Using carbon isotopic signatures of *Ulva* spp. (sea lettuce) as indicators of coastal eutrophication

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Abstract

There has been great interest in using stable isotopes to detect eutrophication in coastal areas. Traditionally, δ¹⁵N is used to trace nitrogen sources and δ¹³C is used to differentiate between C₃ and C₄ plants, but recent studies suggest δ¹³C of algae can also be used as indicators of eutrophication. The present study focuses on whether δ¹³C of Ulva, a genus of bloom-forming macroalgae, can indicate coastal eutrophication. Coexistence of both C₃ and C₄ photosynthetic pathways in Ulva results in a wide range of possible δ¹³C signatures. When exposed to eutrophic conditions, Ulva can alter its carbon metabolism resembling a C₄ plant. To investigate how Ulva’s C isotopic signature responds to different levels of eutrophication, U. lactuca specimens were collected from estuaries subject to different nitrogen loads. Samples were analyzed for biomass and both δ¹³C and δ¹⁵N isotopic signatures. Results suggest that both δ¹³C and δ¹⁵N increase as nitrogen loads increase and δ¹³C of Ulva has the potential to detect difference in nitrogen loads.

Key words: Eutrophication, estuary, macroalgal bloom, N-loading, nitrogen, stable isotopes, δ¹³C, δ¹⁵N, Ulva

Introduction

As nutrient inputs to coastal waters increased due to growing human populations and coastal development, eutrophication has become a major theme of environmental challenges worldwide. There are many undesirable consequences associated with eutrophication. For example, the proliferation of macroalgal blooms, which can subsequently result in development of benthic shading and hypoxia (Valiela et al., 1997; Fox et al., 2008; Rabalais, 2002). Other effects of macroalgal blooms include loss of species diversity, replacement of benthic fauna, nuisance to recreational activities, etc., which not only make coastal environments less pleasant for anthropogenic purposes, but also have huge impact on structure and function of these environments (Hayden et al., 2003).

In temperate coastal waters, macroalgal growth is largely dependent on the nitrogen (N) availability (Howarth et al., 2000; Teichberg et al., 2010). N sources are usually assessed with the ratio of N stable isotopes (expressed as δ¹⁵N), since most macroalgae reflect N isotopic signatures of their sources (McClelland & Valiela, 1998; Thornber et al., 2008). Generally, N from animal sewage and manures has higher δ¹⁵N signatures than that from fertilizer or atmospheric deposition (Heaton, 1986). As eutrophication develops due to increases in one of these N sources, δ¹⁵N signatures of producers also shift.

The ratio of carbon (C) stable isotopes (expressed as δ¹³C) has been conventionally used to distinguish between C₃ and C₄ plants, which have δ¹³C values of about -28.1±2.5‰ and -13.5±1.5‰, respectively (Throughton et al., 1974). However, a recent study by Oczkowski et al. (2014) suggests that in autotrophic, phytoplankton-dominated systems, δ¹³C of particulate matter can also be used to indicate nitrogen pollution and eutrophication. In this study, I focused on whether δ¹³C of macroalgae can be used to study eutrophication.

Among the few bloom-forming taxa of macroalgae, Ulva can be found in many coastal waters and is responsible for some of the largest green tides in the world (Teichberg et al., 2010; Valiela et al., 2018). While most algae perform C₃ photosynthesis, there is evidence that a
bloom-forming alga, *Ulva* spp., is capable of both C$_3$ and C$_4$ photosynthesis, which is shown by the presence of C$_3$ and C$_4$ genes, as well as the enzymes needed for C$_4$ metabolism (Xu et al., 2012, 2013). Previous work has established that *Ulva* is able to take up bicarbonate (HCO$_3^-$), which has higher $\delta^{13}$C than CO$_2$ does, when CO$_2$ concentration is insufficient (Drechsler and Beer, 1991). This evidence is also supported by the wide range of $\delta^{13}$C found in *Ulva*, which covers $\delta^{13}$C of both C$_3$ and C$_4$ plants (Valiela et al., 2018). When *Ulva* is subject to eutrophic conditions, its growth is limited by C uptake and its C metabolism resembles that of C$_4$ plants, which are less selective between lighter (CO$_2$) and heavier (HCO$_3^-$) carbon isotopes. This shift in *Ulva*’s C metabolism drives $\delta^{13}$C higher in its tissue. Previous work has shown that *Ulva* $\delta^{13}$C increases when it is subject to higher N loads (Aguiar et al., 2003). Because of its unique growth dynamic, it is interesting to look at if *Ulva* can be used to detect the difference in the nutrient level, and thus to indicate eutrophication.

