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Trophic transfer of metals from seaweed to shellfish in an aquaculture system

Sophia Boyd
Western Washington University, sophialboyd@gmail.com

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By

Sophia L. Boyd

Accepted in Partial Completion
of the Requirements for the Degree
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ADVISORY COMMITTEE

Dr. Kathryn Van Alstyne, PI

Dr. Ruth Sofield, Co-PI

Dr. Brian Bingham

GRADUATE SCHOOL

David L. Patrick, Dean

Master's Thesis

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Sophia L. Boyd

August 4th, 2023

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

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Sophia L. Boyd
August 2023

Abstract

Seafood, including fish, shellfish, and seaweed, are an important source of nutrients that could meet some of the increasing demand for food globally. In addition to nutrients, chemical contaminants can also be acquired from the environment by primary producers. Seaweeds take up a variety of inorganic and organic contaminants, including metals, that may pose risks to human health. Through trophic transfer, organisms can accumulate elevated levels of contaminants from consuming lower trophic-level organisms. Since particulate organic matter, including seaweed detritus, is a food source for filter-feeding bivalves, contaminants present in seaweed could transfer to shellfish via ingestion. The purpose of my work was to investigate: 1) if seaweeds used in local co-culture operations are accumulating metals, 2) if there is evidence for the transfer of metals from seaweeds to shellfish, and 3) which of these metals are in high enough concentrations to pose human health risks. Given that seaweeds hyperaccumulate metals and bivalves consume particulate matter, we hypothesize that it is possible for the bivalves in co-culture systems with seaweed to have higher metal concentrations than would otherwise be expected and increase risk to human consumers. We conducted both laboratory-based bioaccumulation and feeding experiments, as well as field studies at two sites in the Puget Sound. Results of the bioaccumulation experiment showed that kelp can hyperaccumulate at least three metals (cadmium [Cd], chromium [Cr], and zinc [Zn]). Bioconcentration factors (BCF; L/kg) for Cd, Cr, and Zn were 4.97, 7.32, and 49.5, respectively, indicating that hyperaccumulation occurred (BCF>1). Results of the oyster feeding experiment did not demonstrate transfer of metals from kelp to shellfish, however, questions about ingestion of the kelp disallow us from concluding that the metals cannot be transferred from seaweed to oysters. Evidence that metals are being transferred to shellfish in the types of co-culture operations that currently exist in Washington State, at this point, is not well substantiated. Results of the field studies indicate elevated levels of Cd and copper (Cu) at the IMTA facility in oysters, but no human health-based exceedances in seaweeds or mussels at either site. These risks can be mitigated by reducing consumption rates. To be protective of human health, a new consumption rate was calculated to be 140 g wet weight per week based on the concentrations of Cd

exceeding the screening level. Further work needs to be done to provide a better understanding of the uptake of kelp particulates by oysters and whether this could result in a transfer of metals.

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List of Abbreviations and Acronyms

ARL: Acceptable Risk Level
BCF: Bioconcentration Factor
BW: Body Weight
CR: Consumption Rate
CRM: Certified Reference Material
DL: Detection Limit
DNR: Department of Natural Resources
DW: Dry Weight
EPA: Environmental Protection Agency
IAA: Interagency Agreement
ICP-MS: Inductively Coupled Plasma Mass Spectrometry
IMTA: Integrated Multi-Trophic Aquaculture
IRIS: Integrated Risk Information System
FAO: Food and Agricultural Administration
LOQ: Limit of Quantitation
NOAA: National Oceanic and Atmospheric Administration
POM: Particulate Organic Matter
RfD: Reference Dose
SAM: Stormwater Action Monitoring
SPMC: Shannon Point Marine Center
SL: Screening Level
TMF: Trace Metal Free
TMG: Trace Metal Grade
USDA: United States Department of Agriculture
USDHHS: United States Department of Health and Human Services
WDFW: Washington Department of Fish and Wildlife
WW: Wet Weight
WWU: Western Washington University

