The Use of Stable Nitrogen Isotopes in Evaluating Landscape-Level Nitrogen Inputs to Hood Canal, Washington, USA

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ABSTRACT

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Eutrophication can have profound negative effects on marine ecosystems. The nutrient most associated with marine eutrophication is nitrogen (N). Human activities have increased total nitrogen inputs to US marine ecosystems by an average of six times and approximately two-thirds of US coastal waters are moderately to severely degraded due to N pollution. This study evaluated stable nitrogen isotopes and nitrogen content in marine algae at 9 sites in Hood Canal, Washington, USA. The results from this nitrogen analysis were compared with land use parameters in an attempt to identify anthropogenic sources of nitrogen. This study found that nitrogen isotopes in marine algae were significantly correlated with development, sewage effluent, distance to open ocean water, cultivated land, forested land, and wetlands. This study also found a significant link between nitrogen isotope values and nitrogen content in macroalgae. Values found during this study show that enriched isotope values, typically associated with anthropogenic sources of nitrogen, are associated with lower tissue nitrogen values in algae. A shift in the slope of these relationships may be able to delineate an increase in anthropogenic nitrogen and nitrogen loading, possibly noting a shift to eutrophic conditions. The Puget Sound ecosystem has the potential to provides cultural and subsistence resources to our community, if it is managed appropraitely. This research may provide a cost-effective tool to monitor and evaluate nitrogen cycling in Puget Sound so we may better know how to conserve the valuable resources it provides to us, as well as the habitat and resources it provides to the flora and fauna that call it home.

Table of Contents

Appendices

List of Figures

List of Tables

List of Equations

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Introduction

The Puget Sound is an inland marine waterbody in Washington State, linked with the northern Pacific Ocean through the Strait of Juan de Fuca. The beach ecotone in Puget Sound is closely biogeochemically linked with the marine waters of Puget Sound and the adjacent terrestrial environment (Shipman et al. 2010). This linkage between the marine waters of Puget Sound and adjacent terrestrial watersheds plays an important role in marine water quality that will be examined throughout this thesis.

Hood Canal is a sub-basin within greater Puget Sound (Fig. 1). Hood Canal is a narrow fjord with restricted water flow from Admiralty Inlet to the north due to a glacial sill, shown in Figure 1 with a red line, at the entrance to Hood Canal that comes within 50 – 70 m of the water surface. Once water enters Hood Canal, depths can reach 175 m. Hood Canal was the study area for this thesis.

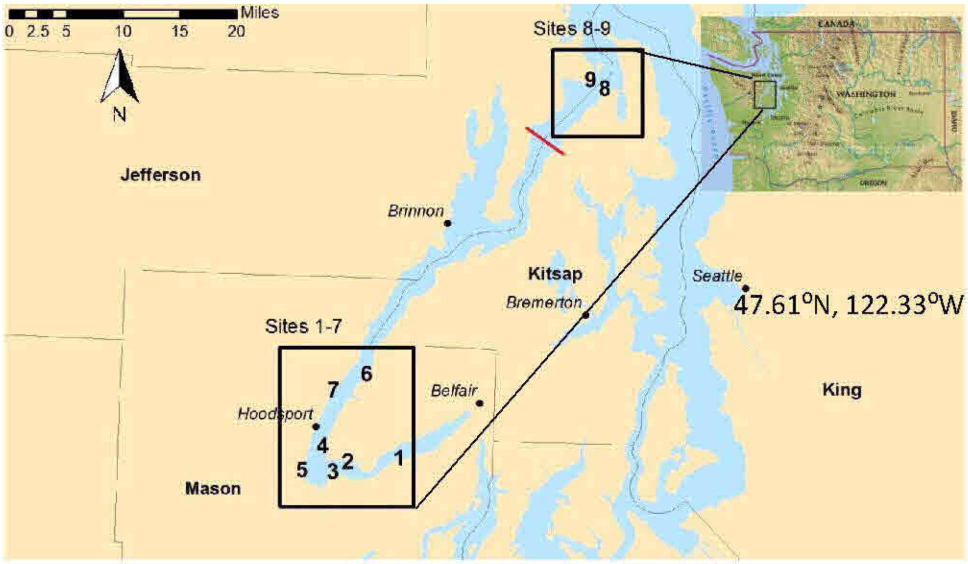

Figure 1. Hood Canal, WA study sites where algal samples were collected. The red line denotes the approximate location of the Hood Canal sill per NOAA bathymetry charts.

Restricted water exchange between Hood Canal and Greater Puget Sound is one factor influencing eutrophication in Hood Canal (Anderson et al. 2008, Dunagan 2009, 2010, 2011, 2012). Eutrophication occurs when algal populations increase due to an increase in nutrients (Sauer et al. 2008). This algal bloom can block sunlight penetration to deeper waters and use up available dissolved oxygen (DO) during subsequent decomposition, lowering dissolved oxygen availability for other flora and fauna. A term commonly used in scientific literature for reduced DO levels is hypoxia, whereas the complete absence of DO is anoxia. Eutrophication and hypoxia can also lead to habitat degradation, changes in food-web structures, and an overall loss of biodiversity (Howarth 1988, Barr et al. 2013)

The nutrient most commonly linked with eutrophication in marine systems is nitrogen (N) (Howarth & Marino 2006, Edwards et al. 2006). Nitrogen comes from natural sources as well as from human activities. Earth's atmosphere is mostly made up of nitrogen gas, which is not readily available to most plant and animal species until it is fixed into another form by bacteria. Humans have roughly doubled the rate of creation of biologically available nitrogen on Earth through the creation of nitrogen fertilizers (Cohen & Fong 2006).

On average in the United States, human activities have increased nitrogen inputs to marine ecosystems six times relative to estimated natural conditions, and approximately two-thirds of the US coastal waters are moderately to severely degraded from nitrogen pollution (Howarth & Marino 2006). Tools that can be used to understand how nitrogen enters the marine system and is then cycled and incorporated throughout the food chain can help land managers better manage nitrogen inputs into this valuable ecosystem. One such tool is the use of stable nitrogen isotopes in marine algae. This method has been used in other areas, but rarely employed in Puget Sound. The following sections of this literature review provide the background knowledge required to understand the key questions examined in this thesis, which are:

1) Do stable nitrogen isotopes in nearshore marine algae reflect terrestrial land use practices in adjacent watersheds to Hood Canal, WA?
2) If so, what is the usefulness of this knowledge in relation to managing episodic eutrophication in Hood Canal?

This first chapter is followed by a second chapter that is a technical manuscript, which includes all data and results. The final chapter includes a summary of interdisciplinary connections to this thesis and final conclusions.

Chapter 1 – Literature Review

1.1. Nitrogen in Marine Waters

1.1.1. Eutrophication and The Nitrogen Cycle

In the late 1960s issues related to nutrient loading into freshwater systems and associated eutrophication and hypoxia were being studied heavily. Although marine eutrophication was not ignored at that time, the focus was clearly on freshwater systems (Kelly 2008). In recent decades marine eutrophication events have been increasing, leading to further research on the causes and implications of increased nutrient loading to marine waters (McClelland et al. 1997, Anderson et al. 2008, Bouillon et al. 2011).

Eutrophication is caused by an increase in nutrients, typically nitrogen in marine systems (Sauer et al. 2008). Other nutrients, such as phosphorus, can play a role in limiting growth in the marine environment, but N plays the main role in Hood Canal and Puget Sound (Newton et al. 2011, Mohamedali et al. 2011, Cope & Roberts 2013). Phytoplankton growth can increase in Puget Sound 300% with the addition of N (Newton et al. 2011). Hypoxic water issues throughout the Puget Sound have also been attributed to nitrogen loading (Mohamedali et al. 2011, Cope & Roberts 2013).

In a study conducted in Narragansett Bay, Rhode Island, USA a group of researchers explored the relationship between phosphorus and nitrogen limitation along a freshwater to saltwater estuarine gradient. They found that limitation shifted from phosphorus to nitrogen as the water became more saline in the estuary (Doering et al. 1995). Additional ecosystem-scale experiments have been conducted in Himmerfjarden Bay and Laholm Bay in Sweden, which also point to nitrogen as the main limiting nutrient in those systems (National Research Council 2000).

A well-cited paper by Robert Howarth addressed the debate around what nutrient is limiting to marine systems (Howarth 1988). Prior to this time scientists had been debating between the importance of phosphorus and nitrogen inputs to eutrophication of marine waters on temporal and spatial scales. Howarth (1988) argued that nitrogen was limiting in northern temperate zones initially based on low nitrogen to phosphorus (N:P) ratios in phytoplankton or water samples. Nitrogen and phosphorus are typically evaluated against the Redfield ratio of 16 moles N to 1 mole of P. Many of the marine studies cited by Howarth (1988) ratios fell below this 16:1 ratio. When the Redfield ratio is below 16:1 phytoplankton growth will be limited by the availability of N. Pedersen and Borum (1996) also confirmed in a study of four algal species that the ratio was always below 16, and below 1 during the entire summer season. Second, there is a slower rate of nitrogen cycling compared to phosphorus cycling in marine waters. Remineralized nitrogen from marine benthos tends to be depleted in nitrogen, as the bacteria in the sediments sequester nitrogen, not readily releasing it back to the water column. Finally, there is a lack of nitrogen fixing cyanobacteria in marine waters, as cyanobacteria may make up only 1% of phytoplankton biomass in marine systems, these would be the only natural supply of nitrogen to marine systems (Howarth 1988).

As of 2008, the case for nitrogen as the main limiting nutrient in marine systems is generally accepted by the scientific community (Howarth & Marino 2006, Edwards et al. 2006, Howarth 2008). It is important to note that other nutrients can play a role in nutrient limitation and eutrophication in marine systems, and these relationships should be examined relative to specific water bodies. It is also important to manage for both nitrogen and phosphorus inputs, since managing for only nitrogen may lead to a case where phosphorus increases and becomes the nutrient controlling eutrophication (National Research Council 2000).

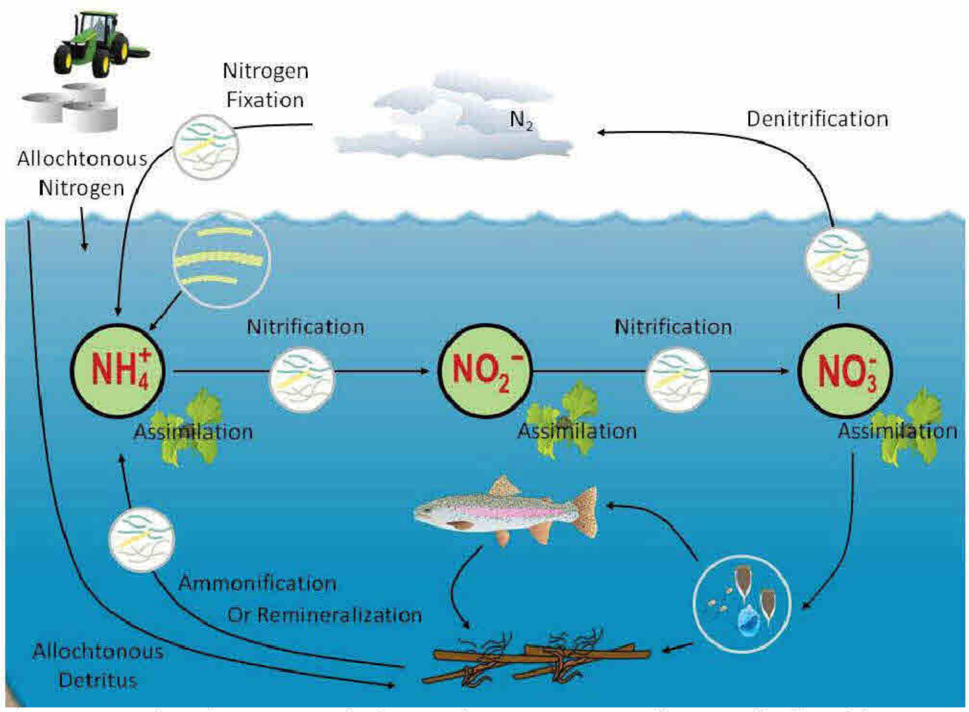

Figure 2. The nitrogen cycle in marine systems. Nitrogen is fixed into a biologically available form by cyanobacteria in the water column or terrestrially by nitrogen fixing bacteria before entering the water. Other sources of nitrogen include detritus and anthropogenic sources from outside the system. Nitrogen is incorporated into algal tissue as ammonium NH_4^+, nitrite NO_2^-, or nitrate NO_3^-, collectively referred to as dissolved inorganic nitrogen (DIN). Assimilated nitrogen may stays in biological tissue until it is cycled back into DIN or denitrified and released

back into the atmosphere as nitrogen gas. Nitrification refers to the oxidation of NH_4^+ to NO_2^- and NO_3^-. Denitrification refers to the reduction of N back to gaseous N_2. Both nitrification and denitrification are facilitated by bacteria. Denitrification occurs in low oxygen environments where the bacteria use nitrogen as a terminal electron acceptor, rather than oxygen.

Nitrogen can come from a variety of anthropogenic (inorganic fertilizers, sewage, farm waste) and natural sources (atmospheric deposition, ocean water, ground water). Nitrogen also comes in a variety of forms, some of which are more labile than others. Dissolved forms can be separated into organic and inorganic forms (Fig. 2). Ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) are all inorganic forms of nitrogen, which are required by plants for growth. Nitrate or ammonium are typically the favored species for algal growth (Lobban & Harrison 1997, Graham et al. 2009). In a laboratory experiment with *Fucus spiralis*, a brown macroalgae, nitrate, ammonium, and nitrite were estimated to have contributed 59, 39, and 2% of the nitrogen to the plant (Topinka 1978).

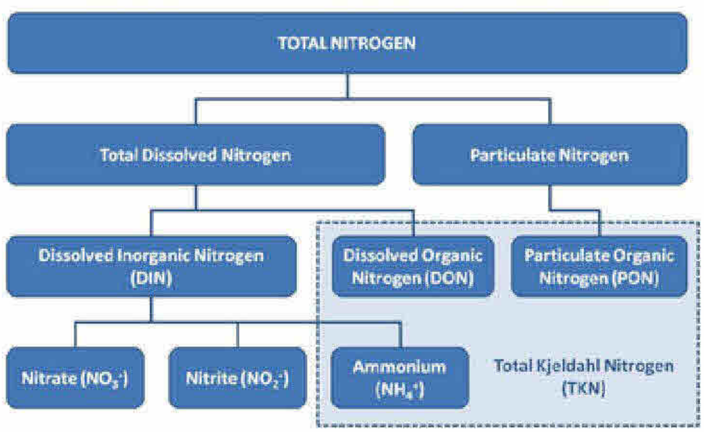

Figure 3. Forms of nitrogen ("Nitrogen in the Puget Sound Ecosystem" 2013).

