Seasonal changes in nutrient limitation and nitrate sources in the green macroalga *Ulva lactuca* at sites
with and without green tides in a northeastern Pacific embayment

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ABSTRACT

In Penn Cove, ulvoid green algal mats occur annually. To examine seasonal variation in their causes, nitrogen and carbon were measured in *Ulva lactuca* in May, July, and September and stable nitrogen and oxygen isotope ratios were quantified in *U. lactuca*, Penn Cove seawater, upwelled water from Saratoga Passage, water near the Skagit River outflow, and effluents from wastewater treatment facilities. Ulvoid growth was nitrogen limited and the sources of nitrogen used by the algae changed during the growing season. Algal nitrogen concentrations were 0.85 - 4.55% and were highest in September and at sites where algae were abundant. Upwelled waters were the primary nitrogen source for the algae, but anthropogenic sources also contributed to algal growth towards the end of the growing season. This study suggests that small nitrogen inputs can result in crossing a “tipping point”, causing the release of nutrient limitation and localized increases in algal growth.

1. Introduction

Accumulations or blooms of seaweeds are becoming increasingly abundant and problematic worldwide (Smetacek and Zingone, 2013; Ye et al., 2011). These blooms occur over a wide range of latitudes, from tropical habitats to cold temperate environments and can be detrimental to other organisms as well as having negative economic impacts on commercial fisheries and recreational and tourist industries (Frankenstein, 2000; Liu et al., 2009; McGlathery, 2001; Teichberg et al., 2010, 2012; Williams and Feagin, 2007). The availability of nutrients plays a role in the development and persistence of seaweed blooms at all latitudes (Teichberg et al., 2012; Valiela et al., 1997); however, at higher latitudes, the importance of seasonal variation in environmental factors, including nutrients, light and temperature, may be more important to the regulation of bloom dynamics than it is at lower latitudes (Thom and Albright, 1990).

In the cold temperate waters of the Salish Sea (the inland marine waters of Washington State USA comprised of Puget Sound, the Strait of Juan de Fuca, and Georgia Strait), the most commonly occurring seaweed blooms consist of large growths or accumulations of ulvoid green algae, which are often referred to as “green tides” (Nelson et al., 2003a). Blooms in this region frequently consist of mixed species assemblages of seaweeds belonging to the genera *Ulva* (including species previously described as *Enteromorpha*), *Monostroma*, and *Ulvaria* (Nelson et al., 2003b). They are problematic because they overgrow ecologically important eelgrasses (*Zostera marina*) (Nelson and Lee, 2001), alter abundances of infaunal organisms (Price and Hylleberg, 1982), introduce toxic natural products and reactive oxygen species into the environment (Van Alstyne et al., 2011a, 2013, 2014; Van Hees and Van Alstyne, 2013), and cause malodorous piles on beaches (Frankenstein, 2000).

Unlike many macroalgal blooms in lower latitudes, the blooms in the Salish Sea have a distinct and relatively short growing season. Typically, these algae are absent or in low densities during the winter months, begin increasing in abundance in mid-spring as water temperatures warm, peak in

abundance in late summer and early autumn, and experience large reductions in biomass following mid-autumn storms, a time of year when light levels become reduced (Nelson et al., 2003b; Thom and Albright, 1990). In the San Juan Islands, ulvoid algal biomasses tend to be positively correlated with day length and water temperature. Dissolved inorganic nitrogen (DIN) concentrations are also strongly and positively correlated with biomass among topographically-similar sites (Nelson et al., 2003b).

The relationship between DIN and ulvoid algal biomasses suggests that anthropogenic inputs of nitrogen could be an important contributor to the development of ulvoid algal mats in the Salish Sea. The fact that blooms tend to be more common in urbanized areas of central and south Puget Sound than in more northern, less urbanized sites (Nelson et al., 2009), supports this possibility. It is assumed that urbanized areas have higher inputs of nitrogen from anthropogenically-generated sources, such as fertilizer runoff and wastewater discharges. However, nutrient levels in the Salish Sea are also strongly impacted by freshwater inputs (Banas et al., 2015) and tidal inputs of upwelled nutrients from the coast (Hickey and Banas, 2003), which vary seasonally and inter-annually (Babson et al., 2006). Because the region as a whole has relatively high seawater nitrogen concentrations (typically 2-20 μM), and because of the high rates of water movement due to tidal exchanges and currents, it is unlikely that anthropogenic nitrogen inputs produce large-scale eutrophication in the system (Mackas and Harrison, 1997); however, the region is topographically complex and anthropogenic inputs may be important in shallow embayments where mixing and water exchanges are limited.