Introduction

Seafood Consumption

As the global human population continues to rise, there is increased demand for food around the world (Godfray et al., 2010; Guillen et al., 2019). Seafood, including fish, shellfish, and seaweed, are an important source of nutrients that could meet some of this demand. For example, to meet daily protein requirements, the United States Department of Agriculture (USDA) and United States Department of Health and Human Services (USDHHS) recommend 8-15 oz/week of seafood for the average adult consuming a 2000 calorie/day diet (USDA and USDHHS, 2020). Although fish are a well-known type of seafood, seaweed and shellfish are also major contributors to the global food market.

Seaweed can be high in vitamins, minerals, and protein (Wells et al., 2017). They are the only non-fish sources of natural omega-3 long-chain fatty acids and contain micronutrient minerals (e.g. iron, calcium, iodine, potassium, and selenium) as well as vitamins (especially A, C, and B-12; FAO, 2020). In addition, many produce bioactive natural products, such as alginates, fucoidans, and phlorotannins, that may have other health benefits (Cherry et al., 2019; Olasehinde et al., 2019).

Historically, seaweed has been used as a food source by many cultures. For example, Coast Salish and First Nation peoples traditionally consumed seaweed dried and toasted, cooked with clams and salmon eggs, or used as a high-value gift item (Turner et al., 2003, 2014; Gaydos et al., 2015). Additionally, seaweed plays an essential role in many Asian diets. For example, Japanese kelp (*Laminaria japonica*) is eaten dried or pickled as a healthy snack, while *nori* (*Pyropia* and *Porphyra* species) is used to wrap sushi (Marshall, 2020). More recently, seaweed has been sought after by chefs and high-end restaurants in pursuit of unique flavors (Allchin, 2017; Griffin and Warner, 2020). Harvested seaweeds are also used as a biofuel alternative to natural gas, bioplastic, fertilizer, fish feed, and in the pharmaceutical and cosmetics industries (Duarte et al., 2021; Marshall, 2020).

The value of seaweed in the US market has increased in recent years, reaching $13.2 billion in 2018, with some sectors continuing to grow and develop as new technologies and uses for seaweed emerge

(FAO, 2020). Seaweed consumption globally was estimated to have grown 8% from 2004 to 2014 (FAO, 2016). Although it has stabilized or decreased slightly since 2014, much of that reduction has been attributed to lower production of tropical species in Southeast Asia. In temperate climates, however, the production of cold-water seaweed species is still increasing (FAO, 2020).

Seaweed consumed by humans or used for commercial purposes can be both cultivated in aquaculture operations or harvested from the wild. However, cultured seaweeds account for more than 95% of the seaweed market (FAO, 2020). In addition to being a source of seaweed for human use, seaweed aquaculture also has environmental benefits, including acting as a localized carbon sink to reduce acidification in close proximity to seaweed farms (Peabody, 2016; Chopin and Tacon, 2021) and a means of removing excess nutrients from coastal waters (Xiao et al., 2017).

Like seaweeds, shellfish, including bivalves such as clams, oysters, mussels, and scallops, offer significant nutritional benefits by providing amino acids, and beneficial fatty acids, while being low in total and saturated fat (Wright et al., 2018). They are also a source of vitamins, such as B_{12}, sodium, potassium, and iodine (Venugopal and Gopakumar, 2017), and are an alternative to land-based animals as a source of protein. As a result, certain communities rely on shellfish as a major part of their diet. One such community is the African country of Senegal, a developing nation heavily dependent on its coastal resources, that relies significantly on marine fish, mollusks, and shellfish consumption to meet 75% of its population's protein requirements (FAO, 2006). As another example, large deposits of clam shells on the coast of Canada suggest the significance of shellfish as a staple food source for Northwest Coast First Nations people for at least 5000 years (Cannon et al., 2008). More locally, tribal communities in the Pacific Northwest have traditionally relied on shellfish harvest as an integral part of their diet, though it is difficult to get an accurate estimate as tribal communities have been under- and misrepresented in consumption surveys (Donatuto and Harper, 2008).

