

# Stable isotopes and oceanographic modeling reveal spatial and trophic connectivity among terrestrial, estuarine, and marine environments

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**ABSTRACT:** Organic matter from autochthonous and allochthonous sources provides energy and nutrients to nearshore food webs including filter-feeding bivalves. In Puget Sound, Washington, USA, the degree to which shellfish rely on these different organic matter subsidies may be important for their management and that of nearshore food webs in general. We explored patterns of terrestrial–marine connectivity in a large, temperate estuary using a combination of oceanographic modeling and isotopic mixing models. We first examined spatial connectivity by modeling freshwater contributions of the major river basins to Puget Sound (potential connectivity), then estimated the relative contribution of terrestrial, nearshore, and marine organic matter sources to nearshore particulate organic matter (POM) (actual connectivity) and to the diets of Pacific oysters *Crassostrea gigas* (realized connectivity). To estimate actual and realized connectivity, we analyzed the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of oyster tissue, POM, and primary producers from intertidal, offshore, salt marsh, and upland habitats across the dry (summer) and wet (fall–winter) seasons. Mixing models indicated that both oyster bed POM and oyster diets were composed largely of intertidal macrophytes and salt marsh plants, with less important contributions of phytoplankton, benthic diatoms, and upland vegetation. Our findings suggest that oyster production may be driven more by coastal and marine primary production than by riverine sources, even in a fjord subject to strong freshwater influences.

**KEY WORDS:** Connectivity · Puget Sound · Estuaries · Stable isotopes · Oysters

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## INTRODUCTION

Understanding ecological connectivity, both within and between ecosystems, is critical to effective natural resource management (Polis et al. 1997, Ruckelshaus et al. 2009). Despite the recognized impor-

tance of connectivity, 2 interdependent issues constrain our ability to implement effective management. The first issue is defining the spatial scale or extent of ecosystem connectivity, especially when ecological boundaries are difficult to delineate. The second issue is quantifying the strength of these con-

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nections; for example, what is the relative importance of energy or nutrient flow from one ecosystem to another? Addressing these issues is especially challenging in estuaries where terrestrial, freshwater, and marine systems come together (Dame & Allen 1996). In estuaries, terrestrial–marine connections may be mediated by the degree of freshwater influence, the spatial and temporal availability and quality of organic matter (Riera 2007, Howe & Simenstad 2011), geomorphology, and habitat modifications. Understanding the ecological connections between freshwater and marine systems has proven to be context-dependent, driven by factors such as species and life stage examined, temporal dynamics, quantity and quality of different carbon resources, and degree of freshwater influence and habitat modification (Page & Lastra 2003, Connolly et al. 2005).

Connectivity in estuarine systems may be thought of in (at least) 3 ways. First, riverine ecosystems provide a conduit for terrestrially-derived particulate organic matter (POM) and other materials and organisms to nearshore ecosystems. For estuarine ecosystems with a single dominant river (i.e. largest volume of fresh water), understanding this spatial connection is relatively straightforward since there is only 1 major freshwater source. However, for estuaries with multiple sources of fresh water, determining the origin of terrestrially derived estuarine POM is much more complicated since the potential input to a given location will be driven by river dynamics as well as by hydrological forcing in the estuary (Banas et al. 2007, 2015). This metric, defined solely by freshwater inputs and transport into an estuary, can be thought of as ‘potential connectivity’. Second, the composition of POM at a given location in an estuary reflects not just freshwater input but also marine and estuarine-derived POM such as phytoplankton, microphytobenthos, and decomposing macrophytes. This POM pool represents energy available to filter-feeding nearshore consumers and can be thought of as ‘actual connectivity’ since it represents the actual mixing of the different components of POM within an estuary. Finally, because estuarine filter feeders are not simply passive reflections of the food available to them, but may selectively ingest some POM components, the relative importance of terrestrial, marine, and estuarine components to their diets may be thought of as ‘realized connectivity’. This is because primary consumers, such as filter feeders, act as a key link between primary producers and higher trophic levels and thus will determine the degree of ecosystem connectivity of the food web as a whole. All 3 forms of connectivity provide insight into the

structure, function, and potential management of nearshore ecosystems (sensu Hamilton et al. 2008).

Organic matter available to estuarine food webs is comprised of phytoplankton and benthic microalgae as well as detritus that may originate from terrestrial, estuarine, or marine sources (Carlier et al. 2007, Marin Leal et al. 2008, Bode et al. 2011, Howe & Simenstad 2015). The relative abundance of these components is influenced by phytoplankton productivity, macrophyte standing crops, and input of organic matter from terrestrial ecosystems mediated by freshwater flow. Together, these sources comprise the pool of POM available for consumption by suspension-feeding primary consumers (Dubois et al. 2007, Marin Leal et al. 2008). Many filter feeders are capable of selectively ingesting their food based on both particle size and food quality (Baldwin & Newell 1995, Ward & Shumway 2004, Beninger et al. 2007) such that the diet of these organisms reflects the interaction between food availability and preferences of consumers.

Freshwater flow and the proximity to different organic matter sources (e.g. upland, salt marsh, and macrophyte vegetation types) can influence the relative importance or magnitude of terrestrial, marine, and estuarine influences in estuarine food webs (Ruckelshaus et al. 1993, Riera & Richard 1996, Ruesink et al. 2003, 2014). In ‘simple’ estuarine ecosystems, freshwater influence can be considered a linear function of distance from the freshwater source, whereby freshwater influence is expected to decrease with increasing distance from the river. However, in more complex estuaries with multiple, geographically distinct sources of fresh water, terrestrial–marine connectivity may instead be thought of as a network driven by hydrologic transport. In the simplest scenario, a nearshore habitat with little or no freshwater influence, consumers may primarily consume marine phytoplankton, with lower contributions of other organic matter sources such as salt marsh vegetation and benthic diatoms (Riera 2007). In estuaries with multiple sources of riverine input, nearshore consumer populations may receive relatively more energy from terrestrial sources, particularly during high freshwater flow (Riera & Richard 1997, Marin Leal et al. 2008) or in locations located closest to riverine inputs (Bucci et al. 2007). Additionally, in complex estuaries, connectivity between land and sea will be determined by the interaction between the availability of fresh water and transport of this fresh water in the estuarine environment. Because of the dynamic nature of freshwater transport, land–sea connectivity in such complex, non-

linear systems has rarely been explored (but see Simenstad & Wissmar 1985, Phillips et al. 2014).

Here, we take a multi-pronged approach toward understanding both the degree and mechanism of connectivity in a complex estuarine ecosystem, Puget Sound, Washington, USA, which is fed by a geographically diverse network of major rivers including the Fraser River in Canada which lies to the north of Puget Sound in the greater Salish Sea (Fig. 1). We used oceanographic modeling to understand the potential connectivity of freshwater sources to nearshore habitats in Puget Sound and stable isotopic analysis of POM and nearshore benthic consumers, viz. Pacific oysters *Crassostrea gigas* Thunberg, to understand the actual and realized connectivity, respectively.