The purpose of this study was to examine the importance of nitrogen limitation and nitrogen sources to ulvoid algae throughout a growing season in a small embayment, Penn Cove, Whidbey Island, in the Salish Sea. In Penn Cove (Fig. 1a), thick accumulations or blooms composed of *Ulva* spp. and *Ulvaria obscura* occur annually in some areas of the embayment, but are absent in others. In the northwest corner of Penn Cove (hereafter referred to as NW Penn Cove; Fig. 1b), summertime accumulations cover over 70% of the surface and do so consistently from one year to the next, unlike

accumulations in many areas in Puget Sound that undergo substantial inter-annual fluctuations in size (Nelson et al. 2009). In 2006, the only year for which sufficient data are available for comparison, the ulvoid algal cover at a site in NW Penn Cove was 610% higher than the average cover in the Saratoga Passage and eastern Whidbey Island region, and 113% higher than the average for Central Puget Sound, the region most heavily impacted by algal accumulations (Nelson et al. 2009). Published summertime concentrations of seawater nitrogen taken from surface waters near the NW Penn Cove sampling site (Fig. 1) are 23 μM nitrate, 0.3 μM nitrite, and 11 μM ammonium (Van Alstyne et al., 2011b).

The causes of the ulvoid algal blooms in Penn Cove have not been directly examined but may be due to the high concentrations of nitrogen in Penn Cove's waters, which could result, in part, from anthropogenic inputs. Inorganic and organic nitrogen and *ortho*-phosphate can promote the growth of ulvoid algae (Fong et al., 2003; Valiela et al., 1997); however, nitrogen is the nutrient that most limits the growth of *Ulva* in Penn Cove. The molar ratio of nitrogen to phosphorus in *Ulva lactuca* tissues in Aug 2007 at NW Penn Cove was approximately 12 (Van Alstyne et al., 2011b), which is within the range expected for nitrogen-limited seaweeds (Björnsäter and Wheeler, 1990). In lab experiments, both *Ulva* and *Ulvaria* from a nearby site grew better when nitrogen concentrations in the media were increased (Nelson et al., 2008; Van Alstyne et al., submitted), but growth did not change when media were supplemented with phosphorus (Van Alstyne et al., submitted).

There are several potential sources of nutrients that could be affecting algal abundance. In the east end of the embayment, there are two wastewater outflows that discharge nutrient-rich effluents: 1) the Coupeville wastewater treatment facility (CVTP in Fig. 1c) on the southern shore and 2) the smaller Penn Cove treatment facility (PCTV in Fig. 1c) on the northern shore. The Coupeville facility is estimated to discharge 45 pounds of nitrogen daily. However, there are alternative sources of nutrients that could promote algal growth. These include upwelled waters transiting from Rosario Strait through Deception Pass between Fidalgo and Whidbey Islands into Saratoga Passage (Fig. 1) and freshwater from

the Skagit River (SR in Fig. 1b), which drains a watershed that includes agricultural lands. Furthermore, ulvoid growth in Penn Cove can be promoted by remineralized nutrients that have accumulated in intertidal and shallow subtidal sediments and are later resuspended into the water column when the sediments are disturbed (Van Alstyne et al., 2011b).

Because marine algae take up nutrients in the same isotopic ratios that are present in the surrounding seawater, the proportions of isotopes that are present in the algae can provide information about the sources of the elements used by the algae (Michener and Lajtha, 2007; Fry, 2008). The ratio of the dominant form of nitrogen, ^{14}N , to its stable isotope ^{15}N (calculated as $\delta^{15}\text{N}$) is commonly used to track the influence of wastewater on algal growth in marine environments (e.g., Costanzo, et al. 2001, 2005; Carmichael et al., 2004; Cohen and Fong, 2004; Cole et al., 2004; Cole et al., 2005; Daskin et al., 2008). The ratio of ^{16}O to its stable isotope ^{18}O (calculated as $\delta^{18}\text{O}$), while not as useful as $\delta^{15}\text{N}$, can also provide information about potential sources of nitrate (Seiler, 2005).