Sources that are not from a specific location, known as non-point sources, include septic systems, lawn fertilization, and surface runoff. Non-point sources are estimated to contribute to 65% of the total pollution load in inland surface waters (Narumalani et al. 1997). Manure N sources are hard to estimate because content is based on livestock type, health, and age (Follett 2008). Similarly human sewage waste is typically estimated based on average values, which may inaccurately predict the contribution. Point sources originate from a precise location, i.e. rivers, streams, and outfalls. All of these sources need to be estimated for inclusion into nutrient cycling models. It is estimated that human sources of N have doubled the amount of biologically available N to 120 Tg/yr (Chapin et al. 2002, Cohen & Fong 2006).

Evaluating N inputs and subsequent effects from increased nutrients can be difficult. As N is a limiting nutrient it may be rapidly taken up by biota and thus not accurately detected with in situ water samples (Peterson et al. 2007). Computer modeling is a common method for evaluating N loading and its effects on marine systems and many values presented here are the results of modeling studies (Paulson et al. 2007, Newton et al. 2011, Mohamedali et al. 2011). This may not provide accurate or precise results, as many input variables to the model are estimates and not measured values. There are uncertainties in the estimation of N contributions, which could place actual human contributions higher or lower than current predictions. Some of this uncertainty is also due to water sampling, which can vary significantly due to N processing over short spatial and temporal scales (Cope & Roberts 2013).

Even though there are limitations of modeling, it is a widely used tool, that can provide valuable insights into ecosystem processes that could not be obtained using other methods. Modeling is used extensively at the Washington Department of Ecology to estimate the effects of changes to pollution sources and the subsequent response in water quality. Humans can never know every component of a natural system, and models provide a way to simulate natural systems and perturb and manipulate these system to better understand cause and effect relationships.

Eutrophication can alter the marine ecosystem in a variety of ways, which are just briefly highlighted here. Nutrient enrichment can reorganize the hierarchy of the food web, modifying the bottom tier by favoring fast growing algae species such as *Ulva* or planktonic species over slow growing perennials (Pedersen & Borum 1996, McClelland et al. 1997, Wahl et al. 2011). Fast growing species tend to be smaller and better equipped to incorporate nutrients quickly due to a short life span, in contrast to perennial species which can tolerate periods of non-ideal growing conditions for a time. Nitrogen eutrophication may also favor the growth of invasive species, as invasive species tend to be fast growing and opportunistic, taking advantage of excess nutrients faster than slower growing native species. One study found an increase in invasive plant biomass and density after a manipulative nitrogen addition experiment (Tyler et al. 2007). Bertness et al (2002) found that increased N correlated with the spread of invasive cordgrass in New England, USA (Bertness et al. 2002). Thus, eutrophication may decrease the diversity of photosynthetic species (Dhargalkar 1986). All these effects of eutrophication may lead to less stable food webs (McClelland et al. 1997). In addition, some fast growing algae species may produce toxic chemicals as by-products that can affect human health (Kelly 2008). The Washington State Departments of Ecology and Health monitor for algal blooms and signs at beaches during the Spring and Summer months warning of toxic blooms and toxic shellfish are relatively common.

Marine plankton response to nutrient loading can be linear as shown in figure 4 (Smith 2006, Kelly 2008). From the Washington State Department of Ecology marine water quality monitoring program data total nitrogen in Hood Canal never exceeded 100 μmol N L^{-1} from 2007-2012 at all sample depths. Assuming that phytoplankton in Hood Canal are limited by N, this data suggests that even at the maximum levels experienced in Hood Canal, phytoplankton may respond with linear growth, which may lead to eutrophication and hypoxia.

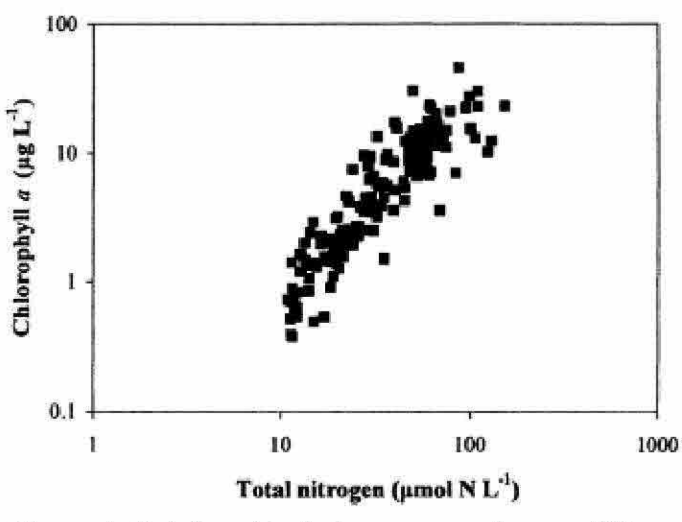

Figure 4. Relationship between annual mean Chl α and annual mean TN concentration in estuarine and marine ecosystems at 92 sites worldwide (Smith 2006).

1.1.2. Hood Canal

Hood Canal has experienced eutrophication and hypoxic events since the 1930s and fish kills from hypoxia reportedly as far back as the 1920s (Newton et al. 2011). Dissolved oxygen in Hood Canal varies annually, but has shown an overall decreasing trend since 1950 (Newton et al. 2011). This basin was known to have low dissolved oxygen issues naturally (see below for explanatory mechanisms), but since the mid-1990s hypoxia (low dissolved oxygen) events have increased in duration, frequency, and intensity (Newton et al. 2011). Lower Hood Canal, beyond the Great Bend, has had the most severe hypoxic events and the greatest human N contributions (Cope & Roberts 2013). This is most likely due to the basin geography, the channel changes directions at the Great Bend and switches to an angle where winds do not mix the surface as readily.

The Pacific Ocean supplies approximately 68% of the N load to Puget Sound, while local anthropogenic sources supply the remaining 32% based on model results (Mohamedali et al. 2011). In Hood Canal specifically the contribution of oceanic nitrogen is even greater (figure 5). It is known that hypoxic events have occurred historically, but that these events have increased in frequency and severity, likely associated with increased development (Newton et al. 2011). This suggests that even though the Pacific Ocean is the main source of N, the smaller, local anthropogenic contributions may play an important role in triggering eutrophication.

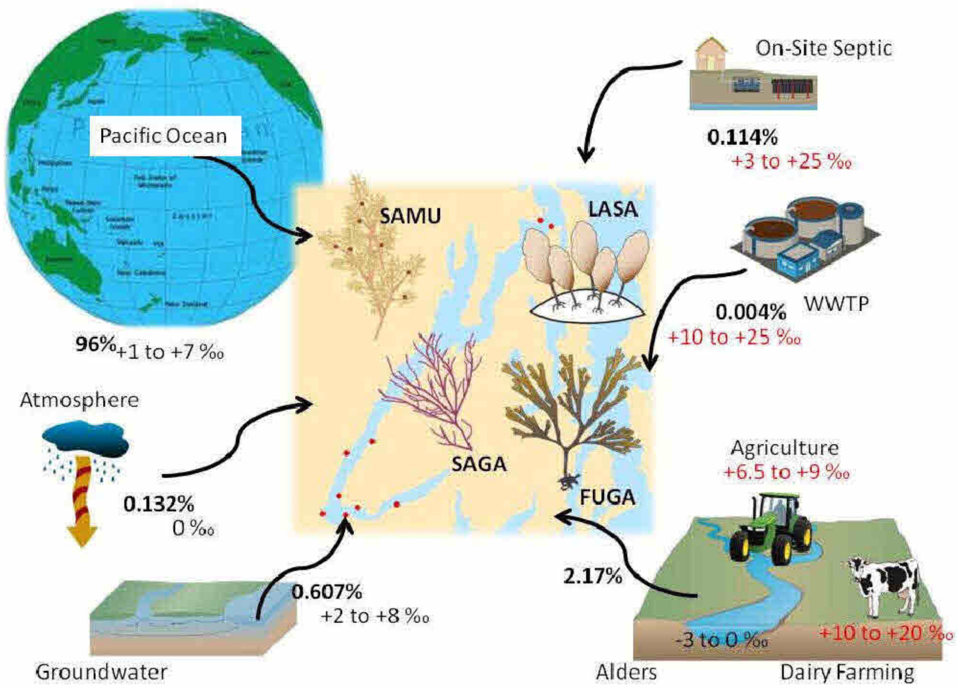

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Figure 5. Sources of nitrogen to Hood Canal. The percentage values in bold represent the percent contribution of the source to the total nitrogen load in Hood Canal. The percent per mil (‰) values are the estimated $\delta^{15}N$ values of that source during at the time of incorporation into algal tissue. A summary table of references for these values are presented in Appendix B.

Hood Canal's primary production is N limited, and surface primary production may increase by as much as 300% with an increase in N (Newton et al. 2011). Hypoxic events tend to occur in the late summer and fall when warmer, less dense, oxygen-rich freshwater sits over colder, saline waters from the Pacific, isolating the lower marine layers from mixing and incorporation of dissolved oxygen into the water column (Paulson et al. 2007). Eutrophication and hypoxia are not typically a problem during winter months due to storms that increase mixing and aerate the water column. Paulson et al (2007) also found a lack of Dissolved Inorganic Nitrogen (DIN) in the top 7 m during late summer months, suggesting that DIN was being used by phytoplankton and would eventually produce labile organic matter that would settle out and consume oxygen deeper in the water column due to its subsequent respiration (Paulson et al. 2007).

Wastewater treatment plants (WWTP) and rivers are the two largest point sources of N to Puget Sound. These sources have temporal variability contributing 60% and 40% to the N load annually, but during the summer months WWTP contribute 80% and rivers only 20% (Mohamedali et al. 2011). This makes intuitive sense as rainfall is less during summer months, so less N would be mobilized from the watershed via rivers.

The greatest anthropogenic DIN concentrations overall come from On-site Septic Systems (OSS), WWTP, and stormwater, respectively in the Puget Sound. Direct outflow from OSS ranges from 20 – 60 mg/L DIN, WWTP from 8 – 30 mg/L DIN, and stormwater from 0.3 – 2 mg/L DIN ("Nitrogen in the Puget Sound Ecosystem" 2013). These all happen to be manageable sources. The relative percent contribution of all nitrogen sources to Hood Canal is presented in figure 5.

OSS plays a critical role in hypoxic events in Hood Canal (Newton et al. 2011). Traditional OSS, as are commonly used in Hood Canal, remove only 20% of nitrogen prior to discharge (Swann 2001). Estimates range from 0.2 mg/L – 0.5 mg/L drawdown of DO per month from OSS inputs in Hood Canal (Newton et al. 2011). These drawdown levels can be enough to make fish show avoidance behavior of water masses with DO concentrations that are this low.

To illustrate the area in Hood Canal affected by low DO events, Figure 6 shows the dissolved oxygen levels in Hood Canal in August 2006. Values below 2 mg/L are considered hypoxic, this is also the level at which rockfish would show avoidance. Salmonid species require much higher DO levels than rockfish, at least above 5 mg/L for survival, which only occurs in this figure seaward of the sill (Quinn 2005).

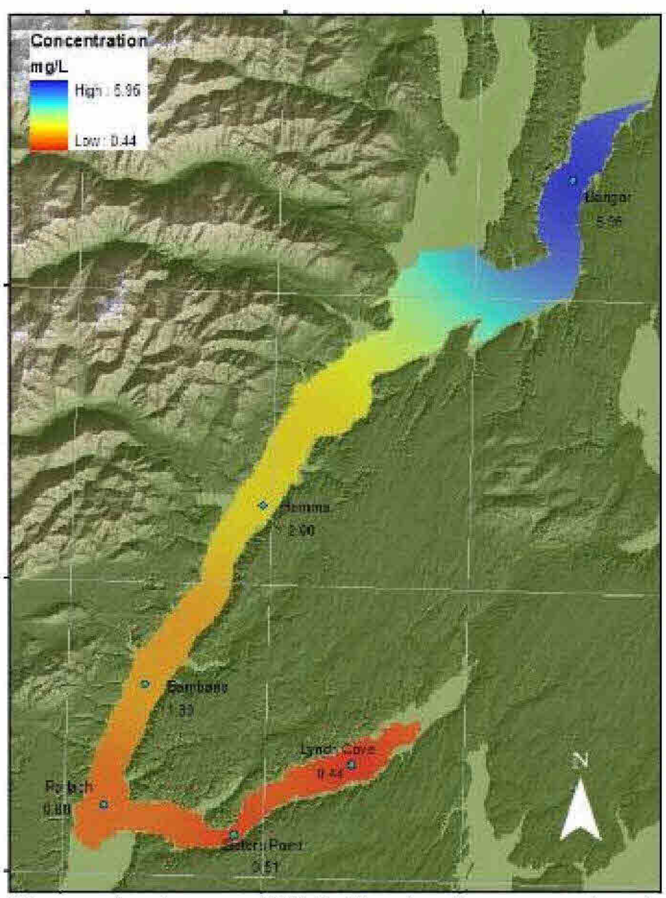

Figure 6. August 2006 dissolved oxygen levels (mg/L) (Newton et al. 2011).

Reduction in DO also occurs from the consumption of DO during decomposition of algae created during eutrophication. Denitrification does not typically occur from OSS discharge that spends little time subsurface, because the water flows laterally to the shoreline rather than entering groundwater first, delivering the N as ammonium. Denitrification does not occur in these situations because N is never exposed to low oxygen levels required for denitrification (Paulson et al. 2007).

The Washington State Legislature sets limits for water quality parameters based on the uses of that water body. For example, excellent quality marine water would have a 1 day minimum DO value of 6.0 mg/L ("Marine water designated uses and criteria" 2014). The one day minimum value means that if a water body is being evaluated for water quality and the minimum on a given day value falls below 6.0 mg/L that water body could not be considered to have excellent water quality. If measured values fall below the minimum level for a given water body including a 0.2 mg/L action level buffer (added to account for potential modeling errors), then the water body is listed on the impaired water bodies list for the state ("Water Quality" 2014). Cope and Roberts (2013) predicted, using modeling, that DO drawdown from anthropogenic sources may be over the 0.2 mg/L action level (Cope & Roberts 2013). This means that anthropogenic N sources may contribute enough N to bump water quality in a given area onto the impaired water bodies listing.

Eutrophication events can be pronounced in confined areas, or areas with long residence times, such as Hood Canal (Brennan & Culverwell 2004). It may take over one year for water in Hood Canal to cycle back to the Pacific Ocean ("Hood Canal Statistics" 2008, Newton et al. 2011). This long residence time is partially due to a sill at the entrance to Hood Canal which rise to within 50 – 70 m of the water surface, where the main body of Hood Canal is up to 175 m deep (Paulson et al. 2007). Hood Canal is the most sensitive basin in Puget Sound to increased N loads and is discussed further in the next section (Newton et al. 2011).

1.2. Nitrogen Isotopes

1.2.1. Introduction

Stable isotopes can be used as a tracer through the environment. For this to be an effective tool information is needed on the chemical isotope being used and how it is processed in the environment. Stable nitrogen isotopes have been used in many studies to trace anthropogenic sources of nitrogen in fresh and salt water systems. The most common way this has been applied is through the tracing of WWTP effluent. The following sections describe basic isotope chemistry, N isotopes in marine systems, tracing anthropogenic sources of N using isotopes, and how marine algae processes and utilizes N.