## MATERIALS AND METHODS

### Study system

Puget Sound is a dynamic, marine-dominated, fjord-like estuary (Moore et al. 2008, Sutherland et al. 2011) receiving freshwater input from 14 major rivers distributed throughout 5 major sub-basins within Puget Sound, as well as from the Fraser River (Fig. 1), all of which serve as conduits linking land and sea. In a companion paper, a model analysis by Banas et al. (2015) demonstrated a high degree of connectivity among freshwater inputs and sub-basins within Puget Sound particularly in summer and fall. Thus, although Puget Sound is strongly influenced by marine hydrology, the influence of rivers on nearshore ecosystems is potentially important and non-local—controlled by the relative volume of each sub-basin and its daily freshwater input, as well as by complex river- and tide-driven transport pathways and dispersion patterns (Banas et al. 2015). The tidal range in Puget Sound is on the order of 4 m, with sub-basin-scale residence times that vary from several weeks to several months (Sutherland et al. 2011). The depth of the main channels is generally 150 to 300 m, although there are extensive shallow areas, especially in South Sound and the Whidbey Basin. In Puget Sound, shellfish are a widely harvested component of the nearshore food web; yet, despite the economic and ecological value of shellfish (Grabowski et al. 2012), little is known

about the sources of organic matter that sustain shellfish and other nearshore filter feeders in this ecosystem. Furthermore, recent efforts to implement ecosystem-based management in Puget Sound have emphasized the importance of understanding links between land and sea in this region (Ruckelshaus et al. 2009), a topic we address in our study of oysters and their energy sources.

### Study organism and site descriptions

Pacific oysters *Crassostrea gigas* are suspension-feeding bivalves that occur in mid- to low intertidal habitats in Puget Sound. They are cultured extensively in this region using primarily bottom culture methods as well as other methods such as long lines and racks. We sampled organic matter sources and oysters at 3 locations in Puget Sound including the Samish Bay, Dosewallips, and Hamma Hamma estuaries (Fig. 1). Samish Bay is located in the Rosario Strait of Puget Sound (Fig. 1); average annual discharge of the nearest freshwater source, the Samish River, is approximately  $7 \text{ m}^3 \text{ s}^{-1}$  (Banas et al. 2015).

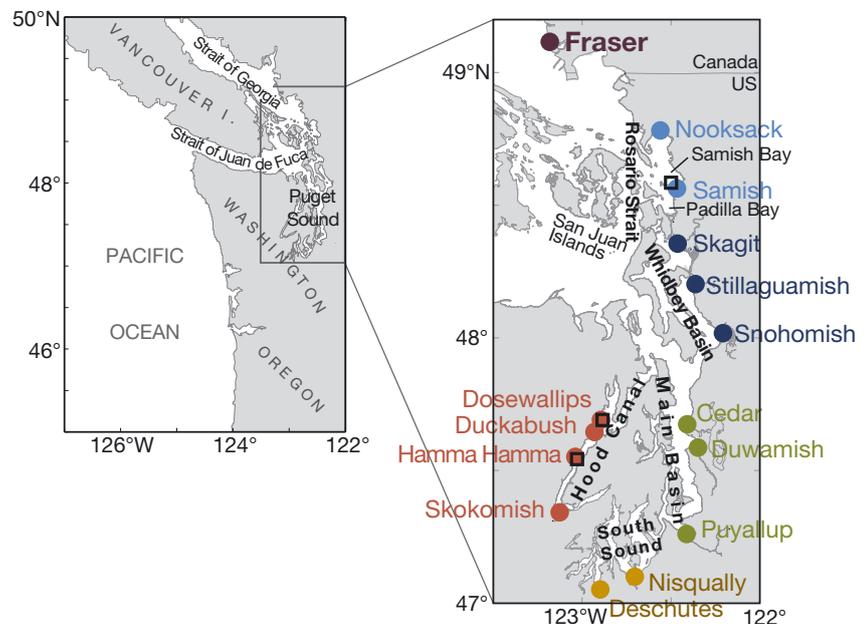


Fig. 1. Salish Sea, showing location of major river deltas and sub-basins within Puget Sound, Washington, USA. Rosario Strait = Nooksack and Samish rivers (light blue), Whidbey Basin = Skagit, Stillaguamish, and Snohomish rivers (dark blue), Main Basin = Cedar, Duwamish, and Puyallup rivers (green), South Sound = Nisqually and Deschutes rivers (orange), Hood Canal = Dosewallips, Duckabush, Hamma Hamma, and Skokomish rivers (red), and the Fraser River, outside of Puget Sound in the broader Salish Sea (purple). Oyster tissue and primary producer collection sites are noted by black squares

Oysters at this site were outplanted as juveniles by local growers since natural recruitment of *C. gigas* does not typically occur in this part of Puget Sound. Oysters included in this study were distributed patchily across an area of 1 km<sup>2</sup>. The Dosewallips and Hamma Hamma River deltas drain the Olympic Mountain range on the west side of Hood Canal (Fig. 1). The Dosewallips oyster bed extends approximately 2 km north of the river outlet and 0.5 km offshore, resulting in an available sampling area of approximately 1 km<sup>2</sup>. In the Hamma Hamma River, our access was restricted to 0.07 km<sup>2</sup> of the available ~0.5 km<sup>2</sup> growing area. These 2 rivers are comparable in flow, with average discharges of ~14 m<sup>3</sup> s<sup>-1</sup> (Banas et al. 2015). All 3 sites experience a similar tidal range (approximately 4 m), and the lower river reaches experience some degree of tidal influence, although this is likely to be less pronounced in the Hamma Hamma and Dosewallips Rivers since the elevation gain there is more steep.

### Oceanographic model

We used the circulation model described in detail by Sutherland et al. (2011) followed by the intensive particle-tracking reanalysis of that model described in detail by Banas et al. (2015) to investigate potential connectivity. The circulation model is implemented using the Regional Ocean Model System (ROMS) in a domain which covers the entire Salish Sea and coastal oceanic waters from central Oregon (USA) to central Vancouver Island (Canada). This model uses 20 terrain-following layers, with horizontal resolution expanding from 280 m in southern Puget Sound to 3.1 km far offshore. Bathymetry for Puget Sound is from Finlayson (2005), and wetting and drying of intertidal areas is not explicitly included in the model. The model was run with a time step of 30 s for a yearlong hindcast of 2006, with output saved hourly. The hindcast was forced by realistic tides, wind, and heat fluxes from the MM5 regional forecast model (Mass et al. 2003), open-ocean boundary conditions from the Navy Coastal Ocean Model (Barron et al. 2006, 2007), and streamflow from the 15 rivers shown in Fig. 1 as well as the Columbia River. We used streamflow data from USGS and Environment Canada gauges on all rivers except the Dosewallips and Hamma Hamma, which are ungauged. For these Hood Canal rivers, flow time series were set equal to that for the nearby Duckabush (Fig. 1) based on their watershed similarities. Banas et al. (2015) described in detail a comprehensive post-pro-

cessing analysis of the 2006 model run, in which 131 000 model particles (1 released per hour at the mouth of each of 15 rivers: Fig. 1) were used to provide a statistical view of pathways and time histories taken by particles representing fresh water from each major river through the system. Here, we describe results from this analysis for 2 of the 3 locations where we collected oyster tissue in Puget Sound: the nearshore environments adjacent to the Dosewallips River (Hood Canal sub-basin) and the Samish River (Rosario sub-basin; Fig. 1). This model incorporates both quantity of freshwater inputs (river discharge) and transport of fresh water throughout the Sound (Banas et al. 2015) to permit the calculation of site-specific estimates of spatial or potential connectivity via fresh water. Sutherland et al. (2011) provided detailed validation of the base model against salinity, temperature, and current observations at a variety of locations. Banas et al. (2015) provided consistency checks on the particle analysis that suggest that total freshwater input is very well accounted for in northern Puget Sound and Samish Bay, but errors increase as one moves landward toward poorly flushed, narrow embayments like southern Hood Canal. The conclusions drawn from the particle-tracking experiment for our northern Hood Canal study areas do not appear to be sensitive to these biases.