To examine the sources of nitrogen that influence the accumulation of ulvoid algae in Penn Cove and their seasonal fluctuations, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ were quantified early the growing season (in May), mid-season (in July), and towards the end of the growing season (in September) in *Ulva lactuca* from four sites in Penn Cove and a site near the outflow of the south fork of the Skagit River (Fig 1b). To characterize the isotope signatures of different water sources, water samples were taken from two locations in Penn Cove, a site near the outflow of the south fork of the Skagit River, Saratoga Passage, and from the Coupeville and Penn Cove wastewater treatment facilities (Fig. 1c) for analyses of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ from nitrate. The algae's tissue nitrogen and carbon concentrations were also measured to determine if the algae were nitrogen limited.

2. Materials and methods

2.1 Sampling sites

Four intertidal sites were selected within Penn Cove for the collection of *Ulva lactuca* (Fig. 1b). These included a site in the northwest (NW) corner of the cove (48° 13.9' N, 122° 43.8' W) where ulvoid blooms occurred consistently during the summers of 2006-2008 (Van Alstyne and Nelson, unpubl. data), a site in the southwest (SW) corner of the cove (48° 12.9' N, 122° 43.1' W), which is close to the site of a reported fish kill in 2003 (Mador 2003), a northeast (NE) site near the Monroe Landing boat launch (48° 14.4' N, 122° 40.9' W), which lacked significant amounts of ulvoid algae during the summers of 2006-2008 (Van Alstyne and Nelson, unpubl. data) and is close to the outflow of the Penn Cove wastewater treatment plant, and a southeast (SE) site (48° 13.6' N, 122° 39.0' W) at Long Point. A fifth site near the outflow of the Skagit River (SR) a (48° 20.3' N, 122° 26.1' W) was also sampled.

Water samples were collected from six locations (Fig. 1c; Table 1). These included two sites from the center of the cove, a western (WPC) site and an eastern (EPC) site, a site from Saratoga Passage (SP) outside of the cove and a site near the outflow of the south fork of the Skagit River (SRW) in a tidal area where waters were brackish and included a mix of freshwater from the river and marine water from Saratoga Passage. Treated wastewater samples were obtained from the Penn Cove wastewater treatment facility, located in Oak Harbor, Washington (WA), near the NE sampling site and the Coupeville wastewater treatment facility located in Coupeville, WA, on the south side of the cove.

2.2 Algal collection

To examine chemical characteristics of the algae, *U. lactuca* were collected in the intertidal zones at the five locations described above (Fig. 1b) in May, July, and September 2010. The algae were transported on ice to SPMC where each individual was identified to species by microscopic examination of sectioned thalli. Twenty pieces of individual *U. lactuca* from each collection were placed into a drying oven (60°C) for at least 48 hr. The dried algae were then ground to a fine powder with a SPEX mixer/mill for 5 min and stored in ultracentrifuge tubes in a desiccator. From each individual, samples of

approximately 3 mg were encapsulated in tin capsules and samples of 1 mg were encapsulated in silver capsules. The encapsulated samples were then shipped to the University of California, Davis (UCD) Stable Isotope Facility for analyses of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$. From each collection, an additional ten samples were weighed (~5 mg) and encapsulated in tin capsules for measurement of tissue nitrogen and carbon concentrations with a CE Elantech 1112 elemental analyzer using atropine as a standard.

$\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ ratios were compared among sites with multivariate analyses of variance (MANOVA; SPSS 19). Tissue nitrogen concentrations were compared among sites where algae were scarce (Skagit River, SE Penn Cove and NE Penn Cove) and sites where algae were common (NW Penn Cove and SW Penn Cove) and among collection dates with a nested analysis of variance (ANOVA) after the tissue nitrogen concentrations were angularly transformed. Pearson's correlation analyses (SPSS 19) were used to determine whether $\delta^{15}\text{N}$ and tissue nitrogen concentrations of Penn Cove algae were significantly correlated within months.

2.3 Water collection

To obtain stable isotope signatures from potential sources of nitrogen, water samples (N=5 per site) were collected with a Van Dorn-type water sampler at the depths given in Table 1. Fifty ml from each sample was filtered through a nylon 0.45 μm syringe filter into an acid-washed 60 ml bottle. The bottles were stored on ice for transport to the Shannon Point Marine Center (SPMC) in Anacortes, WA USA, then stored in darkness at -70°C prior to shipment to the UCD Stable Isotope facility for measurements of nitrate $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$. Measurements of seawater temperature, salinity, and dissolved oxygen were also taken at the same depths and locations from which the seawater samples were taken using a YSI 85 handheld dissolved oxygen meter.