1.2.2. Basic Concepts

An isotope is an atom with the same number of protons and electrons, but differing numbers of neutrons. ^{14}N makes up the majority of N atoms in the world. ^{15}N is a stable N isotope that comprises less than 1 % of the world's nitrogen. The superscript numbers in front of the element reflect the number of neutrons and protons in that isotope, to be the same element there must be the same number of protons, thus ^{15}N has one more neutron than ^{14}N. Delta (δ) notation is used to compare N isotopic content in a sample to N content in a standard. The standard for nitrogen is the ratio of $^{15}N:^{14}N$ in the atmosphere (0.0037).

The delta value for a sample is calculated with the equation below (equation 1):

$$\boldsymbol{\delta(‰) = \left(\frac{Rsample}{Rstandard} - 1\right) \cdot 1000}$$

Equation 1. Difference in sample isotope composition compared to the standard, in parts per thousand (Michener & Lajtha 2007). A positive δ value indicates more of the heavy isotope is present in the sample than the standard, whereas a negative δ value indicates the sample has more of the lighter isotope than the standard.

Variations in isotopic composition occur due to preferential use of isotopes, this process is called fractionation. Typically, heavier isotopes move more slowly through biological processes, but this may be affected by the rarity of a compound and necessity of it for system functionality. Michener and Lajtha (2007) present an excellent overview to stable isotopes and their use in ecology and environmental sciences

1.2.3. Marine

In nitrogen limited systems, such as Puget Sound, extensive mixing in the nearshore and uptake through biological processes can make accurate detection of N fluxes difficult (Dailer et al. 2010). Isotopic signatures may be more meaningful than water quality samples due to temporal and spatial variability in marine waters that obfuscate sources of N input (Cohen & Fong 2006). More specifically, many micro-algae can rapidly take up nutrients, thus water samples may miss short, transient fluxes of N. Also, water quality samples cannot confirm the source of N or the incorporation of N into the food web, as they will simply give you the amount of nitrogen and what the form of nitrogen is (Peterson et al. 2007).

Stable isotopes have been used since the 1970s in marine environments to evaluate anthropogenic sources of N, tracking of sewage wastewater, and food web interactions (Michener & Lajtha 2007, Bouillon et al. 2011). Isotopic signatures between N sources are can be distinct in marine systems, although there is some overlap (McClelland et al. 1997, Costanzo et al. 2001). As shown in figure 5 background isotope values in Hood Canal should range from 0 to 8 ‰. Typical values for marine water samples range 3 to 6 ‰, with a global average of about 4.8 ‰ (Michener & Lajtha 2007). Multiple studies, which will be highlighted in the next section (1.3.3), demonstrate that most anthropogenic sources of N differ significantly from background marine values (Robinson 2001). A summary table of $\delta^{15}N$ values from studies presented in this text is available in Appendix A.

It is important to note that if N sources are mixed or N undergoes fractionation by the organism taking up this nitrogen, any initial difference in source pools may be negated. Nitrogen is the limiting nutrient in Hood Canal (Newton et al. 2011). In theory this means little or no fractionation would occur from the water to assimilation of N into algal tissue, and this is the dominate theory utilized in the studies presented in the next section (1.3.3) (Fogel & Cifuentes 1993).

When comparing land use influence on $\delta^{15}N$, as this thesis presents in Chapter 2, it is important to identify any processing that would occur during transport of N from the initial source to marine waters. As shown in figure 2 the main steps in the nitrogen cycle are fixation, assimilation, mineralization, nitrification, and denitrification. In general, the majority of fractionation would occur during the rate limiting step (Michener & Lajtha 2007). The rate limiting step is the slowest reaction in and chain of chemical reactions, and determines the overall speed at which the complete reaction can occur. In Hood Canal the rate limiting step is most likely the oxidation of NH_4^+ to NO_2^-, during nitrification, based on low NH_4^+ levels in Hood Canal (Michener & Lajtha 2007, "Marine Water Quality Monitoring" 2013). This step happens to be diffusion limited at low NH_4^+ concentrations and is typically associated with a low fractionation factor (Michener & Lajtha 2007). Thus, nitrification is not expected to be associated with fractionation in Hood Canal. Nitrification requires a high oxygen environment, whereas denitrification occurs in low oxygen environments (Chapin et al. 2002). Also, nitrate reductase (the enzyme responsible for nitrate assimilation into plant tissue) and the cellular pathway responsible for assimilation of ammonium both discriminate against ^{15}N, but this is only observed when N supply exceeds N demand in the system (Michener & Lajtha 2007). As Hood Canal is N limited ^{15}N discrimination is most likely not occurring. Bacteria responsible for denitrification may also discriminate against the heavier isotope, with a fractionation factor ranging from 30 – 40 ‰, which would need to be taken into account during any isotope analysis conducted during low DO events (Michener & Lajtha 2007). This would result in enriching the N left behind.

Increased development in Hood Canal should result in enriched $\delta^{15}N$ values and increased total N content (%). This would be due to increased runoff and increased N inputs from anthropogenic sources (i.e., fertilizer), in particular OSS discharge, which have been demonstrated in other studies to be associated with enriched $\delta^{15}N$ values (see section 1.3.3). OSS discharge would be enriched due to denitrification processing by bacteria that preferentially use the lighter isotope.

Increased forest cover may result in enriched $\delta^{15}N$ levels in marine macroalgae due to increased denitrification in forest soils draining to marine waters. This response will vary depending on the extent of denitrification, the amount of N assimilation into forest tissue, and volitization of N back to the atmosphere. Although Pacific Northwest forests are N limited, similar to the N limitation in the marine environment, so it is likely that the fractionation signal in marine macroalgae due to increased forest cover would be weak (Edmonds et al. 1989, Chapin et al. 2002). It is also possible that if the source of N to the forests is predominately from anthropogenic sources (i.e. enriched) and little fractionation occurred, the N output would also be enriched.

As wetland area increases $\delta^{15}N$ may be enriched due to increased denitrification. This may vary similarly to forest cover, depending on the extent of denitrification, the amount of N assimilation into forest tissue, and volitization of N back to the atmosphere. In strongly reducing environments with high sulfide concentrations such as coastal marshes the dissimilatory reduction of NO_3^- to NH_4^+ (DNRA) is an important process and NH_4^+ produced through this process has been shown to be depleted in $\delta^{15}N$ (McCready et al. 1983, Michener & Lajtha 2007). The level of depletion in sulfur rich wetlands has not been well documented and requires further study. It is possible that any wetlands identified in this study may have elevated sulfur levels, due to proximity to marine sediments (Giblin & Weider 2014).

As pastured and cultivated lands increase $\delta^{15}N$ may be enriched, due to an increase if organic fertilizers and livestock wastes. If the fertilizer used was predominately inorganic the signal may be depleted. Total N content (%) should also be increased due to increased runoff.

1.2.4. Anthropogenic Sources

The ability to trace nitrogen sources comes from initial differences in the isotopic composition of nitrogen from different sources. Subsequent processing of nitrogen and the effects on nitrogen isotopic composition need to be well understood to trace nitrogen from source to the biological indicator being analyzed, which in this study was marine macroalgae. As was described previously nitrogen is limiting in the marine environment and fractionation is minimal during the incorporation of nitrogen into algal tissue (Howarth & Marino 2006, Dudley 2007, Barr et al. 2013). This means that isotope values of nitrogen sources presented in figure 5 should be representative of the nitrogen sources mixed into algal tissue (McClelland et al. 1997, McClelland & Valiela 1998a, Savage & Elmgren 2004, Savage 2005). If a $\delta^{15}N$ value is anywhere above the normal range of ocean water, 3 to 6 ‰ or more conservatively anything above 8 ‰, it is very likely that anthropogenic nitrogen was a source to that sample. There is some evidence that algae found in lower light conditions (i.e. deeper in the water column) may have comparatively lighter $\delta^{15}N$ signatures (Dudley 2007). This would be due to lower light levels, thus lower photosynthetic rates. Any fractionation would lead to a reduction in the ability to detect enriched nitrogen signatures from anthropogenic sources. Many studies have traced anthropogenic sources of N in marine and freshwater systems, but only one published study has been conducted in Hood Canal (Paulson et al. 2007).

Human and animal wastes are enriched in ^{15}N, with values ranging from 10 to 20‰ (Aravena et al. 1993, McClelland et al. 1997). This is a result of volitization and microbial processing of the N in the waste material, which favor ^{14}N (McClelland et al. 1997, Michener & Lajtha 2007, Dailer et al. 2010, Bouillon et al. 2011). Some of this variability may be due to variability in composition of the source material. Synthetic, inorganic fertilizers have depleted values ranging from -3 to +3‰ (McClelland et al. 1997). The average $\delta^{15}N$ marine water is 4.8‰ (Michener & Lajtha 2007). There are overlapping ranges for these sources, but the sampling methodology, question being addressed, and understanding of the surrounding landscape still make this approach a useful tool in understanding anthropogenic influence in the marine N cycle. Peterson et al (2007) was able to correlate isotope samples in phytoplankton and benthic invertebrates with land use analysis in the Great Lakes Region, USA, supporting this approach. The Peterson et al (2007) study is similar to the approach in this thesis.

1.2.5. Synthesis of Similar Studies

A number of studies that used stable N isotopes are discussed below, but only one was found that took place in Puget Sound. A United States Geologic Survey (USGS) study in Lynch Cove, South Hood Canal, utilized $\delta^{15}N$ and $\delta^{13}C$ analysis of Particulate Organic Matter (POM) and corresponding water NO_3^- concentrations and $\delta^{15}N$ of the NO_3^-. They found higher ratios of C:N closer to the shoreline, suggesting a greater terrestrial influence. They also found enriched $\delta^{15}N$ at shallower water sites, as shown in figure 7 (Paulson et al. 2007). As noted previously enriched values may represent anthropogenic sources.

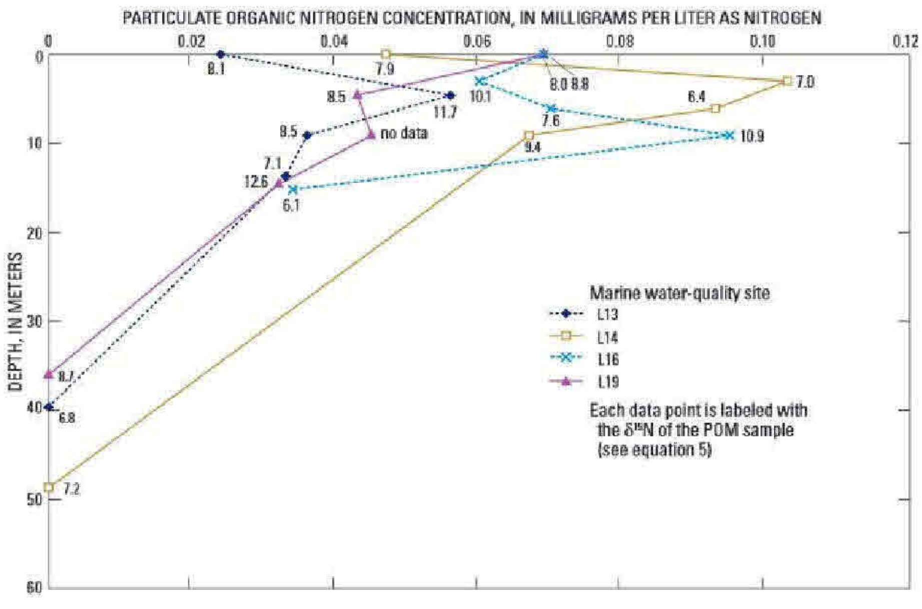

Figure 7. Relation of concentration of particulate organic nitrogen with depth at four water-quality sampling sites and nitrogen isotope data for each particulate nitrogen sample in Lynch Cover, western Washington, September 2004 (Paulson et al. 2007).

Steinberg et al (2011) examined the influence of watershed soils, vegetation, population density, and physical features on N export from tributaries feeding into Hood Canal in a modeling study. They found the highest N export from rivers during the winter (November to January) period. They also found that wastewater (OSS and WWTP) was a significant N source in Lynch Cove (directly contributing 21 to 41% of total N), but a minor source for Hood Canal as a whole. They were able to use a regression model that indicated population could be a predictor of N concentration in Hood Canal. They also found that OSS output was denitrified in the water column, based on N concentration profiles in deeper water. Their sampling sites were in deeper water, farther removed from the initial OSS nitrogen source (Steinberg et al. 2011). This denitrification may not affect the $\delta^{15}N$ values of nearshore algae as nearshore algae is in shallow water when inundated, but if mixing with deeper waters does occur it may influence the N signal in nearshore algae. Valiela et al (1997) estimated that two-thirds of the N from OSS would remain in the water column 200 m from the discharge source, thus sampling macroalgae in the shallow nearshore may yield different results than Steinberg et al study (Valiela et al. 1997).

Pruell et al. (2006) measured stable isotope ratios in six species of flora and fauna in Narragansett Bay, Rhode Island, USA. They collected samples along a sewage gradient monthly from June to October for three years. They found a significant decrease in $\delta^{15}N$ with distance from the contamination source for four of the six species. The two species that did not follow the contamination gradient were ribbed mussels and cord grass. They hypothesized that these two species more closely reflected local groundwater influences than other species sampled. This study also found temporal variability in some of the species sampled and highlights the need to select appropriate species for study (Pruell et al. 2006). This thesis research would have benefited with more lead and preparation time to select sites ahead of time and inventory potential species for sampling, as it turned out species sampled were essentially the only available species at the sampling sites.

Wigand et al (2007) conducted a study looking at nitrogen stable isotopes in New England, USA. They evaluated six species, two green seaweeds and four vascular plants that inhabit different intertidal zones. Five of the studied species showed significant relationships of increasing $\delta^{15}N$ values with increasing wastewater nitrogen. They also found that sites with a vegetated buffer (n=24) significantly reduced the $\delta^{15}N$ mean of *Phragmites autralis*, which is a facultative marsh species (Wigand et al. 2007, "USDA Plants Database" 2013). This suggests that vegetated buffers may sequester nitrogen, reducing the nitrogen released beyond the buffer. The species that showed the strongest relationship with $\delta^{15}N$ were highest in the intertidal zone, this also highlights the importance of species selection.

Savage (2005) and Savage and Elmgren (2004) traced gradients in $\delta^{15}N$ from sewage using the species *Fucus vesiculosis* in the Baltic Sea. The study compared $\delta^{15}N$ values before and after the installation of an enhanced tertiary treatment at a wastewater plant and found the zone of influence reduced from 24 km to 12 km. Two-source mixing models estimated the percent sewage contribution to total N in algal samples near the outfall and found that wastewater contribution was 40% prior to enhanced treatment and 12% after. They also found a strong correlation between total surface water N and macroalgal $\delta^{15}N$ (r^2=0.82) (Savage & Elmgren 2004, Savage 2005).

Rogers (2003) conducted a similar study, tracking the change in $\delta^{15}N$ after the decommissioning of a sewage treatment facility. The study found that $\delta^{15}N$ returned to background levels after 3 months in algal tissue and recovery was slower in consumers sampled, but was notable after 9 months (Rogers 2003). These studies are examples of the most common application of $\delta^{15}N$, which specifically try to trace sewage effluent through the system being studied.