As with seaweed, bivalves also provide environmental benefits. They are an important extractor species because they filter out waste and detritus and use it as food while cycling nutrients, generally increasing water quality (Kreeger et al., 2018). They also offer habitat in the form of hard shells and

structures for other plants and animals. Other ecosystem services provided include protection of coastline from storm surges, nursery habitats, and reduction of shoreline erosion (WA Sea Grant, 2015).

Benefits and Problems with Aquaculture

Farming seaweeds and bivalves has several advantages over wild harvesting. Aquaculture provides more control over production processes and improved market access. As a result, aquaculture has increased the availability of seaweeds and bivalves to regions that would otherwise have little or no access to them, generally at lower prices, resulting in enhanced nutrition and food safety (FAO, 2020). As described above, bivalve and seaweed aquaculture improves water quality by filtering harmful chemicals (Barrington et al., 2010; Boening, 1997; Kreeger et al., 2018), removing excess nutrients such as phosphorus and nitrogen (Xiao et al., 2017), and sequestering carbon dioxide (Dumbauld et al., 2009).

However, seaweed and bivalve aquaculture can also have negative physical and biological impacts on the surrounding environment. For example, the presence of lines and cages change the physical structure of the environment by modifying water flow, building up sediment, altering sediment grain size, and increasing organic content, which indirectly alters oxygen content and nitrogen cycling. These changes are particularly impactful in areas of low flow that lack regular flushing (Dumbauld et al., 2009). Biological impacts include introductions of non-native species (the aquaculture species themselves or hitchhikers), parasites, and diseases (McKindsey et al., 2007; Tano et al., 2015). As the aquaculture industry has continued to grow, a concerted effort has been made to increase sustainability and mitigate aquaculture's negative impacts (Klinger and Naylor, 2012).

Integrated multitrophic aquaculture (IMTA) is one way to mitigate the harmful environmental effects of growing seaweeds and bivalves, while optimizing production. IMTA systems can be defined by organisms from the same food web but occupying different trophic levels, such as seaweeds and bivalves, being grown together (Rosa et al., 2020). The primary producers can be part of the consumers' diet and, in turn, take up nutrients from the consumers' wastes.

Several studies have shown that seaweeds take up nutrients from shellfish. For example, the mussel *Mytilus trossulus* in the Baltic Sea stimulates the growth of seaweed (Kotta et al., 2009). In another example, when the red seaweed *Gracilaria lameneiformis* was cultivated in different ratios with the scallop *Chlamys farreri,* it acted as a carbon sink for the CO_2 that was released by the scallop (Han et al., 2013).

There is also evidence to support that seaweeds provide detritus for filter-feeding bivalves (Bustamante and Branch, 1996). The contribution of detritus to the diet of bivalves can be highly variable, ranging from 12-95% in blue mussels (*Mylitus edulis*) and Pacific oysters (*Crassostrea gigas*) (Hawkins et al., 2013). Furthermore, there is a negative correlation between phytoplankton abundance and ditritus, indicating that when phytoplankton is less available, such as in the winter (Xu et al., 2016), detritus is consumed more (Hawkins et al., 2013). Single-cell detritus from the green seaweed *Ulva* sp. can substitute for up to 50% of the microalgae in cultured *C. gigas* diets while maintaining their productivity (Omont et al., 2021), suggesting that seaweed detritus can provide a substantial amount of nutrients for filter-feeders. Similarly, the kelp *Saccharina japonica* provided much of the dietary intake of the scallop *Chlamys farreri* in an IMTA system in China (Xu et al., 2016).

