2); and (3) higher $\delta^{15}N$ of plankton in the nearshore at Tatoosh (Fig. 3d). Although the quantity and isotopic composition of nitrogen in the ocean has many determinants (Altabet 2005), seawater and the biota revealed patterns consistent with nitrogen assimilation, regeneration, and retention.

The processing of inorganic nitrogen components also differed greatly between the nearshore and the offshore, as indicated by stable nitrogen isotope analysis. First, dual nitrogen and oxygen isotopes of nitrate showed high deviation from a 1:1 line, indicating processes in addition to photoassimilation. Nitrification is expected to generate lower values of $\delta^{15}N_{NO3}$ relative to $\delta^{18}O_{NO3}$ (Lehmann et al. 2004, Wankel et al. 2007), a result consistent with the slope in excess of 1.0 and the especially large deviation from the 1:1 line in samples from TI and 2B (Fig. 4). Thus, points above the 1:1 line in Fig. 4 indicate microbial ammonium uptake and transformation to nitrate (e.g., nitrification; Granger et al. 2004, 2010). Some sampling periods also show $\delta^{15}N_{NO3}$ values significantly greater than expected, a result that might be attributed to uptake by heterotrophic bacteria (Wawrik et al. 2012). Processes other than phototrophic assimilation are also suggested in the fractionation plot of Figs. 4 and 5, where high variation in $\delta^{15}N_{NO3}$ values is seen at more westerly locales. The $\delta^{15}N_{NO2}$ values provide further evidence that microbial nitrogen transformations are important, given that $\delta^{15}N_{NO2}$ values are significantly lower at Tatoosh (Fig. 3c). Purely assimilatory processes would yield higher $\delta^{15}N_{NO2}$ values, whereas a decrease in $\delta^{15}N_{NO2}$ is indicative of microbial processing via nitrification (Horrigan et al. 1990, Casciotti 2009).

The strong signal of assimilatory and regenerative processes that we saw in the nearshore was not as pronounced in the offshore. The $\delta^{15}N$ values of both POM and NO_3^- were lower offshore than the nearshore at Tatoosh, as was $\delta^{15}N_{NO3}$. The dual isotopes of nitrate were tightly correlated and deviated relatively little from the 1:1 line expected from assimilation only. Concen-

trations of nitrate were greater offshore, but ammonium and nitrite were comparatively lower. In other words, across all of the data sources that we utilized for an offshore–onshore comparison, our data indicated greatly increased processing in the nearshore.

There were notable differences in nitrogen processing suggested among the four sites that we studied, with increased $\delta^{15}N$ values of nitrate, surfgrass, and mussels from east to west and with increasing biomass of phototrophs, invertebrates, and vertebrates at these outer coast areas.

This "isoscape" approach (sensu Rascher et al. 2012) revealed higher values for the $\delta^{15}N$ of plant, animal, POM, and nitrate in seawater in the nearshore and with greater animal abundance, consistent with evidence of biological processing that differed on spatial scales related to animal abundance.

Our data are consistent with biotic processing of nitrogen, but also suggest control of stable isotope composition by oceanographic factors. We found some relationships between isotope values and oceanographic variables such as sea surface temperature, upwelling, and nitrate concentrations that support the importance of oceanographic influence (Table 1). Increasing upwelling is associated with decreased $\delta^{15}N_{NO3}$ values. Upwelled water in the region has a relatively low $\delta^{15}N_{NO3}$ value of 6.7‰; upwelling would keep this value low and assimilation and regeneration would cause it to increase. Stable isotope values had differing relationships with SST. Mussel $\delta^{15}N$ decreased with dropping water temperatures, but POM $\delta^{15}N$ values increased. Nitrate, primarily supplied via upwelling, was an explanatory variable for POM, mussel, and dissolved inorganic nitrogen values. Ammonium concentrations were associated with increasing $\delta^{15}N$ values of surfgrass and nitrate, also suggesting the importance of local regeneration. Although only correlative, our analyses indicated both oceanographic and local influences on stable nitrogen isotope values.