Johannsen et al (2008) evaluated the $\delta^{15}N$ of nitrate (NO_3^-) in five German rivers draining into the North Sea for one year. Their study found the maximum nitrate loads in winter and fall when discharge and concentration were highest. Isotope signals in the winter were more depleted, which was attributed to less microbial and assimilative processes. Bacteria are less active in the winter, thus incorporating less of the light ^{14}N into their tissue, which depletes the remaining DIN. In summer months there was an increasing $\delta^{15}N$ and decreasing NO_3^- concentrations, indicating that the assimilation of NO_3^- into biological processes was the main fractionation processes. Although the main fractionation event in this study was microbial processing, the mean $\delta^{15}N$ of NO_3^- in summer ranged from 8.4‰ to 14.3‰ and in the winter from 7.8‰ to 10.8‰, both seasoned were enriched relative to typical marine values (Johannsen et al. 2008). This study suggests that sampling in summer or winter should provide data to reach similar conclusion. Many of the studies that trace sewage effluent collect samples only in the summer, but for this thesis samples were collected in the winter. The

Johannsen et al (2008) study suggests that patterns may be more pronounced during a summer sampling.

Steffy and Kilham (2004) studied an urban freshwater stream system and found enriched $\delta^{15}N$ in biota associated with OSS (Steffy & Kilham 2004). Another study in freshwater found the relationship between $\delta^{15}N$ and DIN concentrations indicated that $\delta^{15}N$ was a valid indicator of eutrophication. They compared DIN concentrations and $\delta^{15}N$ of fish tissue samples (Lake et al. 2001). As OSS is the main sewage disposal method in Hood Canal it is important to note that enrichment has been documented from both OSS and WWTP effluent.

Rolston et al (1993) evaluated nitrate in groundwater. Their study was able to differentiate between animal waste sources, inorganic fertilizers, and organic matter sources of NO_3^-. Their study found that little to no denitrification occurred in the transport of waters through soil to groundwater (Rolston et al. 1996). This is significant to Puget Sound where many rural areas utilize OSS that drain through groundwater to Puget Sound. McClelland and Valiela (1998) found an increase in groundwater $\delta^{15}N$ from -0.9 to 14‰ with a corresponding wastewater input increase from 4% to 86% of the total nitrogen pool (McClelland & Valiela 1998b).

Carmichael et al (2004) suggested that it may be possible to develop methods to detect the beginning stages of eutrophication before negative effects are seen in the ecosystem. Their study evaluated an estuary with relatively low nitrogen loading and concluded that with lower N loads and shorter flushing times, land-derived N sources could be predictably linked to N in biota through the mean $\delta^{15}N$ values (Carmichael et al. 2004). Another study also suggested that shifts in isotopic composition may be detectable at lower loading rates, foreshadowing eutrophication events (McClelland et al. 1997). These results could be very important for Hood Canal, suggesting that a monitoring program could identify the beginning stages of eutrophication, perhaps with enough lead time to put in measures to alleviate the severity or duration of the event.

The use of N isotopes has been field tested in multiple marine and freshwater studies and may show promise for further applications in Puget Sound. As with any methodology, care needs to be taken in drawing conclusions from data. It is particularly important to incorporate fractionation processes and overlapping ranges of $\delta^{15}N$ for anthropogenic source pools (Viana & Bode 2013, Raimonet et al. 2013).

1.2.6. Marine Algae Nitrogen Use

Nitrogen is an essential nutrient for algal growth and is typically the limiting nutrient in marine systems. It is found in amino acids, purines, pyramides, and sugars within algal tissue (Lobban & Harrison 1997). The concentration of nitrogen in algal tissue can be as much as 10^4 to 10^5 greater than concentration in seawater and many species have been found to sequester nitrogen for periods when it will not be readily available (Pedersen & Borum 1996, Lobban & Harrison 1997). Nitrate and ammonium are the most important ions for algal growth, but some species may also utilize nitrite (Lobban & Harrison 1997). Marine algae are unable to utilize atmospheric nitrogen, unless associated with nitrogen-fixing bacteria. Nitrogen is taken up by seaweeds through cellular walls through adsorption, passive transport, facilitated diffusion, or active transport (Lobban & Harrison 1997). Work has demonstrated that several intertidal species may exhibit enhanced short-term nutrient uptake during mild desiccation (Lobban & Harrison 1997). This finding may have implication for $\delta^{15}N$ values of intertidal species associated with properties utilizing OSS that releases to Puget Sound through groundwater.

Selection of an appropriate reference for isotope studies is crucial. Fractionation, the differential use of ^{15}N or ^{14}N, may vary between primary producers (McClelland et al. 1997). Although, as noted previously, nitrogen is the limiting nutrient in Puget Sound and in such situations fractionation should be small. Plants would not have the luxury to use only the lighter isotope, which they prefer (McClelland et al. 1997). Long-lived algal species should reflect the ambient nitrogen supply (Edwards et al. 2006, Dailer et al. 2010).

Waldron et al (2000) found $\delta^{15}N$ values between phytoplankton and effluent from a sewage treatment facility near Edinburgh, Scotland were not significantly different, suggesting that photosynthetic marine algae appear to be a good tracer for of anthropogenic sources in that marine ecosystem (Waldron et al. 2001).

Another study that looked at N fractionation during the assimilation of N by a marine diatom found that fractionation increased with increasing concentration. The cited a fractionation factor of 8 ‰ of NH_4^+ with concentrations above 5 μM. In Hood Canal none of that water samples shallower than 10 m from 2007 – 2012 were even above 1 µM concentration (Waser et al. 1998, "Marine Water Quality Monitoring" 2013). Vavilin and Lokshina (2014) found a similar pattern, also in marine diatoms, with no fractionation below 50 µM concentration of NO^{3-}. There were no samples shallower than 10 m from 2007 – 2012 above 35 µM ("Marine Water Quality Monitoring" 2013). For fractionation to occur N concentrations must be elevated well above levels present in Hood Canal based on 2007 – 2012 water sample data (Fogel & Cifuentes 1993, "Marine Water Quality Monitoring" 2013).

The species selected for this study were *Fucus gardneri* (FUGA, n=5), *Sarcodiotheca gaudichadii* (SAGA, n=10), *Sargassum muticum* (SAMU, n=4), *Laminaria saccharina* (LASA, n=2). These species are all perennial species and were mostly selected based on site accessibility and species availability. It is possible that these species incorporate and fractionate N differently, but literature is not available on these differences and this was not incorporated into this study.

1.3. Conclusion

Nitrogen is an important nutrient for the growth of all marine algae. Marine algae are important to the marine ecosystem as a habitat and food source for other species. It is also important to associated terrestrial ecosystems as an energy source for detrital based food webs (Heerhartz 2013). Excess nitrogen can lead to eutrophication and hypoxia, as has been documented in Hood Canal. Stable nitrogen isotopes have been used in marine systems for multiple purposes including the tracking of anthropogenic sources of N through the marine ecosystem. Geographic Information Systems (GIS) are commonly used for evaluation of land use and can be used to map vegetation. Stable isotopes and GIS are combined together in this study to examine the linkage between nearshore and terrestrial ecosystems (Peterson et al. 2007). If successful, the identification of anthropogenic N sources to the marine environment may allow for more targeted restoration and conservation efforts, modification to water quality policies, and perhaps even lead to methods for detection of eutrophication prior to large-scale hypoxia and anoxia.

2. Scientific Paper in Marine Ecology Progress Series Format

2.1 Introduction

Eutrophication can have profound negative effects on marine ecosystems (Howarth 2008). Eutrophication and associated hypoxia and anoxia may degrade habitat, change food-web structures, and result in a loss of biodiversity (Howarth 1988, Pedersen & Borum 1996, Barr et al. 2013). The nutrient most typically associated with marine eutrophication is nitrogen (N) (Howarth & Marino 2006, Edwards et al. 2006). Humans have roughly doubled the amount of biologically available nitrogen through the creation of nitrogen fertilizers to approximately 120 Tg yr^{-1} (Chapin et al. 2002, Cohen & Fong 2006). Nitrogen fixation by human activities accounts for 45% of all the nitrogen fixed on land and oceans (Michener & Lajtha 2007). Further, human activities have increased total nitrogen inputs to United States marine ecosystems by an average of six time and approximately two-thirds of US coastal waters are moderately to severely degraded due to N pollution (Howarth & Marino 2006). In Puget Sound, Washington, eutrophication is a serious issue. For example, between 2013-2015 $80,000,000 was requested for restoration work, highlighting the concern over this ecosystem ("Puget Sound Partnership" 2013). Coastal ecosystems are rich ecologically and provide many resources to humans, degradation of these systems could have detrimental social, economic, and ecological implications.

This study is the first known attempt to evaluate anthropogenic N loading to Hood Canal, a sub-basin of Puget Sound, using nitrogen isotopes ($\delta^{15}N$) in macroalgae (figure 1). Hood Canal has experienced eutrophication and fish kills from hypoxia since the 1920s (Newton et al. 2011). A glacial sill is present near the entrance to Hood Canal, which restricts water exchange with greater Puget Sound and the Pacific Ocean, leading to residence times for water in Hood Canal ranges from months to years. There is also a floating bridge just south of study sites 8 and 9, which is estimated to reduced water exchange by an additional 10% (Khangaonkar & Wang 2014). Partially due to the natural geography and bathymetry of the basin low dissolved oxygen (DO) issues occur historically, but events have been increasing in duration, frequency, and intensity since the mid-1990s (Paulson et al. 2007, Newton et al. 2011). Lower Hood Canal, beyond the Great Bend, has had the most severe hypoxia. In August 2006 DO values in this area were below 1 mg/L, well below the requirements for most aquatic life to survive (Quinn 2005, Newton et al. 2011, Cope & Roberts 2013).

Nitrogen inputs to Hood Canal were evaluated by a United State Geologic Survey (USGS) study commissioned by Congress in 2003 (Paulson et al. 2007). The study came about after a significant hypoxic event that began in 2002, by October 2003 it was estimated that 30% of the rockfish in the area had been killed. This study confirmed that the dominate source of N to Hood Canal is the Pacific Ocean. Incoming N from the Pacific Ocean is not a source that can be easily filtered or modified. Yet, there are some sources of N that can be managed better, which should lead to improved water quality (figure 6 and appendix B).

In nitrogen limited systems, such as Puget Sound, extensive mixing in the nearshore and uptake through biological processes can make accurate detection of N fluxes difficult (Dailer et al. 2010). Many algal species can rapidly acquire nutrients, removing nutrients from the water column, thus water samples may miss these transient fluxes of N. Isotopic signatures of nitrogen ($\delta^{15}N$) may be more meaningful than water quality samples due to this temporal and spatial variability in marine waters (Cohen & Fong 2006). Also, water quality samples alone cannot suggest potential N sources or confirm the incorporation of N into the food web. Water samples only provide the concentration of nitrogen and what the form (i.e. NH_4^+, NO_3^-, NO_2^-) of nitrogen is (Peterson et al. 2007).

In contrast to water samples, isotopic signatures of N sources to marine systems can be unique, making them traceable, although considerable overlap can occur due to large fractionation factors that occur during biological processes (McClelland et al. 1997a, Costanzo et al. 2001). Typical values for marine water samples range from 3 to 6 ‰, with a global average of about 4.8 ‰ (Michener & Lajtha 2007). Additional N signals to marine waters include: rain water (0‰), groundwater (2 to 8 ‰), Agriculture (6.5 to 9 ‰), Dairy farming (>10‰), Waste Water Treatment Plants (WWTP) (>10‰), and On-Site Septic (OSS) (3 to 25 ‰) (Michener & Lajtha 2007, Paulson et al. 2007, Dailer et al. 2010, Raimonet et al. 2013). Anthropogenic sources of N, WWTP, agriculture, and farming are typically considered enriched in comparison to natural background sources of N.

There is a growing consensus that macroalgae in N limited environments, such as Hood Canal, fractionate little during assimilation, suggesting that the tissue $\delta^{15}N$ will be very similar to the isotopic signature of the inorganic nitrogen assimilated into the algal tissue (Fogel & Cifuentes 1993, Cohen & Fong 2006, Dailer et al. 2010). Also, long-lived, perennial macroalgae species, should be reflective of nitrogen sources and concentrations over time (Raimonet et al. 2013). It is important to note that if N sources are mixed during transit or N undergoes any other fractionation, initial differences in source pools may be negated. Any denitrification that occurs enriches the $\delta^{15}N$ signal due to preferential use of ^{14}N by denitrifying bacteria (Peterson et al. 2007). Denitrification may occur in benthic sediments and during times late summer when portions of Hood Canal become eutrophic, this may enrich a sample by up to 6‰ (Michener & Lajtha 2007). This is the reason for typically enriched values (> 6-7‰) from sewage derived N, organic fertilizers, and livestock (Wigand et al. 2007, Dailer et al. 2010). Inorganic fertilizer may deplete the ^{15}N signal, as inorganic fertilizers range from -3 to +3 ‰, from the process of converting atmospheric N to fertilizer (Wigand et al. 2007). Nitrogen may also be lost prior to entering Puget Sound, either through assimilation into terrestrial tissues or volitization to the atmosphere. Fry et al (2003) suggested that $\delta^{15}N$ values increased with increasing anthropogenic inputs, but varied based on rates of denitrification.

As such, one unique approach to determine the source of N to marine ecosystems is the analysis of N isotopes in marine macroalgae. This method has been successfully applied in other geographic locations, mostly targeting the tracking of WWTP effluent, but also to compare land use influence on marine N. Only one known study has been conducted in Puget Sound, which focused on phytoplankton rather than macroalgae (McClelland et al. 1997b, Valiela et al. 1997, McClelland & Valiela 1998, Rogers 2003, Savage 2005, Pruell et al. 2006, Paulson et al. 2007, Wigand et al. 2007, Peterson et al. 2007, Barr et al. 2013). Tools that can be used to understand how nitrogen enters the marine system and is then cycled and incorporated throughout the food chain can help land managers better manage this valuable ecosystem. This study utilized stable isotope analysis in marine macroalage and land use evaluation to examine the question: can stable nitrogen isotopes in nearshore marine algae be used to trace nutrient sources (either anthropogenic or natural) being delivered by adjacent watersheds to Hood Canal, WA? If so, what is the usefulness of this knowledge in relation to management of episodic eutrophication in Hood Canal.

2.2 Methods

2.2.1 Sample Collection and Processing

Algae samples were collected from nine sites in Hood Canal (figures 1, 8). Initial collection at sites 2, 3, 4, 5, 6, and 7 was conducted on 28-29 December, 2013. A second collection was conducted at sites 1, 8, and 9 on 22-23 February, 2014. During the second collection event duplicate samples were taken at sites 5 and 7 to evaluate the potential to group samples from both sampling events into one analysis. In other studies samples were collected over the duration of one season and compared as one dataset (Wigand et al. 2007, Peterson et al. 2007).