Chemical Contaminants in Seafood

In addition to nutrients, chemical contaminants can also be acquired from the environment by primary producers. Seaweeds take up a variety of inorganic and organic contaminants that may pose risks to human health (Banach et al., 2020; Wells et al., 2017; Hahn et al., 2022). In the Salish Sea, these can include pesticides (e.g. DDTs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and polybrominated diphenyl ethers (PBDEs), and metals such as As, Cd, Hg, and Pb (Contreras-Portia et al., 2017; Hahn et al., 2022). For the purposes of this report, we will focus on metals, which are continually discharged into marine environments, are persistent, and could have health impacts for consumers (Balali-Mood et al., 2021; Stewart et al., 2021).

In the oceans, metals are normally found in low concentrations (Ash and Stone, 2003), but coastal systems tend to have higher concentrations due to natural and anthropogenic sources (Morillo et al., 2004),

including industrial effluents, automobile exhaust, wastes from incineration, and vehicle tire breakdown. Seaweeds hyperaccumulate both natural and anthropogenically sourced metals from their environment (Al-Shwafi and Rushdi, 2008; Sanchez-Quiles et al., 2017) and are commonly used as bioindicators of contaminated sites in coastal systems (Burger et al., 2007; Vasquez and Guerra, 1996).

Like seaweeds, bivalves can also contain contaminants. Bivalves are selective feeders that filter small particulate matter (Purroy et al., 2018), which can include seaweed, from the surrounding water. This particulate matter can be a source of both nutrition and contamination. Metal concentrations in bivalves tend to vary by location and are higher at sites with more people and more industry (Fowler and Oregioni, 1976; Wang et al., 2005). Concentrations also vary seasonally and with the animals' size and/or developmental stage; smaller shellfish generally have higher concentrations of metals because of the metal dilution effect, which means that as shellfish grow, the metals get less concentrated as long as they are not being exposed to higher levels (Bates et al., 2021). Metals can affect the shellfish shell structure and strength, growth, and reproductive success as well as cause an increase in immune responses (Ivanina et al., 2016; Chan and Wang, 2018; Wang et al., 2018; Stewart et al., 2021).

In the U.S. Salish Sea, bivalves have been routinely used to monitor contaminant concentrations as part of the National Oceanic and Atmospheric Administration (NOAA) Mussel Watch program and the Washington State Department of Fish and Wildlife's (WDFW) Mussel Monitoring program (Langness and West, 2022; Apeti et al., 2018). Under these programs, the metals As, Cd, Cu, Pb, Hg, and Zn are monitored biennially in the native mussel *Mytilus* sp. (Langness and West, 2022).

Seaweed/Bivalve IMTA and Metal Contaminants

Trophic transfer, or trophodynamics, can be defined as the way chemicals flow through trophic levels (Luomo and Rainbow, 2008). Through this process, organisms can accumulate elevated levels of contaminants from consuming lower trophic-level organisms. Since particulate organic matter, including seaweed detritus, is a food source for filter-feeding bivalves, contaminants present in seaweed could transfer to shellfish via ingestion.

The potential for changes in metals concentrations because of co-culturing bivalves with seaweed may increase health risks to the people that consume the bivalves. All organisms need a variety of trace metals to stabilize protein structures, facilitate electron transfer, and catalyze enzyme reactions. However, some metals in environmentally realistic concentrations can be harmful (Sarmiento et al., 2011). For example, Pb, Hg, and Cd can displace or substitute necessary trace metals, causing enzymes to malfunction (Ash and Stone, 2003). Examples of other effects of metals include disrupting liver function, carcinogenicity (Lasfer et al., 2007), increased autoimmunity, and neurological and psychological effects (Ali and Khan, 2019).

Given that seaweeds hyperaccumulate metals and bivalves consume particulate matter, we hypothesize that it is possible for the bivalves co-cultured in IMTA systems to have higher metal concentrations than would otherwise be expected and increase risk to human consumers. Here, we address the following questions:

1) Are seaweeds used in local co-culture operations accumulating these metals?
2) Is there evidence for the transfer of these metals from seaweeds to shellfish, particularly in IMTA systems?
3) Which metals in seaweed and shellfish are high enough to pose human health risks?