### Isotope mixing model

Natural abundance levels of stable isotopes are a key tool for understanding the cycling of elements through an ecosystem. For example, they allow us to estimate the relative importance of different organic matter sources to consumer diets if these sources display distinct isotopic composition (Fry & Sherr 1984, Cloern et al. 2002). We applied the Bayesian mixing model MixSIR (Moore & Semmens 2008) to measure stable isotope values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) to assess the contributions of marine, terrestrial, and estuarine primary producers to nearshore POM (oyster bed POM, hereafter POM-O<sub>ys</sub>) and estuarine bivalves (Pacific oysters) at the mouth of 3 river basins (Samish, Hamma Hamma, and Dosewallips) in Puget Sound.

### Oyster collection and preparation

Six adult oysters of standardized size (110–130 mm in length) were haphazardly collected from 3 equally sized portions (approximately 300 × 300 m) of the

Samish and Dosewallips shellfish growing areas (12–18 oysters total per site at each sample interval). In the Hamma Hamma growing area, access was restricted; so, collection was from a smaller portion of the growing area (0.07 km<sup>2</sup> of the available ~0.5 km<sup>2</sup>). For consistency across sites, only oysters growing on the primary substrate (bottom cultured) were collected. Oysters were placed into clean plastic bags and put on ice immediately following collection and frozen at –20°C at the end of each sampling day. We collected oyster tissue in June 2011, August 2011, and January 2012 (Table 1).

In Samish Bay, commercial harvest periodically removes all oysters from a given location; therefore, in early August 2011, 30 oysters were relocated within each subarea and placed in 2 m × 2 m staked plots. Subsequent collections (6 ind. subarea<sup>-1</sup> at each sample interval, as above) were made from these staked plots, ensuring that oysters of the appropriate size could be located during subsequent sampling events.

Prior to dissection, oysters were rinsed thoroughly with dilute (10 %) hydrochloric acid (HCl) and deionized (DI) water. The adductor muscle tissue was removed and placed into 50 ml glass scintillation vials and then stored in a –20°C freezer. Individual samples were freeze-dried for 24 h, homogenized to a fine powder with stainless steel scissors, and stored in a desiccator. A microbalance was used to weigh tissue (0.5–0.8 mg), which was then placed into 5 × 9 mm tin capsules and a sealed 96-well tray (Washington State University Stable Isotope Core Laboratory). Stable isotope composition (ratios of <sup>13</sup>C:<sup>12</sup>C and <sup>15</sup>N:<sup>14</sup>N) and quantitative elemental composition (%C, %N) were determined using a Costech ECS 4010 elemental analyzer and a Delta Thermo Finnigan continuous flow mass spectrometer at the Washington State University Stable Isotope Core Laboratory. Delta values (units in per mil or ‰) are expressed using the standard notation:

$$\text{Heavy isotope} = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 1000 \quad (1)$$

where  $R_{\text{sample}}$  is the ratio of the heavy to light isotope (<sup>14</sup>C:<sup>13</sup>C or <sup>15</sup>N:<sup>14</sup>N) in the sample and  $R_{\text{standard}}$  is the ratio of <sup>14</sup>C:<sup>13</sup>C or <sup>15</sup>N:<sup>14</sup>N in Vienna Peedee Belemnite for carbon and atmospheric N for nitrogen.

Table 1. Timing of field sample collections from June 2011 to January 2012 in Puget Sound, USA. August Pacific oyster *Crassostrea gigas* and vegetation samples were collected between 27 and 31 August 2011. POM-Oys: oyster bed particulate organic matter; POM-phy: phytoplankton-dominated particulate organic matter

Collection type	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
Oyster adductor muscle tissue	X		X					X
POM-Oys	X	X	X			X	X	
POM-phy	X			X			X	
Intertidal macrophytes	X		X			X		
Salt marsh plants	X		X			X		
Upland vegetation	X		X			X		
Benthic diatoms	X		X			X		

### Primary producer, water sampling, and environmental data

Organic matter sources were collected in June, August, and November 2011, and included phytoplankton (offshore or phytoplankton-dominated POM, hereafter POM-phy), macroalgae and eelgrass, benthic diatoms, leaf material from riparian vegetation, and salt marsh plants (see the section on mixing models below for a complete list of taxa; Table 1). For each source, leaves/blades were haphazardly collected from 3 to 5 individual plants from the oyster bed, salt marsh, and riverine areas for the Samish, Dosewallips, and Hamma Hamma growing areas/watersheds. Collections for all sources (e.g. leaves, macroalgae) followed the methodology described above for oyster tissue. To ensure complete removal, epiphytes were washed from macrophyte samples by rinsing with DI water and visually inspecting under a dissection microscope. Samples collected from intertidal and salt marsh areas were rinsed with 10 % dilute HCl to remove carbonates, followed by a rinse with DI water. Benthic diatoms were collected using 15 cm × 15 cm nitex (20 μm mesh size) squares placed onto the substrate during daytime low tides and allowed to accumulate diatoms for 1 to 2 h (following Cloern et al. 2002, Howe & Simenstad 2011). Diatoms were rinsed from nitex with DI water and allowed to settle in sterile 250 ml glass beakers (1–3 h), during which time they became stratified such that inorganic particles settled to the bottom of the beaker due to density differences. Diatoms were then carefully removed using sterile 20 ml disposable pipettes, and re-suspended in DI water, followed by filtering onto Whatman 47 mm GF/F filters. Diatom samples were then oven dried (60°C for 12 h), exposed to HCl vapor in a glass desiccator for 4 h at

room temperature to remove carbonates (Lorrain et al. 2003), placed in a fume hood (3 h), and oven dried (12 h, 60°C) to remove excess HCl and water. Particulate matter was scraped from filters and processed in the same manner as oyster tissue. To ensure diatom samples met the minimum detection limits for C and N of the mass spectrometer, larger amounts of material (10–12 mg) were submitted for analysis.

Bulk water samples were collected to represent POM over oyster beds monthly from June to December 2011 (Table 1) using small boats at or near high tide. These samples were collected from 1 m below the water surface at 3 evenly distributed locations across each shellfish growing area using a 5 l Niskin® grab sampler. Samples were then filtered onto pre-combusted (450°C for 4 h) filters (Whatman 47 mm GF/F) using a low-pressure (<15 psi) vacuum pump until visible particulate accumulation occurred. Filters were folded using sterile forceps, placed into pre-combusted glass scintillation vials, and frozen at –20°C. When shipboard vacuum pumping was not feasible, water samples were transported on ice and filtered no more than 8 h after collection. Once filtered, POM samples were processed in the same manner as the benthic diatom samples (above).

Temperature, salinity, and chlorophyll *a* (chl *a*) were measured monthly at 3 locations within each oyster bed at or near high tide using a Seabird CTD cast deployed from a small boat.

### Mixing models

We used the Bayesian mixing model MixSIR (Moore & Semmens 2008) to estimate the likely proportionate contributions of different food sources to oyster diets at each site and time period. This modeling approach incorporates source variability and generates probability distributions of likely diet contributions. Potential diet items were as follows: POM-<sub>phy</sub> (defined as phytoplankton-dominated POM collected from offshore locations in Puget Sound); benthic microalgae; terrestrial vegetation, which consisted of leaves of red alder *Alnus rubra* and willow (*Salix* spp.); Douglas-fir *Pseudotsuga menziesii*; salt marsh vegetation (*Salicornia virginica* and *Glaux maritima*); and marine macrophytes (intertidal *Zostera* spp. and macroalgae *Ulva* spp., *Fucus distichus*, *Gracilaria/Gracilariopsis* spp., and *Laminaria* spp.). Not all primary producers were collected at all sites and time intervals; therefore, to keep the model as simple as possible and to reduce overlap among end members (Fry 2013, Semmens et al. 2013), we conser-

vatively pooled diet items by habitat type (riparian vegetation, salt marsh vegetation, intertidal macrophytes, benthic diatoms, and phytoplankton). We used temporally pooled values (means and variances across samples collected in June, August, and November 2011) for each study site for terrestrial vegetation, intertidal macrophytes, and salt marsh plants, allowing us to incorporate temporal variability in end members into the mixing model in a location-specific manner (Dethier et al. 2013). For the other 2 end members, (POM-<sub>phy</sub> and benthic diatoms), we pooled data across sampling locations and time (June–November 2011) since the biomass of individual samples sizes was too low for isotope analysis.