In this study, the species I collected was *Ulva lactuca*, which is common and very abundant in Waquoit Bay, Massachusetts. I examined the relationship of $\delta^{13}$C and $\delta^{15}$N of *U. lactuca* to N loads for a total of five sites, all of which are subestuaries of Waquoit Bay estuarine system. I hypothesize that as N loads increase across the five visited estuaries, both $\delta^{13}$C and $\delta^{15}$N of *Ulva* will increase. Additionally, the relationship of dissolved inorganic nitrogen (DIN) concentrations and biomass to N loads and the relationship of DIN concentrations and biomass to $\delta^{13}$C and $\delta^{15}$N of *U. lactuca* were also assessed.

**Methods**

*Study site*

Our study was done in Waquoit Bay, Massachusetts, USA, an estuarine system of several subestuaries with various levels of human development and land-use patterns, and hence widely different N loads. The estuaries of Waquoit Bay we visited were Sage Lot Pond, Head of Bay, Hamblin Pond, Eel Pond, Quashnet River, and Childs River, with N loads range from 20 kg N ha$^{-1}$ yr$^{-1}$ to 479 kg N ha$^{-1}$ yr$^{-1}$ (Table 1), which cover a very wide range of N loads to estuaries of the World (Valiela et al., 2016). N loads for all sites were 2014 results modeled by a Nitrogen Load Model (NLM) developed by Valiela et al. (1997) and applied in Valiela et al. (2016). To standardize the N loads for comparison among all sites, we divided the total N loads by surface area of each estuary to estimate N loads in kg N ha$^{-1}$ yr$^{-1}$.

*Sample collection*

To cover as much of the system as possible, we collected two samples of *U. lactuca* from Head of Bay and three samples from Sage Lot Pond, Hamblin Pond, Eel Pond, Quashnet River, and Childs River (Figure 1). Samples were collected by hand at Head of Bay and with Ekman grabs at the other visited sites to estimate biomass in g (dry weight) /m$^2$. All *U. lactuca* samples were briefly rinsed in a mesh bag to remove sediment.

At each site, surface water samples were collected, near the stations *U. lactuca* fronds were sampled, with 60-mL syringes, filtered through 20mm non-ashed GF/F filters on Swinnex filter holders into 20-mL scintillation vials, and brought back to lab in a cooler on ice. Dissolved oxygen (DO) level, salinity, and water temperature were measured at where water and *U. lactuca* samples were collected, using a YSI Pro 2030 Multiparameter Instrument. GPS coordinates were
recorded at each station to obtain the accurate sampling locations with a Garmin GPSmap 60CSx.

**Water nutrient analysis**

A total of 17 surface water samples were analyzed for ammonium ($\text{NH}_4^+$), nitrate ($\text{NO}_3^-$), and phosphate ($\text{PO}_4^{3-}$) concentrations to examine the availability of DIN and phosphorus for *Ulva* growth. Ammonium ($\text{NH}_4^+$) concentrations were measured with a spectrophotometer following the phenol-hypochlorite method (Solarzano, 1969; Strickland and Parsons, 1972). Nitrate ($\text{NO}_3^-$) concentrations were measured with a Lachat Flow Injection Analyzer (Wood et al., 1967). Phosphate ($\text{PO}_4^{3-}$) concentrations were measured with a spectrophotometer, using a colorimetric protocol developed by Murphy and Riley, 1962.