Because surfgrass as a phototroph uses dissolved inorganic carbon, the $\delta^{13}C$ of surfgrass can indicate temporal and spatial variations in DIC (dissolved inorganic carbon) source. Deeper water has a relatively lower $\delta^{13}C$ value (Emerson and Hedges 2008), whereas assimilation and regeneration of carbon increase the $\delta^{13}C$ value in surface waters or aged upwelled water. The $\delta^{13}C$ of surfgrass showed distinct geographic patterns that mirrored those of nitrogen (Fig. 2d). Greater $\delta^{13}C$ values in surfgrass were observed in a westerly direction to Tatoosh and as the growing season proceeded. We interpret the lower surfgrass $\delta^{13}C$ values in the months of April and May to be a signature of upwelling, yielding low values at the initiation of the growing season, followed by higher $\delta^{13}C$ values as regeneration and retention became more important. Surfgrass carbon isotope composition can also change depending upon the carbon source used by the plant; bicarbonate yields a greater tissue $\delta^{13}C$ value than CO_2 as a carbon source
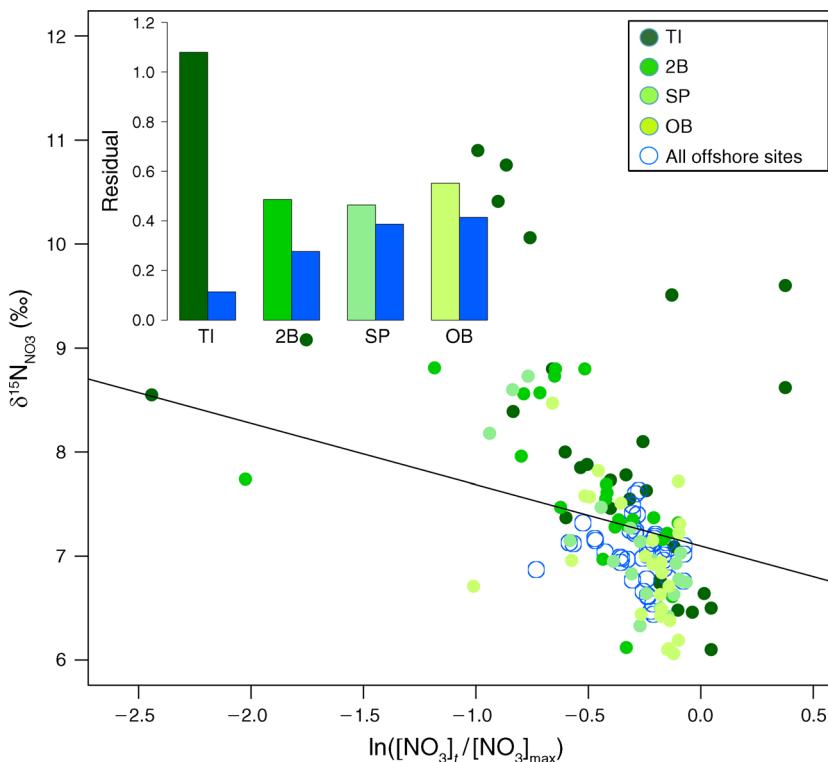
Fig. 5. Isotope $\delta^{15}N_{NO3}$ as a function of nitrate assimilation (ln-transformed $[NO_3]_t/[NO_3]_{max}$) across all locales in surface waters nearshore (green) and offshore (blue), where t is the variable time. The slope estimates the strength of isotope fractionation. The best fit line is shown ($y = -0.59x + 7.10$ (slope SE = 0.08; $r^2 = 0.01$, $F_{1, 146} = 17.00$, $P < 0.001$). Inset: Mean absolute deviation from the estimated line was greatest nearshore relative to the offshore (ANOVA, $P < 0.001$). The color key is as in Fig. 3; means and standard errors are in Appendix B.

(Hemminga and Mateo 1996), and surfgrass may change to this source under conditions of strong CO_2 drawdown. We thus controlled for any variation due to carbon source by collecting all surfgrass outside of tidepools and at a tide height where plants were only emergent at the lowest tides. A positive relationship with surfgrass $\delta^{13}C$ and SST and the negative relationship with upwelling was opposite to that expected under a hypothesis of ocean driving. Thus, the patterns in

carbon usage that we see among sites are likely to reflect seasonal DIC $\delta^{13}C$ differences among sites due to variation in the intensity of biotic activities.

Animals and nutrient regeneration

Consistent with animal regeneration of nitrogen, ammonium concentrations were more than three times greater at the shore than offshore among all four locales (Fig. 1). The elevated ammonium concentrations that we

TABLE 1. Results of linear mixed-effects models testing sea surface temperature (SST, °C), upwelling, nitrate, and ammonium as explanatory (predictor) variables for the $\delta^{15}N$ of filtered POM (particulate organic matter), mussels, surfgrass, and dissolved inorganic nitrogen and the $\delta^{13}C$ of surfgrass, from study sites along the Strait of Juan de Fuca, Washington, USA.

	SST		Upwelling (daily)		Upwelling (monthly)		Nitrate		Ammonium	
Isotope and source	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P	Coeff.	P
Filtered POM $\delta^{15}N$	−0.414	<0.001	−0.003	0.722	−0.013	0.394	0.064	<0.001	−0.072	0.384
Surfgrass $\delta^{15}N$	0.068	0.670	0.002	0.810	0.002	0.894	−0.009	0.487	0.110	0.049
Mussel $\delta^{15}N$	0.179	0.032	−0.005	0.270	0.002	0.785	−0.034	<0.001	−0.022	0.348
Surfgrass $\delta^{13}C$	0.782	0.003	−0.001	0.958	−0.001	0.047	0.020	0.428	−0.058	0.576
$\delta^{15}N_{NO3}$	0.134	<0.001	−0.010	0.028	−0.008	0.315	−0.054	<0.001	0.105	0.001
$\delta^{15}N_{NO2}$	−1.518	0.433	−0.040	0.611	0.081	0.787	0.559	0.036	−0.431	0.509
$\delta^{15}N_{NH4}$	−0.199	0.849	0.067	0.114	0.161	0.254	0.390	0.011	−0.062	0.905

Note: The date of measurement was treated as a random effect and all sampling dates and sites were used; Coeff. denotes the regression coefficient.

quantified in proximity to Tatoosh were a persistent signal over multiple years (Pfister et al. 2007), a result concordant with another locale in the California Current where ammonium increased in association with mussel beds (Aquilino et al. 2009). Increased assimilation of nitrate nearshore was consistently indicated, a difference that was greatest at Tatoosh Island, where nearshore nitrate concentrations were reduced by 26.2% (Fig. 1).