The degree to which consumers become enriched relative to their food sources ( $\Delta$  = fractionation) is an important source of uncertainty associated with using stable isotope values to infer consumer diets since fractionation *in situ* is likely to vary as a function of physiological condition of the consumer, food quantity and quality, growth rates, and ambient temperature (Fry 2006). To address potential variability and uncertainty in fractionation, we adopted the approach of Lefebvre et al. (2009a) by using a whole-body estimate for *C. gigas* (Dubois et al. (2007) of  $\Delta$  1.85 for  $\delta^{13}\text{C}$  and  $\Delta$  3.79 for  $\delta^{15}\text{N}$  as well as a corrected estimate for muscle tissue by adding the fractionation of Dubois et al. (2007) to the difference between whole body and muscle tissue derived from McCutchan et al. (2003). This resulted in using a second trophic enrichment factor of  $\Delta$  2.9 for  $\delta^{13}\text{C}$  and  $\Delta$  4.7 for  $\delta^{15}\text{N}$  (Lefebvre et al. 2009a). We used 1.1 as the standard deviation for both fractionation estimates (Dubois et al. 2007). Because we had no *a priori* data on the consumer diet or relative availability of organic matter sources, we used non-informative priors (Moore & Semmens 2008).

To estimate the contribution of different primary producers to POM collected in oyster beds (POM-<sub>Oys</sub>), we used the same set of primary producer sources described above. We assumed no fractionation between sources (primary producers) and the POM-<sub>Oys</sub>. Low quantities of POM accumulated onto filters combined with analytical difficulties resulted in generally low sample sizes, particularly in Hood Canal. Dosewallips and Hamma Hamma POM-<sub>Oys</sub> data were consequently pooled for the mixing model analysis. No POM-<sub>Oys</sub> data were obtained from the months of September and October at any of the 3 sites (Fig. 2d,e). This resulted in 10 POM-<sub>Oys</sub> samples from Samish Bay and 11 samples from Hood Canal collected in June, July, August, November, and December 2011 (Table 1).

## RESULTS

### Oceanographic model (potential connectivity)

Potential connectivity varied strikingly between Samish Bay and Hood Canal, with a much higher degree of local retention of riverine water in Hood Canal than Samish Bay (Table 2). For Samish Bay,

the Fraser River (Fig. 1) contributed 35 to 66 % of the fresh water (i.e. salinity deficits relative to ocean salinity) found in this area (Table 2). The next largest contributors were rivers in the Whidbey basin (Skagit, Stillaguamish, and Snohomish; 14–37 % of fresh water) (Table 2). By contrast, local rivers were the most important freshwater source in the Hood Canal, with 17 to 54 % of the fresh water originating from Hood Canal rivers (Table 2). However, the Fraser River contributed up to 50 % of the Hood Canal sites' fresh water in the summer months (Table 2). Note that although large, distant rivers like the Fraser and Skagit have a substantial influence on the study locations in volumetric terms, fresh water from distant and local rivers takes very different lengths of time to arrive at a given spot; for example, a particle of Fraser River water in Hood Canal may take more than 200 d to get there after leaving the Fraser mouth (Banas et al. 2015).

### Environmental data

Water temperature in Samish Bay ranged from 14.3°C in August to 7.6°C in December, and from ca. 18°C in August to 7.0°C in December for Hood Canal sites (Fig. 2a). With the exception of September, when weather conditions forced sampling in the Samish Bay closer to the river, water over oyster beds was ~4 to 50 % more saline in Samish Bay than at Hood Canal sites (Fig. 2b). Lower salinity in Hood Canal in June and December potentially reflects increases in river flow from snowmelt and rainfall, respectively. Mean chl *a* was generally low (<2 mg m<sup>-3</sup>) at all 3 sites, with the highest values observed in July, September, and November (Fig. 2c).

### Isotopic composition of organic matter sources and oysters

Marine macrophytes were the most enriched organic matter source (i.e. higher  $\delta^{13}\text{C}$  values) at all 3 study sites

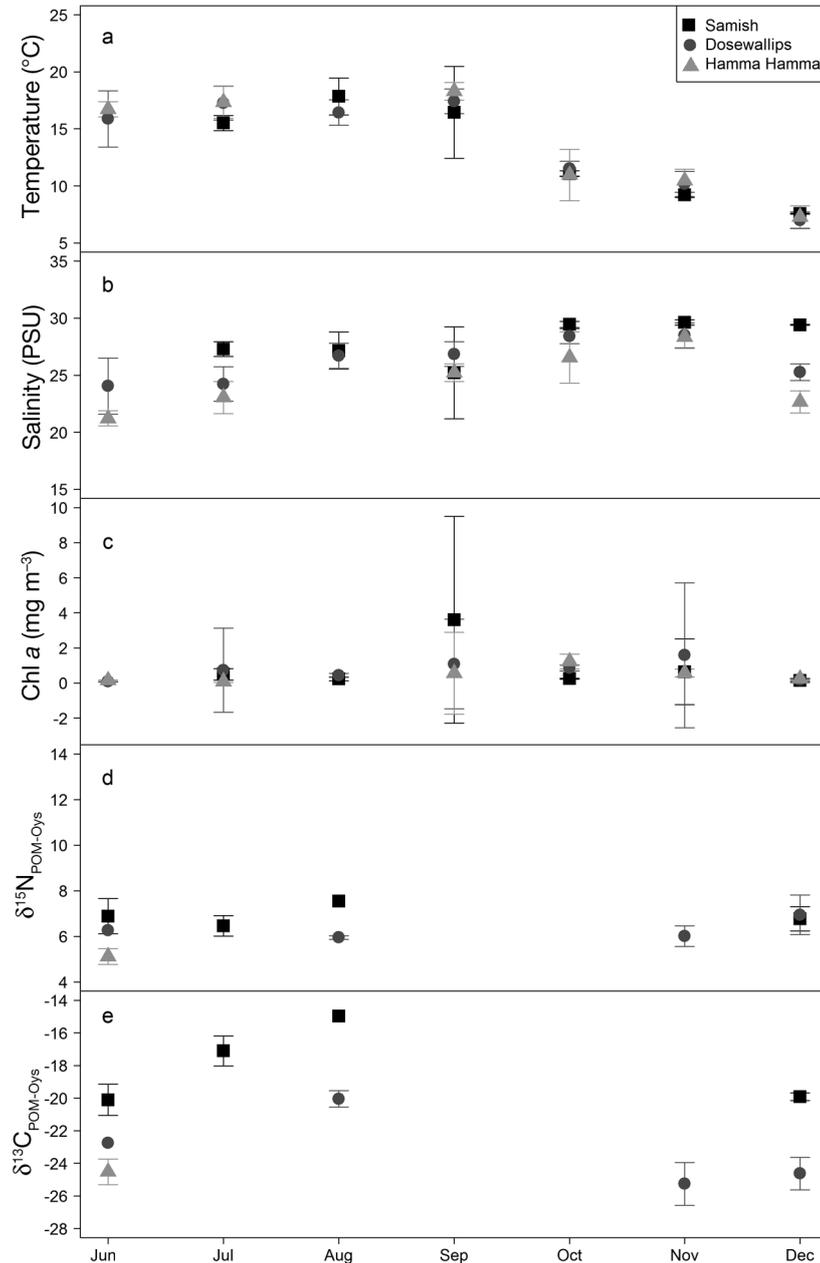


Fig. 2. Monthly mean ( $\pm$ SD) (a) temperature, (b) salinity, (c) chlorophyll *a*, (d)  $\delta^{15}\text{N}$  of the oyster bed particulate organic matter ( $\delta^{15}\text{N}_{\text{POM-Oys}}$ ), and (e)  $\delta^{13}\text{C}$  of the oyster bed POM ( $\delta^{13}\text{C}_{\text{POM-Oys}}$ ) in Pacific oyster *Crassostrea gigas* beds from June to December 2011. Stable isotope units are in ‰