Methods

Field Measurements

To measure concentrations of metals in co-cultured seaweed and bivalves, we collected farm-grown oysters (*Crassostrea gigas,* n = 10 per collection), kelp (*Saccharina latissima,* n = 10 per collection), and wild mussels (*Mytilus* sp.*,* n = 10-20 per collection) four times from February to April 2022 (Table A1) at the IMTA system in Hood Canal. Farmed kelp were obtained during the first three collections, which occurred before the kelp were harvested. Wild kelp near the IMTA system were collected during the 4th sampling event. In 2023, we collected biweekly samples of kelp (n = 5 per collection) and oysters (n = 10

per collection), at the IMTA system from January through May 2023. During the four collections at the IMTA system in 2022, we obtained filtered (45 μm filter) and unfiltered water samples from approximately five cm below the waters surface.

In October of 2022, we established a second site at Penn Cove in the northwest corner of the embayment near Penn Cove Shellfish Farms on Whidbey Island, Washington. At this site, we collected *Ulva fenestrata* (n = 5) and 5-7 cm long mussels (*Mytilus* sp.; n = 8-10) monthly from the mid intertidal zone. All samples were taken to the Shannon Point Marine Center (SPMC), where they were stored at -80ºC for later processing. As of the writing of this thesis, these monthly collections are ongoing.

Mesocosm Experiments

Bioaccumulation of Metals by Kelp

To assess the bioaccumulation of metals by kelp and obtain information about metals concentrations for the subsequent trophic transfer experiment, a mesocosm experiment was set up at SPMC. Fifteen kelp (*Saccharina latissima*) were cut into roughly 10 x 10 cm pieces and divided equally among four buckets, each containing an aquarium bubbler and 12 L of f/2 medium (Guillard and Ryther, 1962). To acclimate the kelp, the buckets were placed into sea tables with seawater flowing around them from July 11th-July 18th, and the culture medium was changed every 3-4 days.

After the acclimation period, individual pieces of kelp were placed in 1 L plastic deli containers containing 800 mL of media designated as the control or metal treatments. The control medium was modified f/4 (Guillard and Ryther, 1962), made without the added trace metals. The four types of treatment media were made by adding zinc chloride, lead (II) perchlorate trihydrate, cadmium chloride, and potassium chromate to the modified f/4 (n = 3 per treatment; Table A2). After the kelp were added, the deli containers were covered with 50% shade cloth, and haphazardly distributed in three outdoor sea tables with ambient seawater (temperature =10.8ºC on July 18) circulating around the outside of the containers. The kelp were kept in the control or treatment media for 48 hours from July 18th-20th. At the end of the exposure, the kelp pieces were rinsed in filtered autoclaved seawater, weighed, and stored in pre-labeled trace-metal free

(TMF) tubes in a -80°C freezer overnight. The kelp pieces were then lyophilized and ground with a mixer/mill for 5 min in methacrylate vials, then kept in a –20ºC freezer until they were processed and analyzed for metals. Filtered (45 μm) and unfiltered water samples were collected at the end of the exposure from each container. To simultaneously measure autogenic changes in metal concentrations (e.g., caused by metals adsorbing to the plastic containers), an autogenic control was set up (n = 3 per treatment level) as above except without kelp; filtered (45 μm) and unfiltered water samples were collected from each control container prior to and after the experiment. The water samples were acidified to 0.15% with trace metal grade (TMG) nitric acid (HNO_3) and stored at room temperature until analysis. Changes in metals from the beginning to the end of the exposure experiment in the autogenic controls were negligible (Table A3) and therefore autogenic metals loss was not considered further.