3. Results

3.1 Algal abundance

Although quadrat measurements were not made of algal abundance, ulvoid percent covers differed greatly among sites, as can be seen in Figure 2. At NW Penn Cove, ulvoid algal covers were between 50-100% during all sampling periods. At SW Penn Cove, covers were estimated to be 5-10% during the May sampling period, 20-50% during July, and 5-10% during September. At the SE Penn Cove, NE Penn Cove, and Skagit River Sites, ulvoid algae were consistently sparse (<5% cover).

3.2 Algal tissue nitrogen and carbon concentrations,

Tissue nitrogen concentrations (Fig. 3a) in individual *Ulva* ranged from 0.85 to 4.55% of the alga's dry mass (DM). They were, on average, 28% lower in algae from the three sites where ulvoid algae were scarce (Skagit River, SE Penn Cove and NE Penn Cove) than they were from the two sites where ulvoid algae were common (NW Penn Cove and SW Penn Cove) (nested ANOVA: $F=33.20$, $df=1$, $P<0.001$). They also varied significantly over time (nested ANOVA: $F=17.0$, $df=2$, $p<0.001$), with average tissue nitrogen concentrations being highest in September 2010 and lowest in July 2010 (Fig. 3). The site x date interaction effect was also significant (nested ANOVA: $F=9.00$, $df=6$, $p<0.001$).

Tissue carbon concentrations in individual *Ulva* (Fig. 3b) ranged from 22.7 to 40.0 % DM. They differed significantly among sites (nested ANOVA: $F=20.8$, $df=3$, $p<0.001$), but concentrations were not significantly different (nested ANOVA: $F=1.17$, $df=1$, $p=0.282$) at sites with and without large amounts of ulvoid algae. Carbon concentrations also did not differ significantly over time (nested ANOVA: $F=0.32$, $df=2$, $p=0.728$). Carbon to nitrogen ratios (Fig. 3c) were 27% higher on average in algae at sites where algae were not abundant than at sites where they were abundant (nested ANOVA: $F=53.72$, $df=1$, $p<0.001$). C:N also differed over time (nested ANOVA: $F=32.72$, $df=2$, $p<0.001$), with the average C:N being 25% lower in September than in May and July.

3.3 Algal stable isotope ratios

Stable isotope ratios in tissues of *Ulva* differed significantly among sites in May (Fig. 4a; MANOVA: Pillai's trace,=1.262, hypothesis df=8, error df=180, $p<0.001$), June (Fig. 4b; Pillai's trace,=24.517, hypothesis df=8, error df=170, $p<0.001$) and September (Fig. 4c; Pillai's trace,=0.821, hypothesis df=8, error df=166, $p<0.001$). This was true for both $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ during all sampling periods (between-subjects effects for both $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$: $p<0.001$). $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ were significantly lower in algae from the Skagit River site than in algae from all Penn Cove sites in May and July (SNK tests: $p>0.05$). In September, $\delta^{15}\text{N}$ values in the Skagit River algae were statistically indistinguishable from those in the NW and SW Penn Cove algae (SNK tests: $P>0.05$), but they were significantly lower than those in the SE and NE Penn Cove algae (SNK tests: $P<0.05$).

Differences in $\delta^{15}\text{N}$ occurred among sites within Penn Cove, but not during all seasons. The $\delta^{15}\text{N}$ ratios of *Ulva* from the Penn Cove sites with high densities of algae (NW and SW Penn Cove) were not significantly different than ratios in *Ulva* from sites with low densities of algae (NE and SE Penn Cove) in May (nested ANOVA, algal density effect : $F=1.80$, $p=0.184$) and July (nested ANOVA, algal density effect: $F=0.57$, $p=0.452$); however, $\delta^{15}\text{N}$ ratios were significantly lower at the high density sites in September (nested ANOVA, algal density effect : $F=44.42$, $p<0.001$). It is noteworthy that the decrease in $\delta^{15}\text{N}$ ratios in algae at NW and SW Penn Cove occurred during the same sampling periods that tissue nitrogen concentrations at these sites increased. $\delta^{15}\text{N}$ and tissue nitrogen concentrations in Penn Cove algae were not significantly correlated in May (Fig. 5a; Pearson's correlation coefficient = 0.014, $p=0.941$) and July (Fig. 5b; Pearson's correlation coefficient = -0.124, $p=0.512$), but were significantly and negatively correlated in September (Fig. 5c; Pearson's correlation coefficient = -0.443, $p=0.014$).