Table 2. Oceanographic model results of relative percent contributions of freshwater inputs from all Puget Sound basins and the Fraser River to the Samish and Hood Canal (Dosewallips River). Entries in **bold** show local percent contributions

	Fraser River	Rosario	Whidbey Basin	Main	South Sound	Hood Canal
<b>Samish Bay</b>						
Winter	35	<b>19</b>	37	2	4	4
Spring	65	<b>17</b>	14	1	2	1
Summer	66	<b>8</b>	20	2	2	0
Mean	56	<b>15</b>	24	2	3	2
<b>Hood Canal</b>						
Winter	18	2	19	4	5	<b>53</b>
Spring	24	1	26	4	8	<b>37</b>
Summer	50	1	24	3	6	<b>17</b>
Mean	31	1	23	4	6	<b>36</b>

(range of mean values from  $-10.4$  to  $-14.0\text{‰}$ ) followed by benthic diatoms ( $-16.9\text{‰}$ ), POM-phy ( $-20.6\text{‰}$ ), and salt marsh plants ( $-26.8$  to  $-28.0\text{‰}$ ), while upland vegetation was the most depleted ( $-31.7$  to  $-30.3\text{‰}$ ; Table 3, Fig. 3). Upland vegetation had the lowest  $\delta^{15}\text{N}$  values (range of means from  $-2.2$  to  $-0.01\text{‰}$ ) while salt marsh vegetation had the highest ( $6.8$  to  $9.1\text{‰}$ ; Table 3, Fig. 3).  $\delta^{13}\text{C}$  values for oysters ranged from  $-16.1$  to  $-19.3\text{‰}$ , while  $\delta^{15}\text{N}$  values ranged from  $10.6$  to  $11.0\text{‰}$

(Table 3, Fig. 3). Using trophic enrichment factors,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of oyster adductor muscle tissue fell within the ranges of potential diet end members at all 3 sites (Fig. 3).

#### Mixing model: POM-Oys (actual connectivity)

Salt marsh plants were key contributors to the nearshore POM-Oys in both Samish Bay (median values are reported to represent central tendency and quartile range to represent dispersion [i.e. median; quartile range];  $17\%$ ;  $13\text{--}21\%$ ) and Hood Canal ( $40\%$ ;

$35\text{--}45\%$ ) along with benthic diatoms and phytoplankton, which together accounted for  $\sim 45\%$  (quartile ranges for Samish =  $25\text{--}64\%$  and Hood Canal =  $31\text{--}59\%$ ) to POM-Oys (Fig. 4). Intertidal macrophytes were estimated to be a less important component of Hood Canal POM ( $5\%$ ;  $3\text{--}9\%$ ) than Samish Bay POM-Oys ( $30\%$ ;  $23\text{--}36\%$ ; Fig. 4). Posterior distributions for source contributions to the POM-Oys were unimodal and had generally informative central tendencies.

Table 3.  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values (units in  $\text{‰}$ ,  $\pm\text{SD}$ ) of potential diet items (upland vegetation, salt marsh plants, intertidal macrophytes, benthic diatoms, and phytoplankton-dominated particulate organic matter [POM-phy]), Pacific oysters *Crassostrea gigas*, and oyster bed POM (POM-Oys) collected in the Samish (Sa), Dosewallips (Do), and Hamma Hamma (HH) river deltas and watersheds. Empty cells indicate that no data were collected. Primary producer group averages and the primary consumer *C. gigas* are listed in **bold**

	$\delta^{13}\text{C}$			$\delta^{15}\text{N}$			Sample size		
	Sa	Do	HH	Sa	Do	HH	Sa	Do	HH
<b>Upland</b>	<b><math>-30.6 \pm 1.2</math></b>	<b><math>-31.7 \pm 2.2</math></b>	<b><math>-30.3 \pm 1.8</math></b>	<b><math>0.0 \pm 2.4</math></b>	<b><math>-1.0 \pm 0.7</math></b>	<b><math>-2.2 \pm 1.6</math></b>	<b>24</b>	<b>24</b>	<b>23</b>
<i>Pseudotsuga menziesii</i>		$-31.5 \pm 2.5$	$-31.8 \pm 0.6$		$-1.0 \pm 0.9$	$-3.6 \pm 1.2$		12	11
<i>Alnus rubra</i>	$-31.1 \pm 0.9$	$-32.0 \pm 2.0$	$-28.9 \pm 1.5$	$-1.5 \pm 0.4$	$-1.1 \pm 0.4$	$-1.0 \pm 0.4$	16	12	12
<i>Salix</i> spp.	$-29.7 \pm 1.3$			$2.9 \pm 2.0$				8	
<b>Salt marsh</b>	<b><math>-26.8 \pm 2.0</math></b>	<b><math>-27.6 \pm 0.9</math></b>	<b><math>-28.0 \pm 0.6</math></b>	<b><math>9.1 \pm 1.1</math></b>	<b><math>6.8 \pm 1.8</math></b>	<b><math>7.4 \pm 1.4</math></b>	<b>19</b>	<b>18</b>	<b>11</b>
<i>Glaux maritima</i>	$-24.7 \pm 0.6$	$-26.6 \pm 0.9$		$8.6 \pm 0.9$	$5.6 \pm 0.7$		8	6	
<i>Salicornia virginica</i>	$-28.4 \pm 0.8$	$-28.1 \pm 0.4$	$-28.0 \pm 0.6$	$9.4 \pm 1.2$	$7.4 \pm 1.9$	$7.4 \pm 1.4$	11	12	11
<b>Macrophytes</b>	<b><math>-10.4 \pm 2.5</math></b>	<b><math>-12.4 \pm 3.3</math></b>	<b><math>-14.0 \pm 3.5</math></b>	<b><math>8.1 \pm 1.0</math></b>	<b><math>7.3 \pm 0.6</math></b>	<b><math>6.8 \pm 0.9</math></b>	<b>38</b>	<b>38</b>	<b>33</b>
<i>Fucus distichus</i>		$-14.4 \pm 1.1$	$-13.7 \pm 0.9$		$7.3 \pm 0.4$	$7.0 \pm 0.4$		12	4
<i>Gracilaria</i> spp.		$-12.0 \pm 1.0$	$-16.0 \pm 1.4$		$7.9 \pm 0.1$	$7.0 \pm 0.5$		4	6
Laminariales	$-15.9 \pm 1.7$			$8.2 \pm 0.9$			4		
<i>Ulva</i> spp.	$-10.4 \pm 1.4$	$-13.4 \pm 4.3$	$-17.1 \pm 2.8$	$8.2 \pm 0.8$	$7.6 \pm 0.4$	$7.4 \pm 0.7$	12	12	10
<i>Zostera</i> spp.			$-9.0$			$5.9$			1
<i>Zostera japonica</i>	$-9.6 \pm 1.6$	$-9.4 \pm 1.3$	$-11.9 \pm 1.5$	$7.6 \pm 1.5$	$6.0 \pm 0.5$	$6.7 \pm 1.2$	12	4	6
<i>Zostera marina</i>	$-9.3 \pm 1.6$	$-8.9 \pm 0.7$	$-9.9 \pm 1.7$	$8.3 \pm 0.5$	$6.8 \pm 0.4$	$6.0 \pm 0.4$	10	6	6
POM-phy (all sites)		$-20.6 \pm 4.7$			$6.4 \pm 1.5$			9	
Benthic diatoms (all sites)		$-16.9 \pm 4.1$			$6.4 \pm 1.4$			7	
<b><i>Crassostrea gigas</i></b>	<b><math>-16.1 \pm 0.7</math></b>	<b><math>-18.7 \pm 0.8</math></b>	<b><math>-19.3 \pm 0.6</math></b>	<b><math>11.0 \pm 0.6</math></b>	<b><math>10.8 \pm 0.7</math></b>	<b><math>10.6 \pm 0.4</math></b>	<b>45</b>	<b>53</b>	<b>47</b>
POM-Oys (Samish)	$-18.5 \pm 1.9$			$6.9 \pm 0.6$			10		
POM-Oys (Hood Canal)		$-23.8 \pm 2.1$			$6.1 \pm 0.8$			11	