Trophic Transfer Experiments

To examine the potential for metals to be transferred from kelp to shellfish when POM generated by kelp is fed upon by shellfish, a second exposure to elevated seawater metals was conducted to generate metals-contaminated kelp using *S. latissima* collected off the Shannon Point Marine Center beach. Kelp were acclimated for three days in modified f/4 as described above. Post acclimation, 90-100 g of kelp were haphazardly placed into one of four buckets containing modified f/4 (ambient kelp) or f/4 with added metals described above (treatments). After 48 hours, kelp were collected and stored in TMF tubes in a -80° C freezer overnight before being lyophilized, ground, sieved to <150 μm, and refrozen at -20ºC. We calculated nominal water concentrations of the four metals in mg/L based on the target measurements in algae. Oysters (n = 6 per mesocosm) obtained from the IMTA system on Aug 25, 2022, were put into 15 mesocosms (20 L plastic buckets) containing 1 μm filtered seawater (Figure 1). The oysters were elevated on plastic mesh platforms 2-3 cm below the water surface. Ambient seawater flowed around the buckets (seawater temperatures: 8/29: 12.4 °C, 9/6: 12.2 °C, 9/13: 11.6 °C). Upon arrival at the lab, an additional ten oysters were shucked and stored at -80 ºC for metals analysis and considered for baseline metal levels. The oysters

in the mesocosms were left to acclimate for 5 days and were fed daily. The food consisted of a mixture of kelp (0.06 g) and *Isochrysis* (0.18 g) soaked in 1 µm filtered seawater immediately prior to feeding. The ratio of microalgae to macroalgae was based on protocols from Rato et al. (2018), and the mass of added feed was approximately 2-4% of the dry weight of an adult oyster as described in Helm et al. (2004).

After the acclimation period, mesocosms without aeration were haphazardly assigned to one of five daily feeding treatments (n = 3 per treatment): *Isochrysis Only* Control (0.24 g *Isochrysis*); *Ambient Kelp* Control (0.24 g *Isochrysis* and 0.06 g ambient kelp); and low, mid, or high *Metals Treatments* (0.24 g *Isochrysis* and 0.06 g metal-treated kelp). During this experiment, oysters were fed their respective diets for 14 days using a 60-mL Luer-lock syringe. Full water changes were done every 3-4 days and unfiltered water samples for metals analysis were collected before and after each water change. After the 14-day exposure, oysters were shucked into 50 mL TMF polypropylene centrifuge tubes (Table A4). They were frozen overnight at -80 °C and lyophilized the next day.