Sites (figures 1, 8) were selected prior to field work based on accessibility, geographic distribution, and differences in adjacent land use patterns between sites. Accessibility was assessed using a shoreline public access database and potential sites were selected further based on aerial photography and proximity to know point sources of pollution (WWTP and fish hatchery). Shorelines in

Washington State are not public property and this limited the ability to access targeted locations. Even with property rights issues it was possible to access sites throughout Hood Canal, which should reflect the geographic distribution of nitrogen isotopic composition and nitrogen content in algal samples. Sites were also selected on opposing sides of the channel to test any variations that may be attributed to circulation patterns or variations in development between the different counties (Kitsap on the east, Mason and Jefferson on the west).

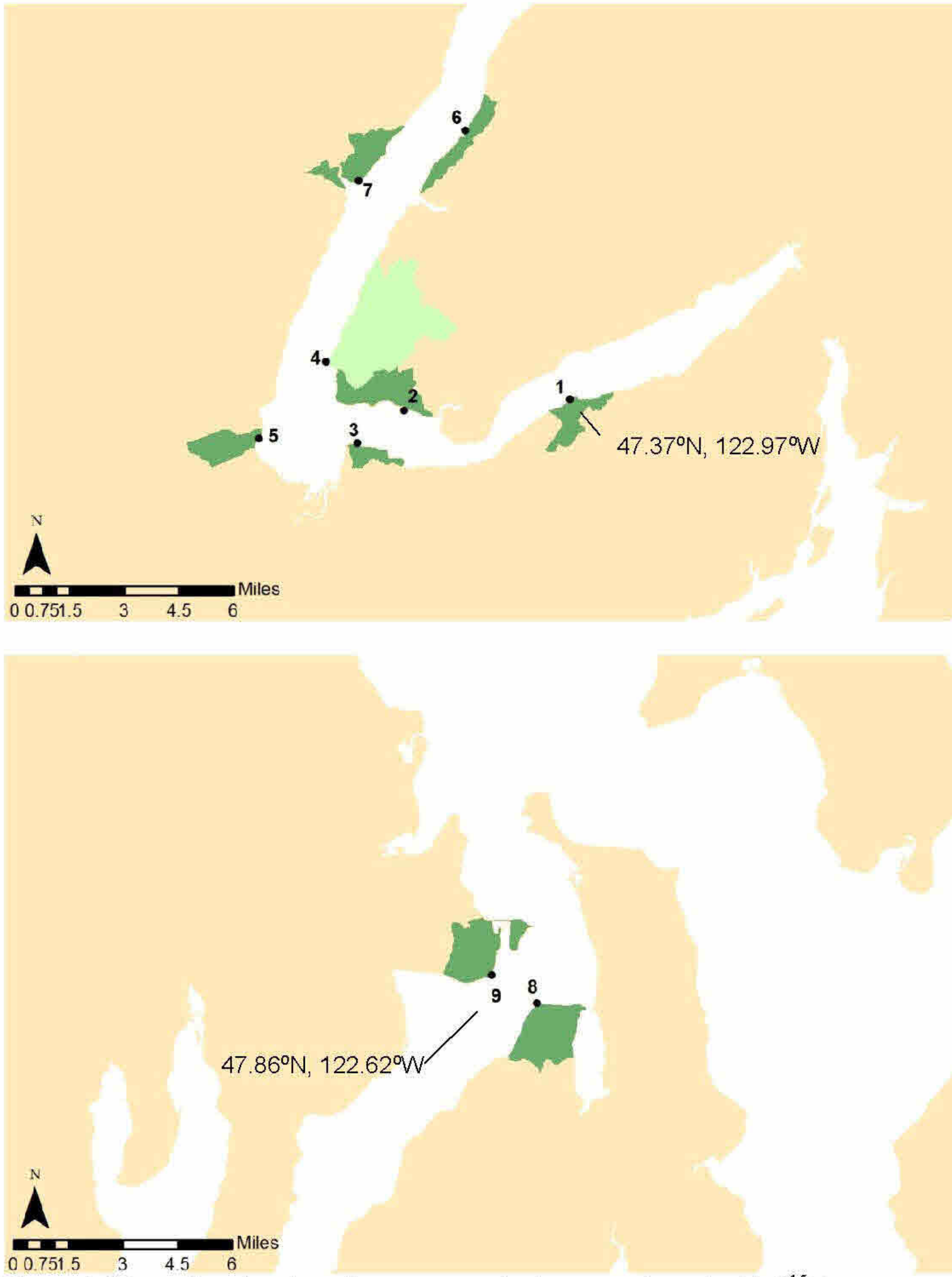

Figure 8. Maps showing the adjacent watershed area evaluated with $\delta^{15}N$ values of macroalgae.

In December algae samples were collected underwater using a Self Contained Underwater Breathing Apparatus (SCUBA) during high tide. Upon reaching the site the diver then entered the water and swam parallel to shore for approximately 50 m, collecting available algae semi-randomly in a mesh bag (Wigand et al. 2007). In some locations algae was sparse which did not allow for a completely randomized sampling methodology. Entire plants were collected to evaluate the nitrogen incorporation over time. Collected algae was placed in a glass jar on ice near freezing, and then frozen upon return to the shore, until further processing for isotope analysis within one week of collection.

In February, algae samples were collected from the shore when the tide was predicted at 0 ft mllw. At each site samples were collected along the water line for approximately 50 m. Collected algae was placed in a glass jar on ice near freezing, and frozen for 1 day to simulate the December collection process, until further processing for isotope analysis the day following freezing.

During the initial sampling event (December) all available species of algae were collected and the same species were targeted during the second collection event in February. Species collected were *Laminaria saccharina* (LASA, n=2), *Sarcodiotheca gaudichadii* (SAGA, n=10), *Sargassum muticum* (SAMU, n=4), and *Fucus gardneri* (FUGA, n=5), which are all perennial intertidal macroalgal species. In this case n is the total number of samples for that species, as there were two sampling events it does not exactly match the number of sites that species was found. The number of plants of each species collected to make a sample varied due to availability and total plant numbers were not documented, but ranged from 2 to 5 individual plants for per sample.

Samples were first rinsed with deionized water and any visible epiphytes were removed. Samples were then oven dried at 60°C overnight. Samples of the same algal species from the same site were then ground and homogenized in a coffee grinder to a fine powder, creating a composite, homogenized sample by species, site, and sampling event. In between samples all equipment was rinsed with deionized water and dried, then sanitized with ethanol. Approximately 3 μg of dried and homogenized samples were placed into 4 x 6 mm tin capsules in triplicate for each sample.

Samples were analyzed by the UC Davis Stable Isotope Lab for $\delta^{15}N$ (‰) and total nitrogen content in the sample using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (http://stableisotopefacility.ucdavis.edu/). Samples were compared to several different laboratory standards that have previously been calibrated against NIST Standard Reference Materials. Final $\delta^{15}N$ values are presented in parts per thousand (‰) relative to the international standard for nitrogen, air (equation 1, chapter 1). Tissue N (wt %) was calculated by taking the N content (μg) of the sample divided by the total sample weight (μg).

2.2.2 Data Analysis

Nitrogen isotope data was lognormally distributed and a log transformation was used prior to any data analysis. Percent nitrogen content was arcsine-square root transformed prior to analysis. Any figures presented show back transformed data and confidence intervals (Zar 2010, Gotelli & Ellison 2013).

Due to low sample numbers only SAMU (n=10) was analyzed as a species individually. An Analysis of Variance (ANOVA) was used to compare the difference in $\delta^{15}N$ between species at each site. This analysis was used to evaluate the potential to group all species into one analysis. This would increase the power of the analysis by increasing the number of samples from 10 (SAGA only) to 21 with all species combined. At sites 3, 5, 6, and 8 there was a significant difference between species ($p < 0.05$). At sites 1, 2, 4, 5, and 7 there was no significant difference in $\delta^{15}N$ between species ($p > 0.05$). All species were considered together to assess if any relationships appeared when all species of macroalgae were combined, with the acknowledgement that variability between species may be an additional source of uncertainty that may make drawing definite conclusions about relationships with $\delta^{15}N$. Some of this variation between species may be explained by a slightly different tidal elevations or differential use of types of N by algal species (Asare & Harlin 1983). Previous studies using different species in similar environments have cited different $\delta^{15}N$ values (Asare & Harlin 1983, Dailer et al. 2010).

An ANOVA was also used to compare the difference in SAGA $\delta^{15}N$ between December and February sampling events at sites 5 and 7, as it was present at these sites during both sampling events. At site 5 the average $\delta^{15}N$ in SAGA significantly decreased from 8.5±0.2 in December to 7.3±0.1 in February ($F_{(1,4)} = 57.1675$, $p = 0.0016$). At site 7 the average $\delta^{15}N$ in SAGA did not significantly change from 5.5±0 in December to 5.9±0.2 in February ($F_{(1,4)} = 4.6887$, $p = 0.0963$). Site 5 is near the mouth of the Skokomish River and as samples were separated temporally during peak flow season there may have been a release that could account for the increase at site 5, whereas site 7 was not subject to the same variability. As other studies have combined samples over the course of a season, and patterns were similar between species, it was determined that data from both sampling events would be evaluated together (Wigand et al. 2007, Peterson et al. 2007, Barr et al. 2013). One important difference in this study was the combining of different species, which has not been done in other studies.

Land use data was extracted from Geographic Information Systems (GIS), using ERSI ArcGIS software v 10.1, through a dataset published by the Washington State Department of Ecology (Ecology) and National Oceanic and Atmospheric Administration (http://www.ecy.wa.gov/services/gis/data/landcover/landcover.htm). Land use area was classified as developed, forested (evergreen, deciduous, mixed, and total), wetlands, and pastured or cultivated. This area was then divided by the total area to obtain a proportion of land use type to total area. All land use data was arcsine-square root transformed prior to analysis (Zar 2010, Gotelli & Ellison 2013).

All land use data were evaluated based on the Assessment Unit (AU) scale presented in Ecology's watershed characterization methodology and are a small to mid-size watershed delineation (Stanley et al. 2012). Assessment units range in size, with smaller areas near the coast and larger areas in the mountains. The median size is 8.8 km^2. Zhou et al (2012) evaluated the difference in land use inputs to freshwater systems based on the different scales that land use could be evaluated and determined that a size similar to the AU was more strongly correlated with water quality data.

Developed land (land that was identified as having constructed surfaces) was classified as low, mid, or high intensity development. The different qualitative descriptors (low, mid, high) were based on the combination of constructed surfaces with vegetation. For example a large industrial park would be classified as high intensity, whereas a residential property with landscaping would fall in the low intensity category. High intensity development had greater than 80% impervious surfaces, mid intensity ranged from 50-80%, and low intensity had less than 50% impervious surfaces. For the purposes of this study high, mid, and low intensity developed land were combined together.

Forested land was classified as either deciduous or evergreen dominate or mixed forest. Deciduous forests had more than 75% of the tree species losing leaves in the fall. Evergreen forests were classified as such if more than 67% of the tree had leaves throughout the year. Mixed forests had both deciduous and evergreen species, and neither could be classified as dominate. Each of these categories was evaluated individually and then combined for total forested cover.

Wetlands combined all different types of wetlands (forested, scrub/shrub, emergent, and estuarine) into one category. Overall wetlands were a very low percentage of any watershed and there were no wetlands identified in watershed adjacent to site 1, 3, and 6. In addition to regression analysis between $\delta^{15}N$, total N content (%), and percent wetland area, two ANOVAs was also used to compare wetland types and presence/absence of wetlands and the response of $\delta^{15}N$ in macroalgae.

The amount of pastured and cultivated land was the final land use parameter used for comparisons. Pastured lands are characterized by grasses for livestock grazing or the production of hay crops. Cultivated lands include herbaceous cropland and woody (i.e. orchards, nurseries, and vineyards). The distribution of cultivated lands in studied watersheds was bimodally distributed, thus it did not allow for parametric regression analysis. Pasture and cultivated lands and pastured lands on their own were used for regression analysis, while cultivated lands were evaluated using an ANOVA based on the presence or absence of cultivated lands within the watershed.

All of these land use parameters were obtained through a dataset that interpreted remotely sensed data. This method of land use analysis may lead to errors in land use estimation due to errors in the data or data interpretation, though it should provide a good general estimation of the type of land use in adjacent watersheds.

In addition to comparisons with land use in adjacent watersheds distances to point source pollution sites and the distance to the Pacific Ocean was evaluated. In Hood Canal there is only one WWTP, just east of site 3. There is also a commercial fish hatchery in Hoodsport, in between sites 7 and 5. Both the fish hatchery and WWTP would be expected to enrich $\delta^{15}N$ values, due to processing by anaerobic bacteria of high N concentration effluent. Rather than using an absolute distance between point sources and algae collection sites a rank distance was used. The site closest to the source (either WWTP, Pacific Ocean, or fish hatchery) was classified with a distance of 1 and the farthest with 9. This was due to the distribution of sites and the fact that sites across the channel from each other would have a similar absolute distance, which skewed the distribution.

Statistical methods used included regression, correlation, ANOVA, and multiple linear regression (MLR). All analyses were completed using JMP v 10.0.2 and graphs were created in Microsoft Excel.

2.3 Results

Summary statistics for all $\delta^{15}N$ (‰) and tissue N (wt %) values are presented in table 1. These values are based on triplicate samples for each species at each site. The wt % N content was more variable between species and with larger errors in comparison to $\delta^{15}N$, this may have been reduced if only one species was studied. There are relatively smaller SE values for $\delta^{15}N$ and values tend to increase from site 9 to site 1 (farthest from ocean). The $\delta^{15}N$ values ranged from 5.5 ± 0.0 at site 7 to 8.9 ± 0.3 at site 3 (nearest the WWTP). The wt % N content values ranged from 1.6 0.3 at site 2 to 4.8 ± 0.2 at sites 1 and 5.