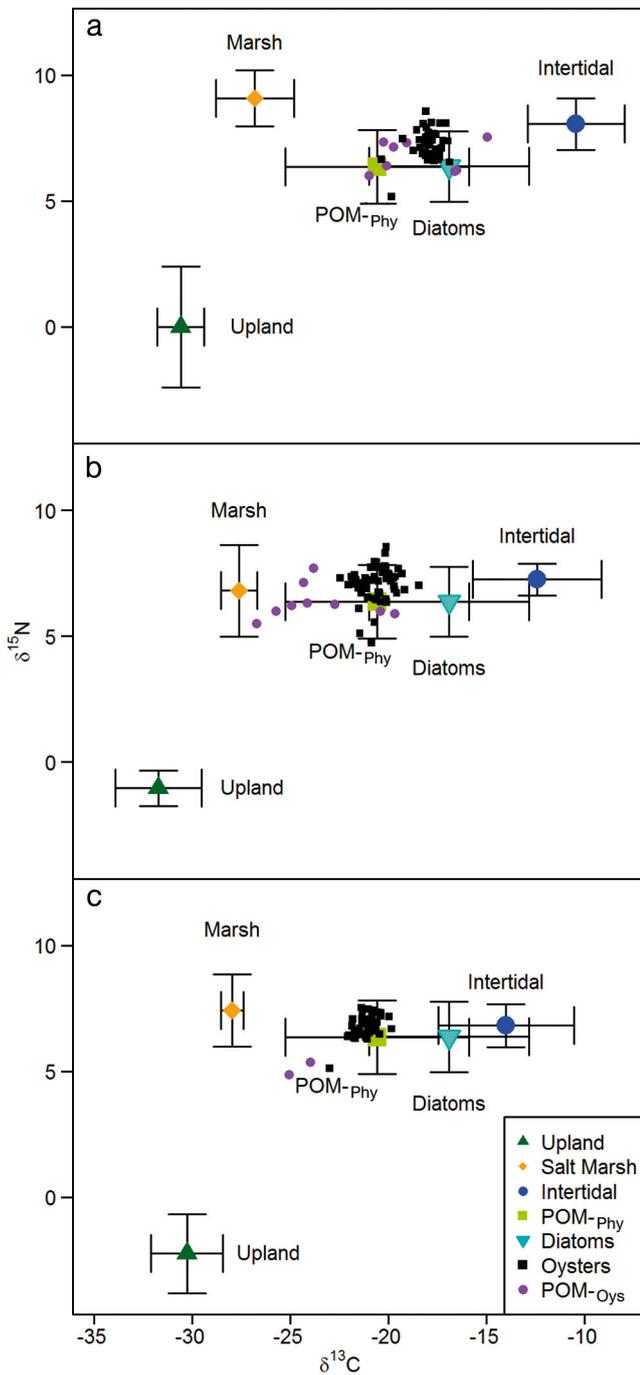


Fig. 3. Carbon and nitrogen stable isotope values (in ‰) of individual Pacific oysters *Crassostrea gigas*, oyster bed particulate organic matter (POM-Oys), and mean ( $\pm$ SD) potential diet items in the (a) Samish, (b) Dosewallips, and (c) Hamma Hamma growing areas. Potential diet items were: upland vegetation (Upland), salt marsh plants (Marsh), intertidal macrophytes (Intertidal), phytoplankton-dominated POM (POM-phy), and benthic diatoms (Diatoms). Oyster values were adjusted using trophic enrichment factors from Dubois et al. (2007): ( $\Delta 1.85$  for carbon and  $\Delta 3.79$  for nitrogen). Benthic diatoms and POM-phy were pooled across all 3 sites but are shown on each individual graph for clarity

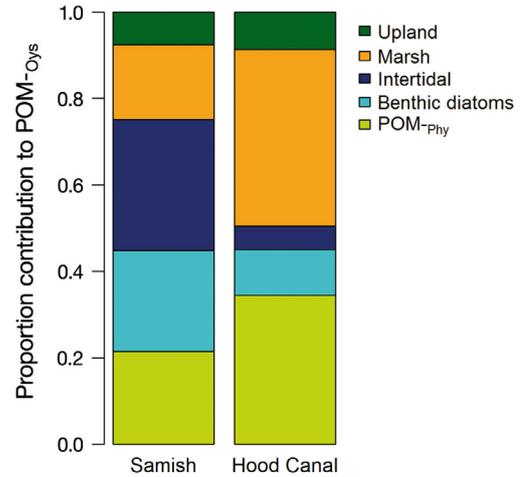


Fig. 4. MixSIR results showing median contributions of organic matter sources (upland vegetation, salt marsh plants, intertidal macrophytes, benthic diatoms, and phytoplankton-dominated particulate organic matter [POM-phy]) to oyster bed POM (POM-Oys) for the Samish and combined Hood Canal sites

#### Mixing model: oysters (realized connectivity)

Results from trophic enrichment factors reported by Dubois et al. (2007) and Lefebvre et al. (2009a) were generally comparable, suggesting that the relative contributions of organic matter sources were fairly robust to this source of uncertainty, with the exception of slightly higher estimates of the contribution of upland vegetation using the fractionation of Lefebvre et al. (2009a), particularly for Samish Bay. Because of this agreement, we report mixing model results using fractionation factors from Dubois et al. (2007), i.e.  $\Delta 1.85$  for carbon and  $\Delta 3.79$  for nitrogen (Figs. 5 & 6). Our models estimated that oysters at all 3 sites received approximately 30% of their energy from benthic diatoms and phytoplankton combined under both fractionation scenarios, while receiving different relative contributions from the other primary producers. Hood Canal oysters had higher combined contributions of terrestrial vegetation with approximately 40% originating in marshes and 2% originating from upland vegetation (Fig. 5). This result suggests particularly strong terrestrial-aquatic coupling between salt marshes and oysters in intertidal habitats of the Dosewallips and Hamma Hamma river deltas (Fig. 6). By contrast, Samish Bay oysters relied more on autochthonous intertidal macrophytes (40%) in addition to salt marsh plants (25%), with approximately 7% of diets originating from terrestrially derived detritus (Fig. 6). Posterior distributions for estimated source contributions to oyster diets were unimodal and with generally informative central tendencies (Fig. 5).