**Stable isotope analysis**

*U. lactuca* samples collected were thoroughly rinsed with DI water, dried at 70°C overnight, and grounded to fine powder with pestle and mortar. All samples were prepared by packing 3-4 mg into individual tin capsules. $\delta^{15}\text{N}$ vs $\delta^{13}\text{C}$ were measured using a Europa 20-20 mass spectrometer interfaced with Europa ANCA-SL elemental analyzer at the Marine Biological Laboratory Stable Isotope Lab. N isotope compositions were expressed as a part per thousand difference ($\delta^{15}\text{N}$) from the reference standard air (AIR) and carbon isotope compositions ($\delta^{13}\text{C}$) from Pee Dee Belemnite (PDB), where $\delta^{15}\text{N}$ or $\delta^{13}\text{C} = [(R_{\text{sample}}/R_{\text{standard}})/R_{\text{standard}}] \times 1000$ and R is $^{15}\text{N}/^{14}\text{N}$ or $^{13}\text{C}/^{12}\text{C}$, respectively.

**Results**

**Nutrient availability and Biomass**

Estimated N loads range widely from 20 to 479 kg N ha$^{-1}$ yr$^{-1}$ across the five estuaries, with Sage Lot Pond being the most “pristine” and Childs River the most “polluted” (Table 1). Except for Sage Lot Pond, wastewater was the main contributor to the total N loads in the other four estuaries (Table 1). Total N load for Head of Bay was available, but unlike the other sites, Head of Bay has a large surface area that will skew the N load on a per hectare basis, so N load for Head of Bay was not included. A linear regression indicates that the DIN concentrations increase significantly as the N loads increase (Figure 2). The regression was statistically significant ($F = 34.1164$, $P < 0.0001$).

The amounts of mean *U. lactuca* biomass vary significantly across the five estuaries, ranging from 16.36 g m$^{-2}$ in Hamblin Pond to 86.69 g m$^{-2}$ in Childs River (Table 2). Samples from Head of Bay were collected by hand, so biomass for Head of Bay was not included. Though standard errors are relatively large for all five sites, especially Eel Pond and Childs River, there is a marked and significant increase in biomass as N loads increase (Table 2, Figure 3). The regression was statistically significant ($F = 7.3225$, $P = 0.0180$). Other factors that might also affect biomass are DO, salinity, and temperature. DO did not vary too much among the five sites. Temperatures of water were very low in all sites, which was expected (Table 3). The N:P ratios of water samples in most stations were smaller than Redfield N:P (16) and exceptions were the sites with high N loads (Table 1, Table 4).

**Isotopic signatures**
Based on stable isotopes results of all specimens collected, there appears to be a positive relationship between δ¹⁵N and δ¹³C (Figure 4). F-test indicates the regression is statistically significant ($F = 9659, P = 0.00274$). *U. lactuca* collected from Sage Lot Pond and Hamblin Pond have lower δ¹⁵N and δ¹³C, while those from Eel Pond, Quashnet River, and Childs River have higher δ¹⁵N and δ¹³C (Figure 4). Both δ¹⁵N and δ¹³C are consistent with N loads modeled for each estuary: δ¹⁵N and δ¹³C appear to be higher in *U. lactuca* found in estuaries subject to more N loads, and lower in estuaries subject to less N loads (Figure 4, Table 1). δ¹³C appears to be very different when subject to lower and higher N loads (Figure 5, Table 1).

There was no significant relationship between δ¹⁵N of *U. lactuca* and DIN concentrations of water ($F = 3.8750, P = 0.0678$), but the relationship between δ¹³C of *U. lactuca* vs. DIN concentrations was significant ($F = 6.9593, P = 0.0186$) and can be expressed with a regression line: $y = 0.24x + 9.5362$. Though no linear relationship is found, δ¹⁵N values increase greatly as DIN concentrations increase from 0 µM to 5 µM and stay consistently high when DIN concentration is higher than 5 µM (Figure 6). δ¹³C values appear to have a more gradual increase as DIN concentrations increase (Figure 6).

Both δ¹⁵N and δ¹³C showed a marked increase as N loads increase (Figure 7). Similar to the pattern of δ¹⁵N of *U. lactuca* vs. DIN concentrations of water, δ¹⁵N increases dramatically at lower N loads and stays high when N loads are high (Figure 7). The relationship between δ¹⁵N of *U. lactuca* vs. modeled N loads for the five of all estuaries is statistically significant ($F = 47.6219, P < 0.0001$) and can be represented by a saturation curve: $y = 1.7112 + 6.0647*(1 - e^{-0.0299x})$. The increases in δ¹³C values are more gradual, which allows us to distinguish between medium N loads and high N loads (Figure 7). A saturation curve best fit to the relationship between δ¹³C and N loads is $y = -15.09 + 7.3574*(1 - e^{-0.0067x})$ and is statistically significant ($F = 9.1861, P = 0.0038$).