$\delta^{15}N$ (‰)									
Site	**Date**	**Species**	**Value**	**SE**	**Site**	**Date**	**Species**	**Value**	**SE**
1	Feb	FUGA	7.8	0.1	**5**	Dec	SAGA	8.5	0.2
1	Feb	SAGA	7.6	0.1	**5**	Feb	SAGA	7.3	0.1
2	Dec	LASA	8.0	0.1	**6**	Dec	SAGA	8.0	0.1
2	Dec	SAGA	7.8	0.1	**6**	Dec	SAMU	6.5	0.1
2	Dec	SAMU	7.8	0.1	**7**	Feb	FUGA	6.1	0.0
3	Dec	LASA	8.0	0.1	**7**	Dec	SAGA	5.5	0.0
3	Dec	SAGA	8.2	0.1	**7**	Feb	SAGA	5.9	0.2
3	Dec	SAMU	8.9	0.3	**8**	Feb	FUGA	6.9	0.1
4	Dec	SAGA	6.8	0.1	**8**	Feb	SAGA	6.5	0.0
4	Dec	SAMU	7.0	0.3	**9**	Feb	FUGA	7.0	0.0
5	Feb	FUGA	7.1	0.0	-	-	-	-	-
Tissue N (%)									
Site	**Date**	**Species**	**Value**	**SE**	**Site**	**Date**	**Species**	**Value**	**SE**
1	Feb	FUGA	1.9	0.0	**5**	Dec	SAGA	4.8	0.2
1	Feb	SAGA	4.8	0.2	**5**	Feb	SAGA	3.0	0.2
2	Dec	LASA	2.5	0.2	**6**	Dec	SAGA	2.7	0.0
2	Dec	SAGA	2.3	0.1	**6**	Dec	SAMU	2.1	0.2
2	Dec	SAMU	1.6	0.3	**7**	Feb	FUGA	2.0	0.1
3	Dec	LASA	1.7	0.2	**7**	Dec	SAGA	3.0	0.2
3	Dec	SAGA	2.9	0.2	**7**	Feb	SAGA	4.7	0.3
3	Dec	SAMU	1.7	0.2	**8**	Feb	FUGA	3.3	0.4
4	Dec	SAGA	3.1	0.2	**8**	Feb	SAGA	3.1	0.3
4	Dec	SAMU	2.3	0.1	**9**	Feb	FUGA	4.1	0.3
5	Feb	FUGA	2.8	0.1	-	-	-	-	-

Table 1. Summary of algal samples collected and mean $\delta^{15}N$ values and tissue N (%) with standard error of the mean (SE). The standard error of the mean was calculated as the standard deviation of the samples divided by the square root of 3 (n = 3). Species are abbreviated *Laminaria saccharina* (LASA), *Sarcodiotheca gaudichadii* (SAGA), *Sargassum muticum* (SAMU), and *Fucus gardneri* (FUGA).

To evaluate the effect of basin topography and bathymetry on $\delta^{15}N$ and tissue N values two ANOVAs were ran, one that compared data between the different sides of the channel and one that compared between values at sites 8-9 (outside the sill and Hood Canal Bridge) to sites 1-7 (inside the sill and Hood Canal Bridge) using average values for all species. There was no significant relationship between side of the channel and $\delta^{15}N$ ($F_{(1,19)} = 0.0309$, $p = 0.8624$). There was also no significant relationship between side of the channel and tissue

N ($F_{(1,19)} = 1.2167$, $p = 0.2838$). There was no significant relationship between sites inside or outside of the Hood Canal sill and $\delta^{15}N$ values ($F_{(1,19)} = 0.6053$, $p = 0.4461$). There was also no significant relationship between sites inside or outside of the Hood Canal sill and tissue N ($F_{(1,19)} = 1.5368$, $p = 0.2302$).

Next, regressions were ran comparing each of the identified land use parameters with average $\delta^{15}N$ values and tissue N of average SAGA values, as well as average values for all species. Some land use parameters had more variability between sites than others. Forest cover ranged between 30 and 80% of land cover, whereas wetland area was either 0, 2, 5, or 6% of land cover. Pastured and cultivated land was a small percentage of land use at any site, always less than 5% of land cover. None of the study area was heavily developed, but developed land coverage did range from 1 to 30%. Table 2 presents a summary of these results. Only regressions that passed a Shapiro-Wilk's test for normalcy on the residuals ($p > 0.05$) are presented in the table.

SAGA Only						
	$\delta^{15}N$			Tissue N		
	r^2	*p*	+/-	r^2	*p*	+/-
Development	0.07	0.45	+	0.0173	0.7169	none
Deciduous Forest	0.00	0.86	none	0.1117	0.2883	-
Evergreen Forest	0.06	0.50	+	0.0325	0.5752	+
Mixed Forest	0.20	0.19	+	0.0172	0.6847	-
All Forest	0.42	*0.04*	+	0.0461	0.5027	-
Wetlands	0.41	*0.04*	-	0.001	0.922	none
Pasture	0.32	0.09	-	0.036	0.5548	-
Pasture and Cultivated	0.33	0.08	-	0.0342	0.5651	-
Distance to Hatchery	0.01	0.78	none	0.0457	0.5048	-
Distance to WWTP	0.21	0.1878	-	0.057	0.4548	+
Distance to Pacific	0.25	0.14	+	0.0402	0.5319	-
All Species						
Development	0.27937	*0.0138*	+	0.08661	0.1953	-
Deciduous Forest	0.12753	0.112	+	0.0823	0.2069	-
Evergreen Forest	0.12874	0.1102	+	0.0282	0.5507	+
Mixed Forest	0.00376	0.7917	none	0.0019	0.8527	none
All Forest	0.10235	0.1574	+	0.02	0.5405	-
Wetlands	0.26297	*0.0174*	-	0.02718	0.4752	+
Pasture	0.11203	0.1381	-	0.0031	0.8091	none
Pasture and Cultivated	0.11949	0.1248	-	0.0029	0.8177	none
Distance to Hatchery	0.0065	0.7283	none	0.0033	0.805	none
Distance to WWTP	0.32148	*0.0074*	-	0.1678	0.0651	+
Distance to Pacific	0.32	*0.01*	+	0.0719	0.2398	-

Table 2. Results of parametric regressions comparing each of the identified land use parameters in section 2.3.2 with average $\delta^{15}N$ values of all algae species, as well as SAGA only. Pearson's r^2, p value (highlighting significant relationships in italics), the direction of the relationship.

Plots for regressions with significant results are presented below. The slope direction of the regressions with all species compare well to SAGA regressions. However, there are more significant relationships when comparing across all species. One interesting deviation is the variance in the comparison to all forested lands. The relationship is significant only using the SAGA data, although the p values is approaching 0.05 for all species, but the r^2 value is much stronger in SAGA only.

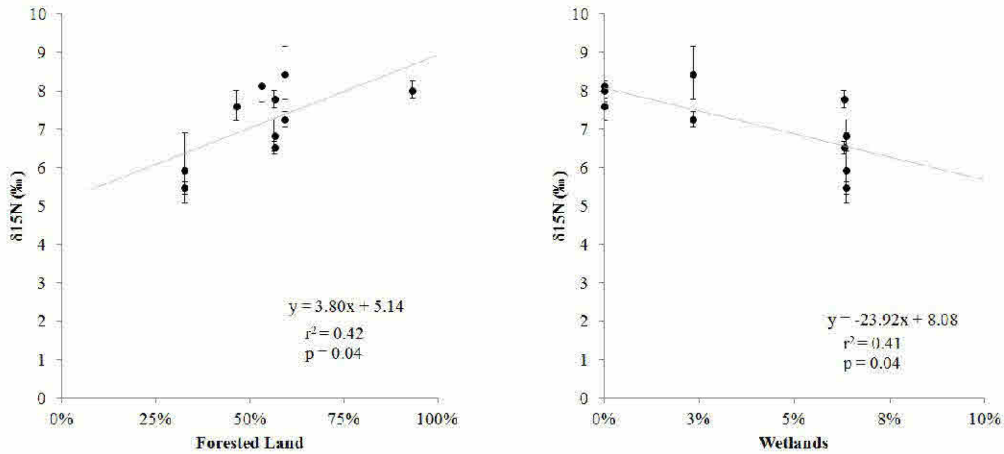

Figure 9. Significant regression plots for multiple land use parameters compared to $\delta^{15}N$ values of SAGA. $\delta^{15}N$ values are presented with 95% confidence intervals.

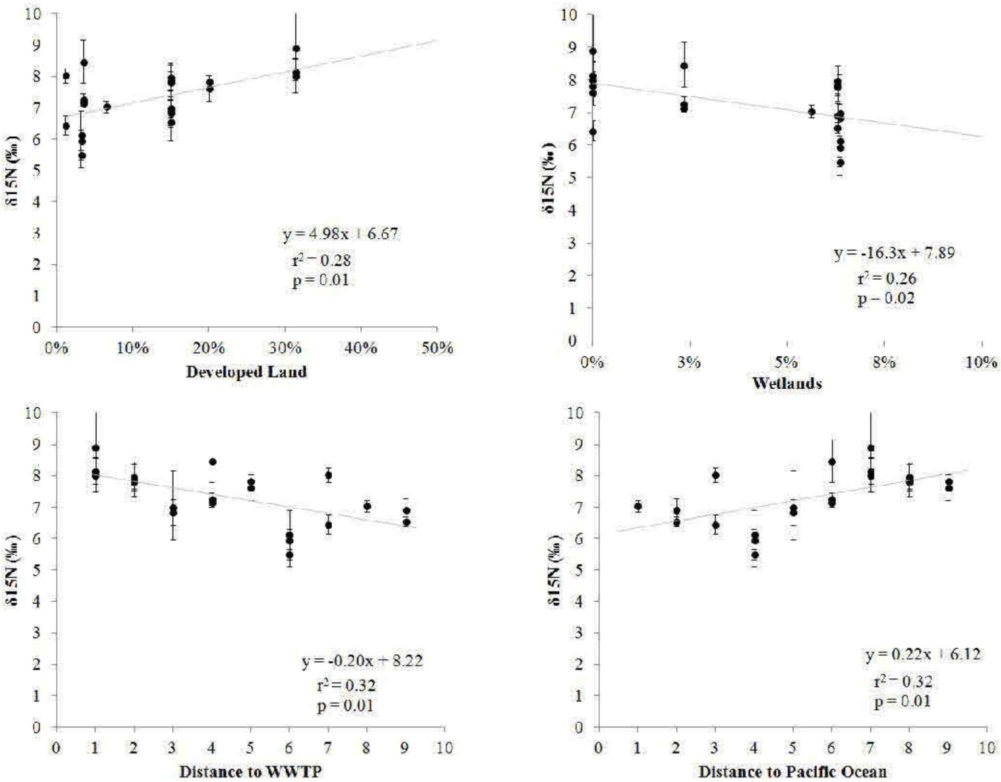

Figure 10. Significant regression plots for multiple land use parameters compared to $\delta^{15}N$ values of all algae species. $\delta^{15}N$ values are presented with 95% confidence intervals.

Due to the non-normal distribution of residuals when comparing cultivated lands to nitrogen values across all species, that comparison was examined by converting the cultivated land to a category (cultivated or not cultivated) and an ANOVA was used. The $\delta^{15}N$ values of all species were significantly enriched when the adjacent watershed had cultivated lands ($F_{(1,19)} = 7.4563$, $p = 0.0133$).

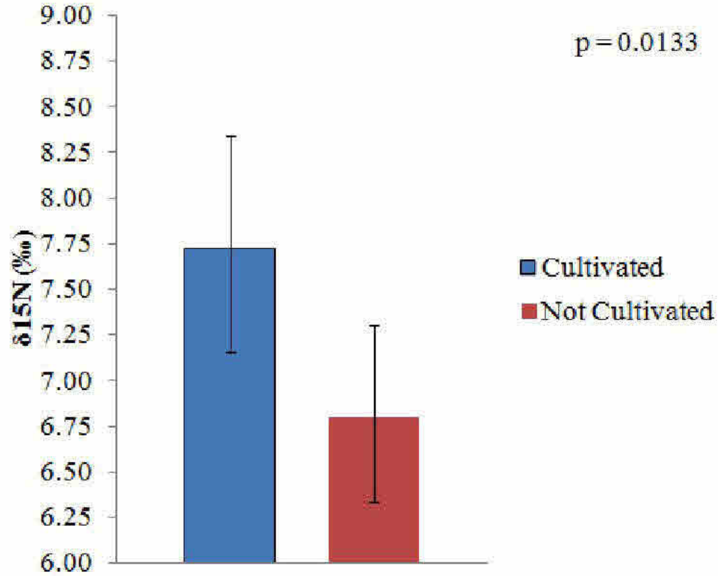

Figure 11. ANOVA results comparing sites with adjacent cultivated land use to sites without cultivated land use. The $\delta^{15}N$ values of all species were significantly enriched when the adjacent land area had cultivation ($F_{(1,19)} = 7.4563$, $p = 0.0133$).

Two ANOVAs examined the relationship between adjacent watersheds with wetlands and without. One simply compared if algae collection sites were adjacent to watersheds with any type of wetlands (figure 12). The other compared wetlands that were classified as both palustrine and estuarine, palustrine only, or if no wetlands were present (figure 13). There was only one site that had a watershed with palustrine wetlands

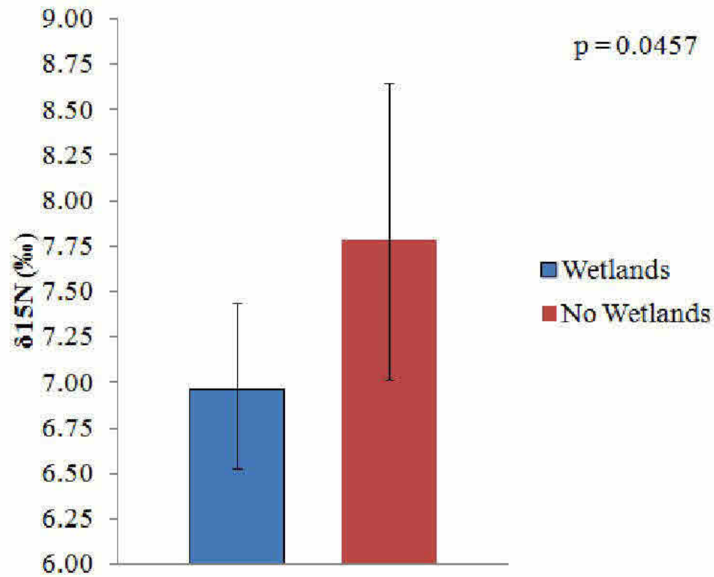

Figure 12. ANOVA results comparing sites with adjacent wetlands use to sites without wetlands. The $\delta^{15}N$ values of all species were significantly enriched when the adjacent land area had wetlands ($F_{(1,19)}$ = 4.5701, p = 0.0457).

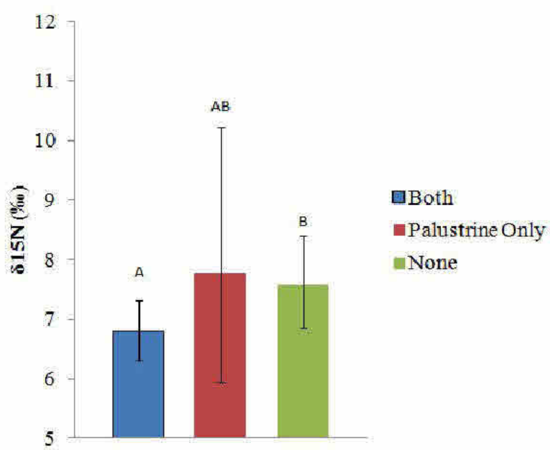

Figure 13. ANOVA results comparing sites with adjacent wetland types that are palustrine only, estuarine and palustrine, or no wetlands present in the watershed. The $\delta^{15}N$ values of all species were significantly enriched when the adjacent land area had wetlands ($F_{(2,20)}$ = 3.6168, p = 0.0478).

A comparison between the δ15N (‰) and the total N in the algal sample (%) show a weak, but significant relationship (Pearson's r = 0.08612, p = 0.0196). There is less N in the sample when the N is enriched.