3.4 Seawater and wastewater stable isotope ratios

Significant differences existed among stable isotope ratios of nitrate from seawater and wastewater sampled in May (Fig. 6a; MANOVA: Pillai's trace=1.190, hypothesis df=10, error df=46, $p<0.001$) and September (Fig. 6b; MANOVA: Pillai's trace=1.367, hypothesis df=10, error df=42, $p<0.001$). Both nitrogen (between subject effects May: $F=8.173$, $p<0.001$; September: $F=33.579$, $p<0.001$) and oxygen (between subject effects May: $F=11.950$, $p<0.001$; September: $F=9.723$, $p<0.001$) isotope ratios differed significantly among the sampled sites. Because of equipment problems, stable isotope data were not obtained for July.

In both May and September, $\delta^{15}\text{N}$ values of nitrate from the effluents of the two wastewater treatment plants were statistically indistinguishable from one another (Fig. 6; SNK tests, $P>0.05$); however, they were significantly lower than the values for all other sites (Fig. 6; SNK tests, $P<0.05$). $\delta^{15}\text{N}$ of nitrate in seawater at east Penn Cove, the site closest to the wastewater discharges, was significantly lower than for the west Penn Cove site in September (Fig. 6b), but otherwise there were no significant differences in $\delta^{15}\text{N}$ among the seawater samples. The overall east Penn Cove $\delta^{15}\text{N}$ values were slightly, but not significantly, lower than at the other sites.

When $\delta^{15}\text{N}$ was compared in algae, seawater, and wastewater, values obtained from seawater from Saratoga Passage and the Skagit River and algae from the Penn Cove sites did not differ significantly (SNK test: $p>0.05$) in May; however, all of these were all significantly higher than the $\delta^{15}\text{N}$ from the two wastewater treatment facilities (SNK test: $p<0.05$). In September, $\delta^{15}\text{N}$ ratios of water from the Saratoga Passage and Skagit River sites were significantly higher than $\delta^{15}\text{N}$ in algae from the Penn Cove sites (SNK test: $p<0.05$), which were, in turn, were significantly higher than $\delta^{15}\text{N}$ in wastewater from the treatment facilities (SNK test: $p<0.05$).

4. Discussion

4.1 Nutrient limitation

Nutrients, especially nitrogen, frequently limit the growth of algae that form green tides and thus may control bloom dynamics (Teichberg et al., 2010; Teichberg et al., 2012; Valiela et al., 1997). In many parts of the world where background nitrogen concentrations are inherently low, additions of nitrogen result in increased rates of ulvoid algal growth (Teichberg et al., 2010), which may contribute to the formation of large accumulations of benthic seaweeds. However, because of the naturally high nitrogen concentrations in the waters of the Salish Sea (Mackas and Harrison, 1997), it is possible that algae are not nitrogen limited and that other factors such as phosphorus availability, light limitation, or herbivory drive bloom dynamics. Conversely, because of the high nitrogen levels already present in the waters, small additional inputs of nitrogen could be enough to provide a “tipping point” that causes the release of nutrient limitation and rapid increases in algal growth rates.

Data from this study suggests that the growth of ulvoid algae along the shores of Penn Cove during the growing season of 2010 was limited by nitrogen availability as tissue nitrogen concentrations were generally low. With the exception of the algae collected from the SW Penn Cove site in May, average tissue nitrogen concentrations in Penn Cove algae were consistently less than 3% and often less than 2% (Fig. 3). Experimental work by Björnsäter and Wheeler (1990) with *U. lactuca* (reported as *U. fenestrata*) has shown that in the absence of phosphorus limitation, *U. lactuca* with tissue nitrogen concentrations of approximately 2.3% or less grow sub-optimally, whereas maximum growths rate occur when tissue nitrogen concentrations are 4% or higher. Tissue nitrogen concentrations are also positively correlated with the growth rates of ulvoid algae at sites from around the world in a variety of *Ulva* species (Teichberg et al., 2010).

Seasonal patterns in tissue nitrogen in *Ulva* tracked expected patterns of nitrogen availability. In the Salish Sea, seawater nitrogen concentrations tend to be lowest in mid-summer when the standing stock of benthic macrophytes is high (Thom and Albright, 1990). In Penn Cove, nitrogen concentrations in *Ulva*, which should integrate short-term variation in seawater nitrogen concentrations and reflect

longer-term trends (Cohen and Fong, 2006), were lowest in July and highest in September. Similar summertime low values in tissue nitrogen concentrations or large decreases in nitrogen content relative to springtime values have been measured at other sites where ulvoid algae occur, including in northwest (Villares and Carballeira, 2003) and southern Spain (Altamirano et al., 2000), along the coast of Oregon, USA (Wheeler and Bjornsater, 1992), in the Po River Delta of Italy (Naldi and Viaroli, 2002), and in Waquoit Bay, Massachusetts, USA (Rivers and Peckol, 1995).