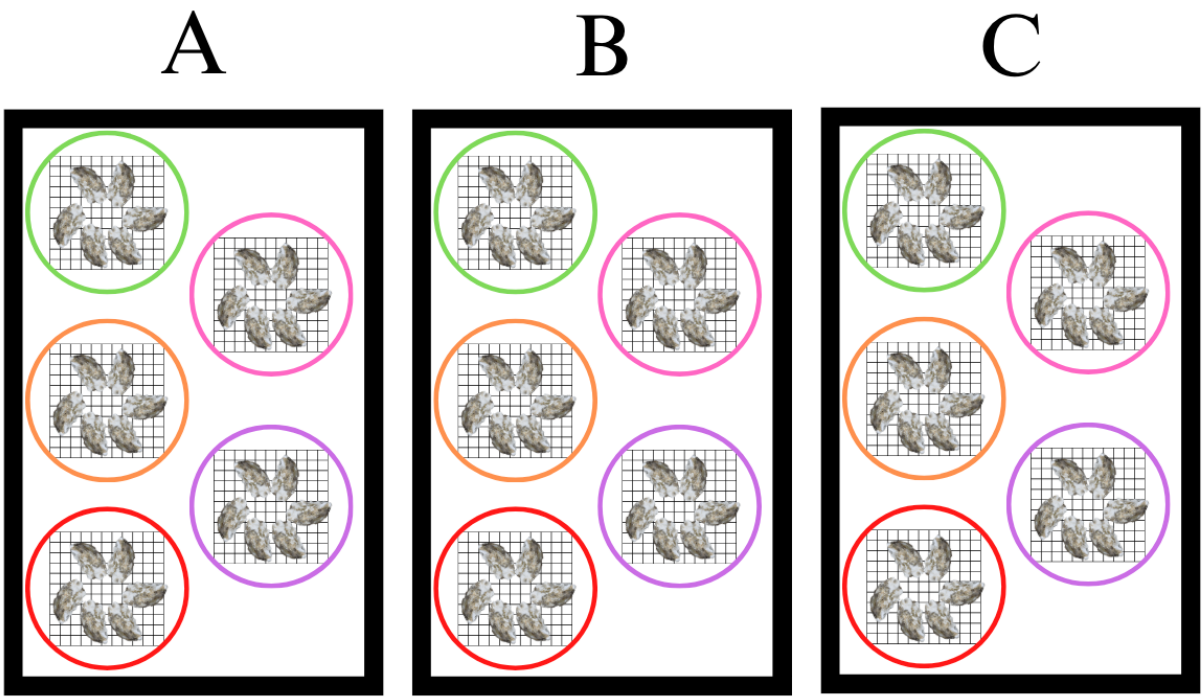

Figure 1. Experimental design of oyster mesocosm experiment. Three sea tables labeled A, B, and C were set up in the above configuration with 6 oysters per mesocosm. Mesocosms consisted of an *Isochrysis* Only control and an Ambient Kelp control, and Metals Treatments groups included kelp with low, medium, and high concentrations of metals.

Sample Processing and Analysis

Chemical Analyses

To prepare kelp and shellfish samples for metals analysis, with the exception of the first round of IMTA system data which were combined into one sample per collection date, samples were processed individually unless their dry weight mass was less than 0.25 grams, in which case two or three individuals were combined to reach the target mass of 0.2 to 0.25 g for digestion. Each dried sample (0.2-0.25 g) was combined with TMG nitric acid and H_2O_2 (9:1 for kelp and 8:2 for shellfish, for 10 mL total liquid volume) in Teflon™ vessels. Samples were digested in a closed-vessel microwave digestion system (Milestone ETHOS 1 Series) using the "Beech Leaves" protocol for seaweed and the "Fish" protocol for shellfish, respectively (Ashoka et al., 2009; EPA, 1996; Table A5). Samples were diluted to 50 mL with UltraPure Type (I) water (Millipore Sigma, Darmstadt, Germany). For metals analysis, they were further diluted to 5% TMG nitric acid with UltraPure water. Digestion blanks with UltraPure water acidified to 5% with TMG nitric acid were included with each digestion run. Certified reference materials were also included in 12 out of 16 of the digestion runs. Bladderwrack (*Fucus vesiculosus;* European Reference Materials-CD200) was prepared and digested following the seaweed procedure and dogfish liver (*Squalus acanthias*; DOLT-3, National Research Council Canada, 2003) was prepared and digested following the shellfish procedure (Table A6).

Metals concentrations were determined with ICP-MS (Agilent 7900), using an inline internal standard, and following Environmental Protection Agency (EPA) Method 200.8, in WWU's Advanced Materials Science & Engineering Center. Calibration standards were made with IV Stock 50 (Inorganic Ventures™, Christiansburg, VA). Instrument blanks (UltraPure at 5% with TMG nitric acid) and blanks spiked with EPA IV Stock 50 were included for every 7-10% of samples. To prepare water samples for ICP-MS, they were acidified to 5% with TMG HNO_3. Samples were analyzed using High Matrix Introduction with a 100x dilution of argon gas on the ICP-MS. Metal concentrations were expressed in mg element/kg tissue dry weight. Any values below the limit of quantitation (LOQ) were replaced with half of the LOQ for statistical analysis.

To help determine whether the oysters consumed the kelp during the feeding experiment, 0.34-0.51 mg of freeze-dried *Isochrysis*, 0.68-1.02 mg of freeze-dried, ground kelp feed, and 0.21-0.32 mg of freeze-dried ground pre- and post-exposures oysters were packaged in 5 x 9 mm tin capsules for ^{12}C:^{13}C and ^{14}N:^{15}N stable isotope analysis at the Washington State University Stable Isotope Core Laboratory (Pullman, WA).