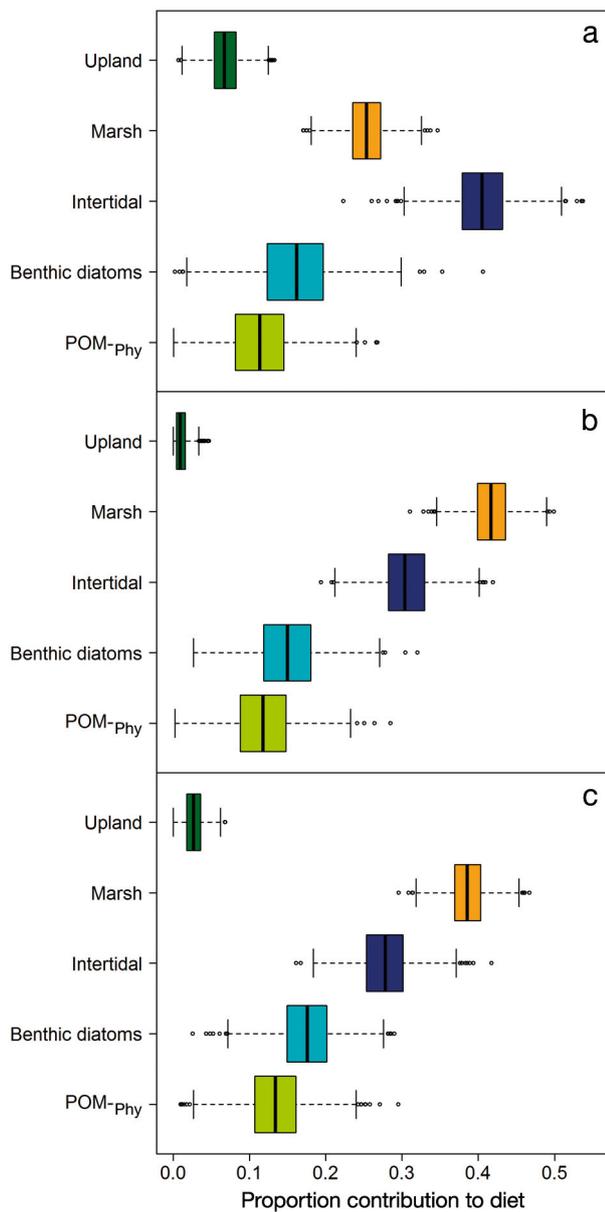


Fig. 5. Distributions of estimated proportion contribution of diet items (upland vegetation, salt marsh plants, intertidal macrophytes, benthic diatoms, and phytoplankton-dominated particulate organic matter [POM-Phy]) in the (a) Samish, (b) Dosewallips, and (c) Hamma Hamma growing areas. Boxes show medians and interquartile distances (IQD), whiskers show values within 1.5 times the IQD, and open circles represent values falling outside of this range

## DISCUSSION

Defining potential, actual, and realized connectivity among terrestrial, marine, and estuarine environments is a key component of understanding and managing nearshore ecosystems, particularly in estuaries that have multiple important sources of

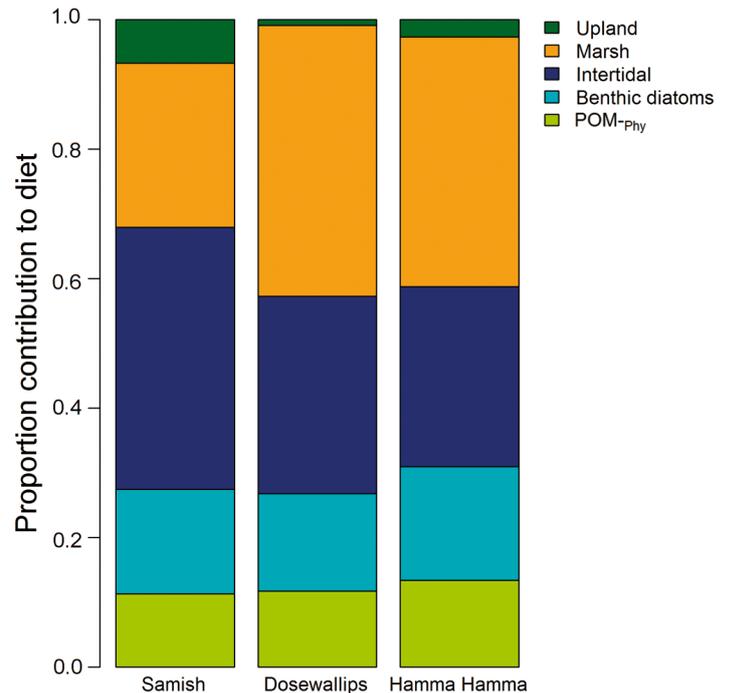


Fig. 6. MixSIR results showing median contributions to Pacific oyster *Crassostrea gigas* diets (upland vegetation, salt marsh plants, intertidal macrophytes, benthic diatoms, and phytoplankton-dominated particulate organic matter [POM-Phy]) for the Samish, Dosewallips, and Hamma Hamma growing areas

freshwater inputs and complex patterns of hydrologic connectivity and flow patterns. We found a strikingly high degree of freshwater transport (potential connectivity) across sub-basins of Puget Sound, particularly for Samish Bay (Table 2), highlighting the potential for strong coupling across broad spatial scales between upland and marine habitats. However, exploring the actual connectivity and realized connectivity between these habitats revealed low contributions of upland detritus and higher contributions of marine, estuarine, and salt marsh organic matter sources to both nearshore POM-Oys and benthic filter feeders. Our analysis helps to define the relationship between the outer limits of the potential for transport of organic matter and the input of organic matter to food webs across a complex and dynamic seascape.

Hood Canal displayed strong local retention of fresh water (potential connectivity) and a high contribution of salt marsh plants to both nearshore POM-Oys (actual connectivity) and oyster diets (realized connectivity). This region has abundant salt marshes, in part because of the expansive Skokomish River delta (Simenstad & Wissmar 1985; Fig. 1), and the ecological importance of salt marshes to filter

feeders in this region has also been recently demonstrated by Howe & Simenstad (2015). The highly comparable contributions of upland vegetation (all median values: POM-O<sub>ys</sub> = 8%; oyster diet ~1–3%), salt marsh plants (POM-O<sub>ys</sub> = 40%; oyster diet = 39–42%) and benthic diatoms (POM-O<sub>ys</sub> = 10%; oyster diet = 15–18%) to both the nearshore POM-O<sub>ys</sub> and oyster diets in Hood Canal suggests that oysters were consuming these diet sources roughly proportionately to their availability in the seston pool (Figs. 4 & 6). We found quite different contributions of both intertidal macrophytes and POM-O<sub>ys</sub> between the POM-O<sub>ys</sub> and oyster diets such that we estimated a relatively high (28–30%) contribution of intertidal macrophytes to Hood Canal oyster diets (realized connectivity) yet only 5% to the nearshore POM-O<sub>ys</sub> (actual connectivity). Conversely, the mixing model predicted a lower (12–13%) contribution of POM-phy to oyster diets while the POM-O<sub>ys</sub> mixing model in Hood Canal estimated a much higher contribution of POM-phy (34%) to the seston pool. Thus, Hood Canal oysters may consume these resources (intertidal macrophytes and POM-phy) in a manner disproportionate to their availability (displaying food preferences; Beninger et al. 2007) by selectively ingesting intertidal macrophyte detritus and rejecting phytoplankton (POM-phy). This selectivity could be accomplished by the initial rejection of some particles upon uptake into the oyster mantle cavity or else by the production of pseudofeces (particles rejected prior to ingestion; Ward & Shumway 2004). It is possible that less palatable species of phytoplankton were more abundant at this time and helped to drive such preferences. However, it is equally possible that the low sample sizes of nearshore POM-O<sub>ys</sub> (11 grab samples collected over 6 mo) from Hood Canal may have led to an underrepresentation of macrophytes in POM-O<sub>ys</sub> samples relative to their actual abundance and availability to nearshore consumers. Species-specific data on phytoplankton as well as sampling a higher volume of nearshore POM-O<sub>ys</sub> and at a higher frequency for at least 1 full annual cycle would help to distinguish between these scenarios. Nevertheless, our data suggest strong connections between the diets of intertidal oysters, nearshore POM-O<sub>ys</sub>, and salt marsh vegetation and intertidal macrophytes.