The potential for animal populations to enhance local productivity via contribution of nutrients has been demonstrated in a diversity of systems, including fish contributions to soils and streams (Drake et al. 2006, Walters et al. 2009), zebra mussels in lakes (Heath et al. 1995), seabirds on marine islands (Wootton 1991, Anderson and Polis 1999, Croll et al. 2005), sponges on coral reefs (de Goeij et al. 2013), and ungulates in the Serengeti (McNaughton et al. 1997). Positive feedbacks between components of the environment and the availability of nitrogen have been further suggested to switch the dominance of terrestrial plant groups over evolutionary time (Berendse and Scheffer 2009). The role of regenerated nitrogen in high-energy coastal upwelling areas, however, has been comparatively overlooked for several reasons. First, the oceanic supply of nitrate via upwelling in the California Current system is such a well-recognized driver of productivity (Chavez and Messie 2009) that the role of regenerative nitrogen processes has been relatively poorly investigated (but see Dugdale and Goering 1967). Second, the physical environment, including high wave action and strong currents, not only makes the system difficult to treat as insular (as in a lake), but also suggests high advection of any excreted nutrients. Thus, unlike insular systems, there must be a mechanism for nutrient retention. The elevated stable isotope values shown here in the nearshore suggest that microbes are a part of the retention, and nitrogen-transforming microbes do reside on some intertidal species (e.g., Pfister et al. 2010). Given the potential for upwelling to be intermittent in this region (Landry and Hickey 1989), animal regeneration may serve to buffer nutrient supply during downwelling events.

The evidence that we have for a large-scale signature of animal-driven regeneration is corroborated by small-scale experiments as well as calculations of the role of animals. Experimental manipulation of mussels in tidepools showed increased ammonium and nitrite concentrations as well as enhanced algal growth with mussels (Pfister 2007). Tracer experiments adding NH_4^+ enriched in ^{15}N revealed ammonium utilization that either kept pace with ammonium regeneration or exceeded it, indicating that the ammonium provided by mussels alone is immediately utilized and can locally enhance productivity (Pather et al. 2014).

We calculated the approximate contribution of mussels to dissolved inorganic nitrogen using published estimates of mussel abundance and individual ammonium excretion rates. On emergent rock benches on Tatoosh Island, mussel abundance can average 4661 individuals/m² (Suchanek 1979). We assumed that mussel sizes are evenly distributed and used per mussel NH_4^+ excretion rates (Bayne et al. 1976), estimating that 1 m² of mussel bed excretes 3.28 g ammonium/d. Assuming that ammonium is diffusing into the water 1 m above the mussel bed, this nets 3.28 mg/L in that volume, an order of magnitude greater than nitrate concentrations during upwelling periods. Although we note that these calculations ignore the complexity of water flow in nearshore areas, they do indicate the great potential for animals, including mussels, to impact coastal nutrient availability. The utility of this excreted ammonium was demonstrated by enrichment experiments in which ammonium produced by mussels in tidepools was almost entirely taken up by benthic primary producers (Pather et al. 2014).

Given the rich diversity and high abundance of other animal species in the nearshore, there is a great potential for productivity to be enhanced by local animal populations. As animal populations continue to decline in the ocean, especially those species at higher trophic levels (Worm et al. 2006), the non-trophic contributions that they are likely to make via nutrient regeneration also decline. Estimates of the productivity needed to maintain fisheries (e.g., Pauly and Christensen 1994) may require the contribution of that exploited species itself. Thus, in demonstrating a large-scale signature of biotic nitrogen isotope processing in coastal regions, we suggest that there is a unique non-trophic role for animals.

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SUPPLEMENTAL MATERIAL

Appendix A

Concentrations of dissolved inorganic nitrogen forms at each of the four sites along the Strait of Juan de Fuca, Washington, USA, both onshore and offshore (*Ecological Archives* E095-243-A1).

Appendix B

Mean and standard error for all nutrient concentrations, stable isotope values of POM (particulate organic matter), surfgrass, and mussels, and stable isotope values of ammonium, nitrate, and nitrite (*Ecological Archives* E095-243-A2).

Appendix C

ANOVA tests of the effects of site, shore location, and month on the dissolved inorganic nitrogen concentration (*Ecological Archives* E095-243-A3).

Appendix D

ANOVA tests of the effect of site and month on the stable isotopes of surfgrass, mussel, and POM (*Ecological Archives* E095-243-A4).