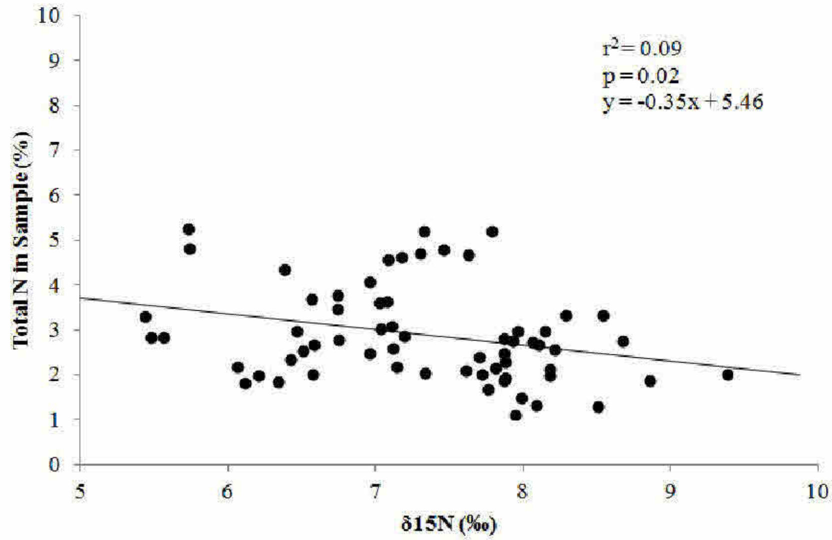

Figure 14. Correlation of $\delta^{15}N$ (‰) of all species at all sites compared to corresponding nitrogen content (%) in each sample. Results show that as nitrogen loading decreases $\delta^{15}N$ increases (Pearson's r = 0.086, p = 0.02, $\beta_1 = -0.35$).

Finally, forward stepwise multiple linear regression (MLR) was used to examine the effects of multiple land use parameters on $\delta^{15}N$ values. These analyses used $\delta^{15}N$ data across all species. Independent variables were tested for correlation with each other and rejected for use in MLR if $r > 0.7$ (Ellis et al. 2012). Standard linear regression results presented above were used to determine combinations for inclusion in MLR. Standard assumptions for MLR were followed and tests for normal distribution of residuals was completed for each analysis (Zar 2010, Gotelli & Ellison 2013).

Independent Variables	Model	r^2	F	df	*p*
Forest, Pacific, Dev	0.5801 + 0.1479*Dev + 0.0107 * Pacific + 0.2232 * Forest	0.6698	11.4925	3,17	0.0002
Forest, Pacific, Wet	0.6628 + 0.1706*Forest + 0.0138*Pacific - 0.0657*Wet	0.5586	7.1702	3,17	0.0026
Forest, Pacific	0.6224 + 0.1997*Forest + 0.0152*Pacific	0.5452	10.7907	2,18	0.0008
Dev, Wet, WWTP	0.8913 + 0.8393*Dev - 0.1734*Wet - 0.0073*WWTP	0.5203	6.154	3,17	0.005
Dev, Forest	0.6289 + 0.2315*Dev + 0.1996*Forest	0.5032	9.1147	2,18	0.0018
Dev, Wet, Pacific	0.8134 + 0.1003*Dev + 0.0068*Pacific - 0.1560*Wet	0.496	5.5758	3,17	0.0075

Table 3. Forward stepwise MLR results. Independent variables are Dev = developed land, Wet = wetland area, WWTP = distance to wastewater treatment plant at Alderbrook resort, Pacific = distance to the Pacific Ocean, Pas/Cul = pasture and cultivation, Forest = all forested land. Independent variables were compared against all species average $\delta^{15}N$ at all sites in the study area.

2.4 Discussion

Multiple linear regression (MLR) analysis suggests that development and preservation of natural N sinks are key components to reduced N loading in Hood Canal. The MLR analyses that showed the strongest explanation of $\delta^{15}N$ values were combinations of forest, wetland, and developed land uses and distances to the Pacific Ocean and the WWTP at Alderbrook Resort.

Isotope values in this study are not as enriched compared with other studies that have used this method, some of which have reflected WWTP effluent assimilation with values near 20‰ (Dailer et al. 2010, Raimonet et al. 2013). Average $\delta^{15}N$ values in Hood Canal during this study ranged from 6.13 to 8.91.

Although this is not as significantly enriched as other studies, this is greater than the global average marine value, which ranges from 3 to 6 ‰ (Michener & Lajtha 2007). This values fluctuates little in the deep ocean (Hartwell 2013).This slight enrichment may represent anthropogenic nitrogen sources and the trends discussed further below suggest some influence of human development in the watersheds surrounding Hood Canal.

First, as development increased $\delta^{15}N$ also increased (β_1 = 4.9759, p = 0.0138), but this pattern was only significant when comparing all algal samples, likely due to an increased sample number. This enrichment is likely in response to increased sewage from OSS that is processed by bacteria with a preference for the lighter N isotope, as described in the introduction. OSS is the main method for sewage disposal in Hood Canal, thus as development increases so does the use of OSS. The only WWTP in the vicinity serves only the Alderbrook Resort and discharges between 0.17 and 1.8 MT/yr in comparison to 26 ± 15 MT/yr for OSS (Paulson et al. 2007). In contrast, increasing development may lead to more fertilizer application, especially in residential areas. The effect of increased use is variable, with inorganic fertilizers depleting the signal, whereas organic fertilizers would increase the signal. Thus it is difficult to tease apart any direct influence of fertilization. There is no data available on fertilizer loading in Hood Canal to help determine if inorganic fertilizer application is reducing the enrichment signal. A number of studies have looked at the influence of OSS on water chemistry and found similar results (Aravena et al. 1993, Steffy & Kilham 2004). Peterson et al (2007) used a parameter for development in their study that correlated higher $\delta^{15}N$

values in the biota in the Great Lakes to higher development in watersheds adjacent to their study sites.

Forested land also showed a significant increase in $\delta^{15}N$ when comparing with SAGA only (β_1 = 3.7999, p = 0.0438). The difference in relational significance may be due to a stronger relationship between SAGA and the terrestrial community, or related to a reduction in variability when only examining one species. Forested land occurs is interspersed with development in the study area, and thus may be receiving enriched N from anthropogenic sources. Forests in the Pacific Northwest are N limited and little fractionation should occur through the forests, so it is reasonable that N exiting forested land would be similar in isotopic composition to the source of N entering the forest (Edmonds et al. 1989, Chapin et al. 2002, Michener & Lajtha 2007). In Peterson et al (2007) the land use parameter was on a scale that had natural, forested landscapes on one end and development, urbanization on the other. This land use parameter was used in correlation analysis with $\delta^{15}N$ values, they found that forested areas had lower $\delta^{15}N$ values and developed areas had higher $\delta^{15}N$ values. The forested results in their study do not match the results from this study, this could be due to different forest types in the study area. They also used a development land use parameter that was inclusive of development and undisturbed natural landscapes, whereas in this study forests, development, and wetlands were all separated during analysis.

The wetland area compared well to both SAGA and all species. $\delta^{15}N$ was reduced significantly when compared to SAGA (β_1 = -23.923, p = 0.0449) and all algal species (β_1 = -16.3, p = 0.0174). The depletion response may be related to sulfur content in the wetlands and the dissimilatory nitrate reduction to ammonium DRNA process, which has been shown to result in depleted $\delta^{15}N$ values (McCready et al. 1983, Michener & Lajtha 2007). Hydrogen sulfide is used as an indicator of hydric soils in the western U.S. and estuarine wetlands are known to have high levels of sulfur ("Wetlands Delineation Manual" 1987, Giblin & Weider 2014). Fractionation during DRNA ranged from -40 to -5‰ in one published study (Lehmann et al. 2003). An ANOVA comparison between sites with palustrine, both palustrine and estuarine type wetlands, and without wetlands there was a significant response in $\delta^{15}N$ ($F_{(2,20)}$ = 3.6168, p = 0.0478). Sites 1, 3, and 6 had no wetlands and two of these sites are within Lynch Cove, where eutrophication issues are more pronounced. If even the presence of a small amount of wetlands influences N cycling significantly it would highlight the importance of wetlands to marine water quality due the potentially large fractionation effect. It is also important to note that the statistical significance of this relationship may not be representative of actual land-sea connections as the greatest wetland area coverage in any watershed is only 6%.

The distance to the WWTP at Alderbrook resort reflected a depletion in $\delta^{15}N$ with increasing distance (β_1 = -0.2041, p = 0.0074). The site with the most enriched value was nearest the WWTP, 8.9 ± 0.3‰. The relationship between enriched $\delta^{15}N$ values and WWTP effluent is strongly documented in the literature. These studies tend to have more enriched values relative to the results in this study, but this is likely due to the small size of the WWTP in Hood Canal (McClelland et al. 1997a, Savage 2005, Wigand et al. 2007, Dailer et al. 2010).

There was also an enrichment in $\delta^{15}N$ with increasing distance from the Pacific Ocean (β_1 = 0.2163, p = 0.0074). The relationship between $\delta^{15}N$ and the Pacific Ocean may highlight the effect of residence time of water in Hood Canal, which exacerbates eutrophication through a reduced tidal exchange. Longer residence time may allow for increased denitrification in the sediments and water column, which would enrich the $\delta^{15}N$ signal. Waters from the Pacific Ocean should be depleted in $\delta^{15}N$ relative to most anthropogenic sources, so areas where water does not exchange with the ocean as frequently may be more enriched relative to areas closer to the ocean (site 8-9).

There were no significant patterns between tissue N and land use parameters. Tissue N values ranged from 1.56 to 4.82%. Some of this is likely due to variability from the use of multiple species. Although there were no relationships identified between land use and tissue N, there may be an elevated N level in algae in Hood Canal when compared with other studied areas. This would require more study and a better understanding of N use by specific algal species (Hein et al. 1995, Costanzo et al. 2000).

There was a weak significant negative relationship between $\delta^{15}N$ and tissue N values (Pearson's r = 0.08612, p = 0.0196). As $\delta^{15}N$ was depleted, tissue N values also decreased. Depleted $\delta^{15}N$ is typically associated with natural marine conditions (where nitrogen is limited) and enriched $\delta^{15}N$ is typically associated with an anthropogenic nitrogen source in similar studies (Wigand et al.

2007, Peterson et al. 2007, Dailer et al. 2010). Other studies have suggested that relationship between $\delta^{15}N$ and tissue N values shows that as anthropogenic N increase (enriched $\delta^{15}N$ values) the tissue N also increases, suggested an increases N load (Barr et al. 2013).

Trends in this analysis highlight the importance of sample distribution and number, with more significant relationships being identified with more algae samples included in the analysis. Also, a clear understanding of the local nitrogen processes and use of N by the species being analyzed is needed, which was sometimes oversimplified in other studies. The selection of species needs to be evaluated prior to data collection, with perennial species being useful to understand average long-term N patterns and short-lived annual species being useful in understanding rapid changes in N inputs. Species selection requires and understanding of N use by the algae of interest.

This study found that marine algae can highlight some important relationships between land use, geography, and nitrogen in Hood Canal. This short experiment suggests that N isotope analysis may be a cost effective tool for long term monitoring to detect changes in N loading and sources to Hood Canal. It seems reasonable that a picture of algal N content and isotopic composition over time may be useful in identifying a critical point, after which eutrophication will occur in Hood Canal. This information could be used to assist managers with policy development to deal with episodic eutrophication. This method may also be useful in detecting malfunctioning sewage systems, without the need for more costly underground exploration. More work needs to be done, but this study suggests some of the potential benefits of this method, as well as modifications and additional studies that should be conducted to make the methodology more robust.

3. Interdisciplinary Connections and Conclusion

3.1 Introduction

This thesis has argued that eutrophication is a problem for marine systems and attempted to understand anthropogenic inputs that affect nutrient loads in Hood Canal with the use of stable nitrogen isotopes in marine macroalgae. Chapter One provided background knowledge on the nitrogen cycle, Hood Canal, and nitrogen isotopes. Chapter Two was a technical manuscript, formatted for submission to the Marine Ecology Progress Series scientific publication. This final chapter aims to summarize important results from this study and put them into context with local issues. This chapter will also suggest ideas for ways to improve similar studies in the future.

Hood Canal may be more prone to issues with eutrophication due to the natural shape of the basin and the presence of the Hood Canal floating bridge, but it is certainly not the only area in the Salish Sea that may be negatively affected by anthropogenic nitrogen loading and eutrophication. Wastewater loads for Vancouver, BC and Seattle, WA, USA are estimated at 23,125 kg/day and 19,324 kg/day and these values will only increase with increasing population (Mohamedali et al. 2011). Low dissolved oxygen (hypoxic) events have been noted throughout the Puget Sound, not only in Hood Canal, and have been associated with nitrogen inputs ("Nitrogen in the Puget Sound Ecosystem" 2013).

3.2 Interdisciplinary Approach

This thesis required an interdisciplinary approach, combing physical and chemical oceanography to understand the marine nitrogen cycle, and using ecology and biology to evaluate the use of nitrogen by marine algae. To understand the linkage between natural systems and human systems, this thesis briefly attempts to incorporate the unique cultural, social, and economic systems linked with Hood Canal in this final chapter.

3.3 Study Results and Environmental Implications

Results from this study suggest that maintaining natural landscapes and managing development are key to mitigating negative effects of anthropogenic nitrogen inputs to Hood Canal, possibly reducing eutrophic events in the area. Restoration and preservation of existing forested and wetland areas should be a high priority for drainages leading to Hood Canal. Any modifications to existing OSS and requirements for future OSS or WWTP should consider technology that reduces nitrogen outputs, which is discussed further in section 3.5. Increasing the ability of Hood Canal to exchange water with the Pacific Ocean may also improve water quality, which may be difficult due to the natural bathymetry and glacial sill. This flow issue could be addressed with modifications to the Hood Canal floating bridge, or with considerations in the future when the bridge will inevitably need to be replaced.

Increased nitrogen may also be linked with increases in harmful algal blooms, which in turn may be associated with ocean acidification ("Nitrogen in the Puget Sound Ecosystem" 2013). The growth of planktonic marine algae, in response to increased nitrogen inputs, may temporarily decrease the CO_2 concentration in the surface water. This would result in an initial increase in pH. As the algae die and settle in deeper waters CO_2 is released back to water driving down the pH (acidifying) the water ("Nitrogen in the Puget Sound Ecosystem" 2013). Ocean acidification and harmful algal blooms may have a profound negative effect on the multi-million dollar shellfish industry in Washington State ("Sea Change" 2014). Nitrogen is not only changing dissolved oxygen concentrations in Puget Sound, but may be changing the pH of the water, and modifying the trophic structure of the ecosystem.

3.4 Social, Cultural, and Economic Implications

Changes to the Puget Sound ecosystem will also negatively affect local tribes, whose cultures revolve around the fish and shellfish resources that the Puget Sound/Salish Sea provide. In Hood Canal there are two tribes, the Skokomish and Port Gamble S'Klallam ("Port Gamble S'Klallam Tribe" 2014, "The Skokomish Tribal Nation"). The Skokomish culture centers around the Skokomish river, which drains into the south end of Hood Canal, at the Great Bend. Salmon play an important role in this tribes culture, and restrictions to salmon migration from dams on the Skokomish and poor water quality in the marine environment may lead to more salmon species being listed under the Endangered Species Act. The Port Gamble S'Klallam is located at the north end of Hood Canal and the reservation is located on the last remaining commercial and domestic shellfish harvesting area in Kitsap County. This tribe also runs a fish hatchery on Little Boston Creek.