To obtain a better estimate of how δ¹⁵N and δ¹³C relate to biomass, which is used as a proxy for growth rate, I plotted data from Lloret et al., in prep. with my measured data. The data shows no significant relationship between δ¹⁵N and biomass (Figure 8). δ¹³C varies at low biomass but stays consistently high when biomass exceeds 40 g/m². The change in δ¹³C in response to biomass is significant ($F = 6.8314, P = 0.0025$) and is indicated by a saturation curve: $y = -16.03 + 5.4183*(1 - e^{-0.072x})$.

**Discussion**

The positive relationship between DIN concentrations and N loads indicated that higher N loads provided more N to the water column (Figure 2). This N is available for algae to grow, as it is reflected by the increase in *Ulva* biomass in the estuaries with higher N loads (Figure 3). Although significant, the scatter of the biomass measurements suggest that more measurements at different times of the year, especially during growing season, may improve the statistical power and provide more robust results.

While a significant linear relationship between δ¹³C and DIN concentrations was found, it is important to note that DIN concentrations were measured only from water samples collected in October and November. Considering the seasonal tourists influx in Cape Cod during warm months, I would expect there were more wastewater input and higher DIN concentrations when *Ulva* was growing. Therefore, the relationship of δ¹⁵N and δ¹³C to DIN concentrations depicted
in Figure 6 could be better assessed by including more observations collected during the growing season.

The comparison of \(\delta^{15}N\) and \(\delta^{13}C\) to N loads was more straightforward and comprehensive, as it integrates the changes in N supply to algae occurring throughout the entire year. Both \(\delta^{15}N\) and \(\delta^{13}C\) seem to saturate as N loads increased. From looking at the two curves, it seems like \(\delta^{13}C\) allows us to differentiate sites with intermediate and high N loads, as opposed to \(\delta^{15}N\), which saturates faster, but this can also be ascribed to the larger scatter of the \(\delta^{13}C\) data. The variation is especially protruding in sites with intermediate N loads. Whether \(\delta^{13}C\) is able to distinguish between sites with intermediate and high N loads requires more data and further investigation. However, \(\delta^{13}C\) does show the same trend as \(\delta^{15}N\) do in relation to N loads.

\(\delta^{13}C\) of *Ulva* showed a marked shift when N loads increased. *U. lactuca* collected from sites subject to lower N loads (Sage Lot Pond, Hamblin Pond, and Head of Bay) have \(\delta^{13}C\) similar to that of C\(_3\) plants and those from sites subject to higher N loads (Eel Pond, Quashnet River, and Childs River) have \(\delta^{13}C\) similar to that of C\(_4\) plants (Figure 5). This pattern is consistent with observations found in other literatures (Valiela et al., 2018). Such results suggest \(\delta^{13}C\) can be used to indicate difference in N loads.

While \(\delta^{15}N\) does not seem to correlate with biomass, \(\delta^{13}C\) interestingly shows a consistent high signature around -11‰, which is similar to that of a C\(_4\) plant, when biomass is high. Since \(\delta^{15}N\) is rather representing the main source of N that contributes to N loads, it does not directly relate to biomass or growth rate. Based on the significant relationship found between \(\delta^{13}C\) and biomass, \(\delta^{13}C\) can potentially be used as a proxy for growth rate. However, the sole evidence found in this study is not sufficient to support this hypothesis and further incubation experiments to directly measure how growth rate relate to shift in \(\delta^{13}C\) would need to be conducted.