Nitrogen limitation in this study is also suggested by the fact that tissue nitrogen concentrations were lower at the three sites where ulvoid algae were scarce, relative to the two sites where algae were abundant, although the abundance of the algae may have been affected by the topography of the cove and water flow patterns. The ulvoid algal accumulations at the west end of Penn Cove include both attached and free-living algae. *U. lactuca* in Penn Cove can form large floating mats, which can be deposited suddenly at the west end of the embayment during the summer (K. Van Alstyne, pers. observ.). Thus, not all of the biomass at the west end of the Cove may originate from there. Similar floating mats, albeit on much larger scales, are responsible for accumulations of ulvoid algae in other areas, such as the large green tides that occur annually in Qingdao, China, which originate at aquaculture facilities 180 km south of the embayment where the algae is deposited (Hu et al., 2010; Liu et al., 2009).

The tissue nitrogen concentrations that we measured at Penn Cove were low relative to concentrations measured from other sites in the Salish Sea and comparable to others, suggesting that inter-site or inter-annual variation is common. The concentrations measured in this study were 20-50% lower than those reported from *U. lactuca* in August of 2008 at Ship Harbor (Van Alstyne et al. submitted) and 55 to 70% lower than concentrations in *U. lactuca* at the Shannon Point Beach in July of 2001 (Van Alstyne et al., 2009); both sites are about 30 km from Penn Cove. Of the two sites, only Ship Harbor has frequent green tides. They are comparable to those reported by Nelson and Melton (2011)

for Dumas Bay, Federal Way, WA during June and August, 2010, another site where green tides commonly occur. Previous measurements of average tissue nitrogen concentrations in *U. lactuca* in August 2008 from NW Penn Cove (Van Alstyne et al., 2011b) were within 10% of the concentrations that we measured in this study, suggesting that there may be little inter-annual variation in nitrogen concentrations at the Penn Cove sites.

4.2 Sources of nitrogen

The isotope signatures in Penn Cove algae were consistent with upwelled or Skagit River water being the primary sources of nitrogen supporting algal growth. In May, average $\delta^{15}\text{N}$'s for Saratoga Passage and Skagit River waters were close to 9 (Fig. 6). It is likely that these averages reflect inputs of nutrients from both transiting upwelled water and water from the Skagit River. Water sampled near the outflow of the Skagit River water had a large variance because half of the samples had values that ranged from 5.1 to 7.0 and the other half had values ranging from 13.1 to 13.5. This suggests that these samples were either taken near the boundary of two different water masses or that a pulse of water with a different source of nitrate was included in the samples. The water at Saratoga Passage in May was likely a mix of upwelled and Skagit River water as well. The $\delta^{15}\text{N}$ values of water of waters from the Saratoga Passage and Skagit River sites both rose in September when discharge rates from the Skagit River were substantially lower (Fig. 7a) as a result of low rates of precipitation during the summer months (Fig. 7b). Measurements of salinity (Table 1) also support a relatively higher freshwater input at the Skagit River site in May than September.

The stable isotope signatures of the water samples collected for this study provide a snapshot of the isotope ratios on the day of collection, whereas the isotope signatures in *Ulva* integrate the signatures of the nutrients taken up by the algae over a longer period of time. The lower $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values in algae collected at the Skagit River sites in May and July (Fig. 4a, b) better reflect the influence

of freshwater from the river during the days and weeks preceding the collection of the algal samples and give a better picture of isotope ratios in the waters influenced by Skagit River inputs than water collected on a single day. The large differences between isotope signatures in the Skagit River and Penn Cove algae during these two sampling periods show that there is little influence of river water on Penn Cove algae during these months and that the primary nitrogen source for the Penn Cove algae was upwelled water from Saratoga Passage. Further support is provided by the similarities in the isotope signatures of algae from Penn Cove and the Skagit River in September (Fig. 4c), a time when the river discharge rate had dropped dramatically (Fig. 7a), leaving Saratoga Passage waters as the primary source of nitrate for algae growing near the Skagit River outflow.