Non-tribal members in the area are also typically linked with the Salish Sea through recreation fishing and the consumption of local fish at restaurants throughout the area. During the 2009-2010 fishing season approximately 729,000 catch record cards were issued by the Washington State Department of Fish and Wildlife to track salmon, steelhead, sturgeon, and shellfish harvesting by recreational fishermen (Kraig 2013). The population during this time was just under 6 million and the number of catch record cards does not take into account fishermen that do not get the proper license, or are fishing for species that are not tracked, thus it is reasonable to estimate that one sixth of the population in Washington fishes recreationally. This is a large group of the population that may be able rally behind tougher nutrient control measures, if they understood the risks that eutrophication pose to their recreational fishery.

The Salish Sea also dominates the geography of Western Washington, leading to the largest ferry service in the United States. The quintessential image of Seattle is the skyline of the city from the perspective of someone on Puget Sound. Changes to the Salish Sea will not only have scientific consequences, but many other social, cultural, and economic repercussions.

3.5 Nitrogen Management in Hood Canal

Anthropogenically derived nitrogen is a source that could be managed through policy and public education, in comparison to naturally occurring atmospheric or oceanic nitrogen sources, which cannot be easily managed. If the Pacific Ocean is a consistent source of nitrogen to Hood Canal, it stands to reason that humans may be the cause of the excess that leads to eutrophic conditions, and the ability to identify anthropogenically derived nitrogen sources could be useful for management purposes. Managers need to understand how the relatively small

N (approximately 4%) input from humans in Hood Canal plays a role in the marine system, to best evaluate methods for management.

The Washington State Department of Ecology (Ecology) is responsible for the enforcement of water quality standards in the Washington State Administrative Code (WAC). Nutrient management currently focuses on restrictions to phosphorus content in detergents, nutrient management plans for dairy farms, and improvement programs for new and expanding wastewater treatment facilities. Nitrogen also plays a role in dissolved oxygen and is typically incorporated into management plans for water bodies that do not meet water quality standards for dissolved oxygen. Based on some of the results in this study and other studies that suggest septic systems may play an important role in nutrient loading, it seems that increased monitoring and regulations for their use should be implemented.

A pilot study currently being conducted in Hood Canal, to evaluate the potential for an additional filter system attached to an OSS, found a 92% removal of nitrogen from the OSS during the 12 month verification period (The University of Washington & Washington State Department of Health 2013). The filter system functioned similar to a wetland, reducing flow and providing time for denitrification and volatilization of N back to the atmosphere. Future work combining the use of isotopes and this filtration technology could prove useful as an additional tool to track nitrogen from the OSS to the marine environment. Also, as mentioned previously in this thesis, due to fast biological uptake of nitrogen in marine systems if water quality measurements were solely used to evaluate nitrogen reduction, the flux of nitrogen being incorporated into biological tissue may be missed. Water quality measurements are an important tool for understanding water quality, and in combination with isotope analysis may provide a more complete picture of nutrient concentration and cycling.

A similar concept was proposed in another study to reduce nitrogen loads in rivers (Craig et al. 2008). In essence, reestablishing wetlands and reducing flow in some areas to increase denitrification and volatilization, thus reducing the amount of N. This thesis also suggested that the presence of wetlands was correlated with the nitrogen content in Hood Canal. In the Pacific Northwest this would need to be approached with caution, to try and also maintain structural habitat for salmonids. Although, the most basic requirement for salmonid survival is clean, oxygen rich water, which may not be available when nitrogen is in excess. It seems prudent to explore all potential avenues to reduce nitrogen loading to Hood Canal.

3.6 Suggestions for Future Studies

This thesis strived to evaluate the potential to identify nitrogen sources to Hood Canal through the analysis of N stable isotopes in macroalgae. This study also tried to evaluate if this methodology could serve as a tool to reduce the severity, frequency, and duration of eutrophication and hypoxia in Hood Canal. This study suggests that isotopic analysis has the potential to be a cost-effective tool that could prove useful for tracing of point and non-point sources that are increasing nitrogen in Hood Canal, as well as a method to identify the tipping point in nitrogen load, where Hood Canal becomes eutrophic. It seems like monitoring of fast growing species, such as *Ulva sp.*, at regular intervals over time may identify the loading point where eutrophication occurs. Fast-growing species quickly incorporate nitrogen into their tissues and should be able to highlight changes in water chemistry over short period of time, in comparison to long-lived perennial species which do not respond as quickly to changes in water chemistry. To monitor the functionality of OSS this method may be applied to identify areas where nitrogen values are enriched along a shoreline and target properties for further investigation. For this isotope method to be more successful careful evaluation of sample number and species selection should be taken into consideration.

During the course of this research many faults in the isotope approach were noted that should be incorporated in future studies. First, there is a lack of research on use of nitrogen by specific algal species. It is always assumed that no fractionation occurs during the incorporation of nitrogen into algal tissue, but this is not strongly corroborated by scientific evidence. There should also be more work on the use of different types of nitrogen (i.e. NH_4^+, NO_3^-, NO_2^-) by different algal species. Second, with regards to the lack of data on nitrogen use by algae, some species are more studied than others and the ability to identify sites ahead of time and know the species present at the site is beneficial. If more time is allowed for site reconnaissance, sites may be selected where species that have been well studied are available for collection, thus reducing unknowns in the fractionation by algal species. There is also the potential to relocate algae to specified locations and track the change in nitrogen content over time, this would remove the need to select sites based on algal availability (Costanzo et al. 2001). Finally, there could be more research on the processing of nitrogen in terrestrial watersheds prior to N release into marine systems, and this work should be area specific. Studies oversimplify these processes when attempting to correlate watershed level sources with marine macroalgae N content and isotopic composition. More collaboration between terrestrial and marine scientists could improve the understanding of fluxes across this ecotone.

3.7 Conclusion

The Puget Sound ecosystem has the potential to provide cultural and subsistence resources to our community, and the cost of trying to preserve these services is a multi-million dollar business. Future work to address issues with eutrophication will require interdisciplinary collaboration between scientists to better understand the linkages between terrestrial and marine ecosystems. I hope my research may spark an interest in someone to continue on with this idea or in some other way continue to better understand the Puget Sound, so we may better know how to conserve the valuable resources it provides to us, as well as the habitat and resources it provides to the flora and fauna that call it home.

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Appendix A

Algal Species	$\delta^{15}N$ Algae	N Source	$\delta^{15}N$ Source	Habitat	Location
-	-	STE	>11.8	Monitoring Well	Florida, USA
-	-	STE	>7.3	Monitoring Well	Florida, USA
Catenella nipae	4.0-7.3	SFE	-	Estuarine	Australia
Catenella nipae	4.0-11.3	WWTP SE	-	Estuarine	Australia
Fucus vesiculosus	25.7	Anthropogenic	-	Estuarine	Netherlands
Fucus vesiculosus	8.0-13	BNR WWTP SE	38	Estuarine	Sweden
Fucus vesiculosus	3.0-4.0	Natural	-	Estuarine	Sweden
Fucus vesiculosus	6.3	Natural	-	Estuarine	Netherlands
Fucus vesiculosus	8.0-10.5	WWTP SE	24	Estuarine	Sweden
Hypnea musciformis	25.6	20% BNR WWTP SE	-	Lab Experiment	Hawaii, USA
Livestock Farming	-	-	12	Effluent Sample	Mexico
Macroalgae	6.7	Sewer	-	Estuarine	Pennsylvania, USA
Macroalgae	12.3	STE	-	Estuarine	Pennsylvania, USA
Multiple genera	0.01-1.4	Natural	-	Lava Flow	Hawaii, USA
Multiple genera	1.2-2.0	Natural	-	Offshore Reef	Jamaica
Multiple genera	6.0-12.0	SE,STE,PP	-	Nearshore Reef	Florida, USA
Poultry Farmin	-	-	13	Effluent Sample	Mexico
Ulva fasciata	30.3	20% BNR WWTP SE	-	Lab Experiment	Hawaii, USA
Ulva sp.	9.3	WWTP 2nd SE	13.5-23.5	Lab Experiment	Australia
Vidalia sp.	9.3	WWTP 2nd SE	13.5-23.5	Lab Experiment	Australia

Summary of results from similar isotope analysis studies from Dailer et al 2010. Nitrogen source abbreviations are WWTP=Wastewater Treatment Plant, SE=Sewage Effluent, BNR=Biological Nitrogen Removal, SFE=Shrimp Farm Effluent, PP=Percolation Ponds, STE=Septic Tank Effluent, 2^{nd}=Secondary Treatment.

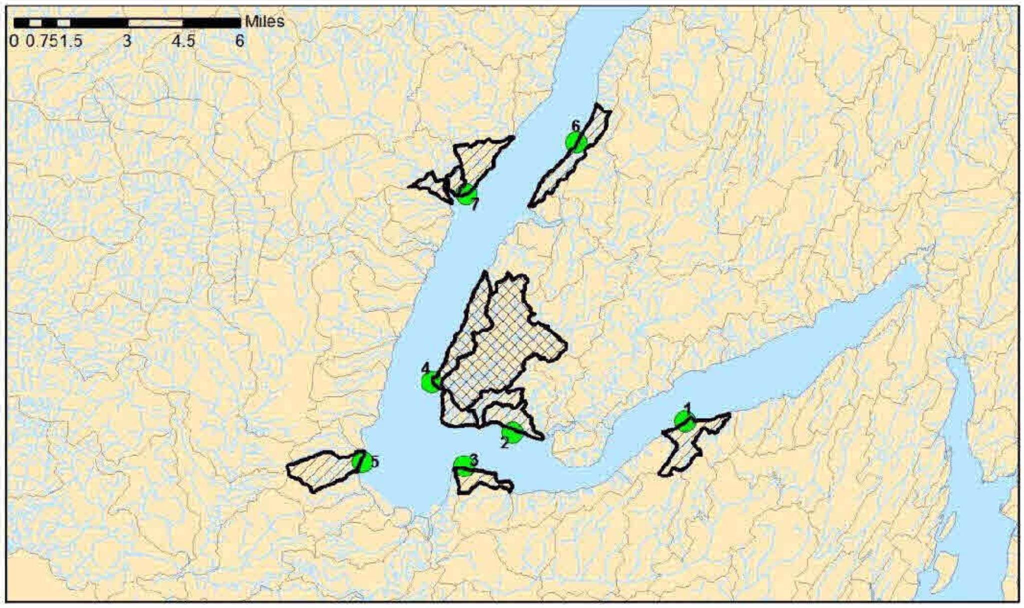
Appendix B

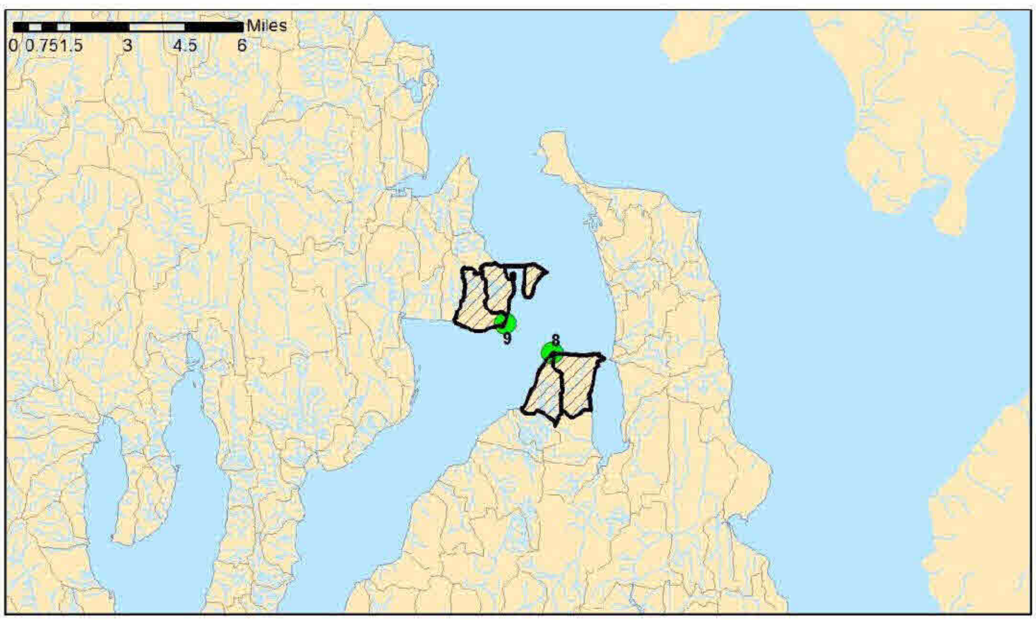
Source	Annual Contribution	Error	Units	Enriched/Depleted
WWTP	0.985	-	MT/yr	E
Agriculture	-	-	-	E
Dairy Farming	-	-	-	E
On-Site Septic	26	15	MT/yr	E
Rivers	493	170	MT/yr	-
Atmospheric Deposition	30	11	MT/yr	Background
Groundwater	138	77	MT/yr	Background
Ocean	22050	-	MT/yr	Background
Synthetic Fertilizer	0.4	-	MT/month	D
Alders	-	-	-	D

Source	$\delta_{15}N$ (‰)	References
WWTP	+10 to +25	McClelland et al 1997, Raimonet et al 2013, Paulson et al 2007
Agriculture	+6.5 to +9	Raimonet et al 2013
Dairy Farming	+10 to +20	McClelland et al 1997, Raimonet et al 2013
On-Site Septic	+3 to +25	McClelland et al 1997, Paulson et al 2007
Rivers	Depends on upstream activity	Paulson et al 2007
Atmospheric Deposition	0	Dailer et al 2010, Paulson et al 2007
Groundwater	+2 to +8	McClelland et al 1997, Paulson et al 2007
Ocean	+1 to +7	Michener and Lajtha 2007, Raimonet et al 2013, Dailer et al 2010, Paulson et al 2007
Synthetic Fertilizer	-3 to +3	McClelland et al 1997, Dailer at al 2010, Paulson et al 2007
Alders	-3 to 0	Michener and Lajtha 2007

Summary of sources and $\delta_{15}N$ values presented in Figure 5. The references used to obtain values are also listed.

Appendix C





Maps showing the adjacent land area evaluated for regression analysis with sample site $\delta^{15}N$ values with a table of watershed land area.

Site #	Watershed Area (sq ft)	Watershed Area (sq m)	Watershed Area (hectare)
1	30034277	2790274	279
2	52116327	4841763	484
3	15465891	1436828	144
4	176097827	16360016	1636
5	34965806	3248428	325
6	30954887	2875802	288
7	44260554	4111938	411
8	67957858	6313489	631
9	63817839	5928869	593

Summary table of watershed area adjacent to study sites, presented in multiple units.