As in Hood Canal, we found generally comparable estimated contributions of seston sources to POM-O<sub>ys</sub> collected in Samish Bay (Rosario sub-basin; actual connectivity) and oyster diets (realized connectivity). Intertidal macrophytes (eelgrass and macroalgae) appeared to be particularly abundant components of

both POM-O<sub>ys</sub> (30%) and oyster diets (40%). Samish Bay and adjacent Padilla Bay have some of the largest continuous beds of eelgrass in Puget Sound (Gaeckle et al. 2009), which may help explain the high proportion of intertidal macrophytes contributing to both consumer diets and POM-O<sub>ys</sub> at this site. Furthermore, the oceanographic model predicted high potential connectivity between Whidbey Basin, where Padilla Bay is located, and Samish Bay, suggesting that freshwater flow between these basins could provide a mechanism for transport of organic matter from Whidbey Basin to Samish Bay.

We found similar patterns in the estimated relative importance of organic matter sources to oyster diets (realized connectivity) in Samish Bay and Hood Canal despite substantial differences in the degree of potential connectivity with other Puget Sound sub-basins between the 2 locations. That we found better agreement between the estimated contributions to oyster diets and nearshore POM (POM-O<sub>ys</sub>) in Samish Bay than Hood Canal raises the possibility that oysters in Hood Canal may be feeding more selectively than those in Samish Bay, although other physiological processes such as tissue turnover rates could also play a role in driving this pattern (Lefebvre et al. 2009b). The degree of oyster feeding selectivity and filtration rates can be influenced by environmental variables such as temperature, salinity, and turbidity as well as by food availability (Barille et al. 1997, Ren et al. 2000, Ward & Shumway 2004, Guzman-Aguero et al. 2013), so site-specific differences in selectivity are theoretically possible. However, given the low frequency of sampling for POM-O<sub>ys</sub>, particularly in Hood Canal and the generally low chl *a* at all 3 sites, it is equally possible that oysters in general feed opportunistically at all 3 sites and that the Hood Canal POM-O<sub>ys</sub> samples may not have captured the full extent of the seston available to the oysters.

In both Samish Bay and Hood Canal, salt marsh vegetation and intertidal macrophytes were estimated to be more important components of oyster diets than phytoplankton-dominated POM (POM-phy), with salt marsh most important in Hood Canal and macrophytes most important in Samish Bay. This strong contribution of detritus to oyster diets seems reasonable given the variable yet low chl *a* concentrations observed in the oyster beds at all 3 sites and the high contribution of these organic matter sources to nearshore POM (POM-O<sub>ys</sub>). Previous work in both laboratory and field settings has found that macrophyte detritus (of both algal and vascular derivation) can contribute to bivalve growth, particularly in situations where phytoplankton is less abundant

(Williams 1981, Alber & Valiela 1995, Arambalza et al. 2010). Moreover, it has been demonstrated that salt marsh vegetation, eelgrass beds, and macroalgae are highly productive and thus may help sustain nearshore benthic food webs in this region and elsewhere (Simenstad & Wissmar 1985, Ruckelshaus et al. 1993, Duarte 2002, Riera 2007, Tallis 2009, Howe & Simenstad 2015). Taken together, these findings suggest that oyster productivity in Puget Sound is dependent upon adjacent habitats such as salt marshes and eelgrass beds. As such, maintaining connectivity among salt marsh vegetation, eelgrass beds, macroalgae, and oysters may be a key condition of sustainable nearshore shellfish production, particularly when phytoplankton availability is low.

Stable isotopes used in conjunction with mixing models are a powerful approach for understanding resource partitioning (Layman et al. 2012, but results should be interpreted with caution (Fry 2013, Phillips et al. 2014). Because we only used 2 isotopic tracers, we were unable to distinguish the relative importance of different intertidal macrophytes as carbon sources for oysters, although the use of fatty acids and  $\delta^{34}\text{S}$  may aid future studies in discerning the relative importance of eelgrass *Zostera marina* as well as different species of macroalgae (Galloway et al. 2013). Trophic fractionation also presents a large source of uncertainty in stable isotope mixing models. We partly addressed this problem by applying 2 published fractionation estimates for *Crassostrea gigas* and were reassured by the general agreement between them. Another potential source of error for mixing models is uncertainty in the fractionation of primary producers during decomposition (Sosik & Simenstad 2013). We assume here that the isotopic signatures of plant detritus are not appreciably different from living plants and thus that decomposition is primarily mechanical rather than microbial. This assumption seems reasonable given that the marine vegetation we collected was from intertidal habitats and thus potentially subject to more physical degradation through wave energy and abrasion (Kharlamenko et al. 2001); however, it is possible that some (unknown) degree of microbial decomposition was present (Crosby et al. 1990), thus making our estimates of stable isotope values of macro-vegetation end members less precise. Similarly, we assumed no fractionation between macro-vegetation end members and POM- $\text{Oys}$  samples, and therefore did not account for potential microbial decomposition between end members and the POM- $\text{Oys}$  mixture. Additionally, while we selected vegetation to include as end members in the mixing model based on our

visual assessment of its abundance and potential importance to nearshore POM pools (Marin Leal et al. 2008, Howe & Simenstad 2015), we recognize that having quantitative abundance or biomass data for all potential diet sources would strengthen this effort and possibly permit the use of informative priors. However, as we have shown, this presents a complex problem given the variable potential for transport of water within and among sub-basins of Puget Sound, thus justifying the more conservative approach of non-informative priors. Finally, others have emphasized the importance of incorporating both temporal and spatial variation in the isotopic baseline when making inferences about consumer diets (Vander Zanden & Rasmussen 2001, Post 2002, Solomon et al. 2011, Dethier et al. 2013). We used site-specific vegetation sources collected over multiple seasons to capture both the spatial and temporal variation in end members into our mixing models. Because we observed variation in both the consumers and the end members (Table 3), we believe this approach may have been particularly appropriate for this study.

## CONCLUSIONS

We found that Pacific oysters, which are important nearshore benthic consumers in Puget Sound, rely on autochthonously derived organic matter originating in estuarine habitats as well as salt marshes, with lower contributions from upland vegetation, phytoplankton, and benthic diatoms, providing evidence for strong physical, biological, and chemical linkages within and between these adjacent ecosystems. That oysters were highly dependent on energy from autochthonous production by macrophytes as well as salt marsh habitats suggests that changes (e.g. land conversion, shoreline modifications) in these source habitats may affect the secondary productivity of the estuarine benthic food web. This hypothesis is supported by multiple lines of evidence including the hydrologic (potential) connectivity of major organic matter sources to the shellfish beds we studied and the strong contribution of these sources to independently sampled POM above oyster beds. This evidence for local-scale connectivity between estuarine habitats (intertidal shellfish beds, eelgrass beds, and salt marshes) adds to the emerging picture of estuaries as a heterogeneous mosaic of interconnected habitats and emphasizes the importance of management across ecosystem boundaries to maintain ecological function and linkages.

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