The lower $\delta^{15}\text{N}$ values in the Penn Cove algae in July and September relative to the Saratoga Passage water values suggests that an additional source of nitrogen with a lower $\delta^{15}\text{N}$ signature was also being used by the algae at this time. Algae from the western end of the cove, especially the NW site, tended to have higher nitrogen concentrations (Fig. 3c) and lower $\delta^{15}\text{N}$ values (Fig. 4c) than algae from the eastern end of the cove in September, suggesting that the low $\delta^{15}\text{N}$ nitrate source was contributing both to the high algal nitrogen concentrations and the high algal biomasses at the sites. Given that inputs from the Skagit River are unlikely to be the source because of the low discharge rates occurring at the time (Fig. 7a), the most likely sources of nitrogen with low $\delta^{15}\text{N}$ values were the two wastewater treatment plants, which had very low $\delta^{15}\text{N}$ values in September (Fig. 6b). Algae in the west end tended to have lower $\delta^{15}\text{N}$ values than algae in the east end in September, a pattern that did not occur in May or July. The magnitudes of spring tides in August are lower than those in May through July (NOAA, 2010). Thus, tidal exchanges of high- $\delta^{15}\text{N}$ water from Saratoga Passage would be reduced, especially at the western end of the cove and $\delta^{15}\text{N}$ values in the algae could be lower because the algae are exposed to relatively more of the low- $\delta^{15}\text{N}$ water from the treatment plants.

Low $\delta^{15}\text{N}$ values are also associated with urea (Choi et al., 2006) and ammonium-based fertilizers (Kendall et al., 2005) as well as some manure-based fertilizers (Choi et al., 2006) and agricultural land surrounds a large portion of Penn Cove, especially on the northern side. However, given the low amounts of rainfall that occurred prior to the September measurements, it is unlikely that runoff from these farms was the source of the low $\delta^{15}\text{N}$ nitrate. Most manures and septic nitrogen have higher $\delta^{15}\text{N}$ (Kendall et al., 2005), which suggests that these also were not a significant source of nitrogen for the algae at the west end of the cove in September. The $\delta^{15}\text{N}$ values reported here for wastewater from the Coupeville and Penn Cove treatment facilities are consistent with $\delta^{15}\text{N}$ values for wastewater with a low level of processing, which tends to have low $\delta^{15}\text{N}$ (Daskin et al., 2008).

4.3 Conclusions

The results of this study showed that the growth of ulvoid algae in and near Penn Cove was nitrogen limited and that the relative importance of the sources of nitrogen used by the algae changed seasonally, especially at the Skagit River site. Upwelled water from Saratoga Passage appeared to be the primary source of nitrogen for Penn Cove algae throughout the entire growing season, but another source of nutrients, most likely effluents from wastewater treatment, was contributing to algal growth later in the growing season at the west end of the embayment. The shifts in nitrogen sources were a result of changes in the drainage rates of the Skagit River due to summertime decreases in precipitation coupled with seasonal changes in the magnitudes of the tides.

Bioindicators, such as stable isotope ratios, are valuable methods for elucidating factors contributing to the formation and development of seaweed blooms (Lapointe, 1997; LaPointe et al., 2005; Piñón-Gimate et al., 2009; Thornber et al., 2008). These indicators are most useful when background levels of nitrogen are low relative to anthropogenic inputs and when there are few potential nitrogen sources to support algal growth (Ochoa-Izaguirre and Soto-Jiménez, 2015). However, even

when there are many potential sources of nitrogen, natural concentrations of nitrogen are high, and water flow patterns are complex and variable, these indicators can help tease out the relative contributions of different nitrogen sources and how they change over time.

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Table 1

Seawater temperature, salinity and dissolved oxygen at four sampling locations. Seawater samples were also collected at these depths for measurements of nitrogen and oxygen isotopes.

Location	Date	Depth (m)	Temperature (°C)	Salinity (PSU)	Dissolved
					Oxygen (mg/L)
West Penn Cove	11 May 2010	7	10.0	28.7	9.7
(48° 26' N, 122° 43'W)	9 Sept 2010	6	12.4	27.6	9.0
East Penn Cove	11 May 2010	10	9.6	28.9	9.0
(48° 14' N, 122° 41'W)	9 Sept 2010	10	11.8	28.7	7.6
Saratoga Passage	11 May 2010	10	9.7	28.4	10.0
(48° 15' N, 122° 35'W)	9 Sept 2010	10	12.0	27.8	8.44
Skagit River	11 May 2010	1	12.4	13.0	13.3
(48° 18' N, 122° 24'W)	9 Sept 2010	1	14.5	17.1	8.8