As expected, we observed an increase in \(\delta^{15}N\) of *Ulva* with increasing N loads. The parallel increase in \(\delta^{13}C\) is explained by *Ulva*’s ability to alter its C metabolism under those high N conditions. As N loads increase, there is more DIN in the system and *Ulva* can adapt to and take advantage of the different nutrient regimes by changing the way it photosynthesizes, resembling a C\(_4\) plant. \(\delta^{13}C\) of *Ulva* is useful as an indicator of elevated N loads in coastal waters, which provides us a potential new method to detect eutrophication.

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Literature Cited


Tables and Figures

**Table 1.** Modeled N loads arriving at each estuary for 2014 (Valiela et al., 2016). All loads in kg N ha\(^{-1}\) yr\(^{-1}\).

**Table 2.** Average *U. lactuca* biomass ± standard errors in each estuary.

**Table 3.** Ranges of dissolved oxygen, salinity, and temperature measured in each estuary.

**Table 4.** DIN concentrations, phosphate concentrations, and N:P of water samples collected from each station. SL stands for Sage Lot, HP stands for Hamblin Pond, QR stands for Quashnet River, HB stands for Head of Bay, CR stands for Childs River, and EP stands for Eel Pond. *N:P is higher than Redfield ratio.

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**Figure 5.** \(\delta^{13}C\) of *U. lactuca* collected from Sage Lot Pond, Hamblin Pond, Head of Bay, Eel Pond, Quashnet River, and Childs River.

**Figure 6.** \(\delta^{15}N\) (top panel) and \(\delta^{13}C\) (bottom panel) of *U. lactuca* vs. DIN concentrations of water at each station in all six estuaries. No significant relationship was found between \(\delta^{15}N\) and DIN concentrations.

**Figure 7.** \(\delta^{15}N\) (top panel) and \(\delta^{13}C\) (bottom panel) of *U. lactuca* vs. modeled N loads for Sage Lot Pond, Hamblin Pond, Eel Pond, Quashnet River, and Childs River.

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<table>
<thead>
<tr>
<th></th>
<th>Sage Lot Pond</th>
<th>Hamblin Pond</th>
<th>Eel Pond</th>
<th>Quashnet River</th>
<th>Childs River</th>
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</tr>
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<td>20</td>
<td>51</td>
<td>146</td>
<td>304</td>
<td>479</td>
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Table 2. Average biomass ± standard errors in each estuary.

<table>
<thead>
<tr>
<th>Site</th>
<th>Biomass (g/m²)</th>
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</thead>
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<tr>
<td>Sage Lot Pond</td>
<td>23.11 ± 9.54</td>
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<tr>
<td>Hamblin Pond</td>
<td>16.36 ± 2.21</td>
</tr>
<tr>
<td>Eel Pond</td>
<td>36.88 ± 22.41</td>
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<tr>
<td>Quashnet River</td>
<td>18.34 ± 9.61</td>
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<tr>
<td>Childs River</td>
<td>86.69 ± 22.42</td>
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Table 3. Ranges of dissolved oxygen, salinity, and temperature of each estuary.

<table>
<thead>
<tr>
<th>Site</th>
<th>DO (mg/L)</th>
<th>Salinity (ppt)</th>
<th>Temp (°C)</th>
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</tr>
<tr>
<td>Childs River</td>
<td>7.52-9.48</td>
<td>16.2-26.5</td>
<td>10.9-13.8</td>
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Table 4. DIN concentrations, phosphate concentrations, and N:P of water samples collected from each station. SL stands for Sage Lot, HP stands for Hamblin Pond, QR stands for Quashnet River, HB stands for Head of Bay, CR stands for Childs River, and EP stands for Eel Pond.
*N:P is higher than Redfield ratio.

<table>
<thead>
<tr>
<th>Station ID</th>
<th>DIN conc. (µM)</th>
<th>PO$_4^{3-}$ conc. (µM)</th>
<th>N:P</th>
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<tr>
<td>CR8</td>
<td>13.41</td>
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<td>5.59</td>
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\[ y = 0.0484x + 0.5637 \]

\[ F = 34.1164 \]

\[ P < 0.0001 \]
Figure 3. Relationship between measured biomass and modeled N loads in Sage Lot Pond, Hamblin Pond, Eel Pond, Quashnet River, and Childs River.

\[ y = 0.1198x + 12.334 \]

\[ F = 7.3225 \]

\[ P = 0.0180 \]
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