Fig. 1. a) Map of western Washington USA showing the location of Penn Cove. b) Sites where *Ulva lactuca* was sampled. NE: northeast Penn Cove (Monroe Landing), NW: northwest Penn Cove (Grasser's Lagoon), SE: southeast Penn Cove (Long Point), SR: Skagit River, SW: Southwest Penn Cove. c) Sites where seawater and wastewater were collected. CVTP: Coupeville Wastewater Treatment Plant, EPC: East Penn Cove, PCTP: Penn Cove Wastewater Treatment Plant, SP: Saratoga Passage, SR: Skagit River, WPC: West Penn Cove.

Fig. 2. Photographs of the four sites in Penn Cove where *Ulva lactuca* was sampled: a) NW Penn Cove showing the high percent cover of *Ulva* that occurred throughout the study period, b) SW Penn Cove showing a dense cover of *Ulva*, c) SE Penn Cove where eelgrass (*Zostera marina*), was abundant but ulvoid algae were scarce, and d) NE Penn Cove where all macrophytic vegetation was scarce.

Fig. 3. Concentrations of a) tissue nitrogen (as mean percent of dry mass $\pm$ 1 SD), b) tissue carbon (as mean percent of dry mass $\pm$ 1 SD), and c) the carbon to nitrogen ratio (mean $\pm$ 1SD) in algae from Penn Cove and the Skagit River outflow area during May (black bars), July (grey bars), and September (open bars) of 2010. Asterisks designate sites are where ulvoid algae were common based on visual estimates. Ulvoid algae were scarce at the other three sites. NW: northwest Penn Cove, SW: southwest Penn Cove, NE: northeast Penn Cove, SE: southeast Penn Cove, SR: Skagit River outflow.

Fig. 4. Stable oxygen isotope ratios ($\delta^{18}\text{O}$) as a function of stable nitrogen isotope ratios ($\delta^{15}\text{N}$) in *Ulva lactuca* tissues from four sites in Penn Cove (northwest Penn Cove, closed circles; southwest Penn Cove, closed diamonds, northeast Penn Cove, open circles; southeast Penn Cove, open diamonds) and a site near the Skagit River outflow (open squares) during a) May, b) July and c) September of 2010. Data are means $\pm$ 1 SD. Asterisks designate sites are where ulvoid algae were common based on visual estimates.

Fig. 5. $\delta^{15}\text{N}$ as a function of tissue nitrogen concentration (as % of dry mass) in *Ulva lactuca* from four sites in Penn Cove (northwest Penn Cove, closed circles; southwest Penn Cove, closed diamonds, northeast Penn Cove, open circles; southeast Penn Cove, open diamonds) during a) May, b) July, and c) September of 2010. Asterisks designate sites are where ulvoid algae were common. $\delta^{15}\text{N}$ is not significantly correlated with nitrogen concentration in May (Pearson's correlation coefficient = 0.014, $p=0.941$) and July (Pearson's correlation coefficient = -0.124, $p=0.512$), but is negatively correlated with nitrogen concentration in September (linear regression line: $\delta^{15}\text{N} = 9.61 - 0.291 [\text{nitrogen}]$; Pearson's correlation coefficient = -0.374, $p=0.02$).

Fig. 6. Stable oxygen ($\delta^{18}\text{O}$) and nitrogen isotope ratios ($\delta^{15}\text{N}$) in seawater from the eastern (EPC: open circles) and western (WPC: closed circles) ends of Penn Cove, Saratoga Passage (SP: open diamonds) and the Skagit River (SR: open squares) as well as wastewater from the Penn Cove (PCWWTP: open upward-pointing triangles) and Coupeville (CVWWTP: open downward-pointing triangles) wastewater treatment facilities in a) May and b) September 2010. Data are means $\pm$ 1 SD. An asterisk designates the site closest to shorelines where ulvoid algae were common.

Fig. 7. a) Skagit River discharge rate (m^3s^{-1}) from Apr 15, 2010 to Sept 15, 2010 at the United States Geological Survey (USGS) hydrological station #12200500 at Mount Vernon, Washington (22 km upriver of the SR sampling location). Data were obtained courtesy of the United States Geological Survey. b) Daily rainfall amounts (in cm) for Coupeville, Washington from Apr 15 to Sept 15 2010. Data are courtesy of the National Oceanic and Atmospheric Administration National Center for Environmental Information.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7