Bioconcentration Factor (BCF) Calculations

To determine whether kelp hyperaccumulate metals, BCFs were calculated for kelp from the bioaccumulation experiments as:

$$BCF = \frac{C}{C_W}$$

where BCF is the bioconcentration factor in L/kg tissue, C is the metal concentration in the kelp (mg chemical/kg tissue dry weight (DW), and C_W is the metal concentration in the water (mg/L). BCFs greater than one are indicative of hyperaccumulation (Usman et al., 2019). Metals concentrations in the kelp were assumed to have reached equilibrium with the surrounding seawater.

Screening Level Calculations

The EPA method for fish consumption advisories is a risk-based approach for determining whether concentrations of metals in fish pose health risks to humans consuming it (EPA, 2000); these approaches can be used for other foods. To conduct similar assessments with our data, measured levels of contaminants in seaweed and shellfish were compared to risk-based screening levels (SLs); these were based on reference doses (RfDs) indicating the daily quantity of a contaminant that an individual can be exposed to without significant risks of non-carcinogenic effects (EPA, 2000). To evaluate the possible health hazards to consumers of seaweed and shellfish, health-based SLs were calculated and compared to concentrations measured from field-collected seaweed and shellfish.

The screening level was based on reference doses (RfD [mg/kg/day]) for adults and is used to assess non-carcinogenic effects. Reference doses were obtained from the Integrated Risk Information System (IRIS) database provided by EPA and was calculated as:

$$SL_{RfD} = \left(\frac{(RfD \times BW)}{CR}\right) \cdot 1000$$

where SL_{RfD} (in mg chemical/kg DW of seafood) is the reference dose-based screening level, BW is body weight (assumed to be 80 kg), CR is the consumption rate (in g DW seafood/day), and 1000 is a unit correction. CRs used to calculate SLs were 5 g dry weight of seaweed per day, 27.5 g dry weight of oysters, and 9.9 g dry weight mussels per day, which is the amount customarily consumed in a meal (USFDA, 2023). These consumption rates for shellfish were originally provided on a wet weight (WW) basis, so to convert to dry weight, the percent moisture (mussel = 91%, n = 30; oyster = 75%, n = 101 [1- ($WW - DW)/WW * 100$]) was calculated for each and the new dry weight-based consumption rate was calculated.

In cases where the metals concentrations exceeded the SL, a new consumption rate was calculated to be protective of human health:

$$CR_{New} = \frac{RfD \times BW}{(SL_{RfD}/1000)}$$

Statistical Analysis

Field Data

Median values from each collection date were calculated, and summary statistics were done based on those median values; therefore, the replicates are the collection date.

Bioaccumulation Experiment

To quantify how kelp accumulate metals, a linear regression was run with the metal concentration in the kelp (mg/kg WW) as a function of the metal concentration in the water (mg/L; Figure 2). To get wet weight of the kelp, the dry weight was divided by 0.102, as the average water content in local kelp has been measured at 89.8% (K. Van Alstyne, unpublished data).

Trophic Transfer Experiments

A linear regression of metal concentration in oysters (median of the n = 6 per mesocosm) as a function of metal concentration in the *Metals Treatments* was performed, as well as a one-way analysis of variance (ANOVA) between the same variables. A Levene's test was done to ensure the equal variance had been met and a Shapiro-Wilk test was done to verify normality of the variables prior to the analyses. In cases where normality (Shapiro-Wilk) test failed, ($p < 0.05$), log transformation (natural log) of the non-normal metal data was performed, and an ANOVA was run using the log-transformed data. In cases where normality failed after log transformation, a Kruskal-Wallis One Way ANOVA on Ranks was performed. Post-hoc comparisons were made using Tukey's HSD test.

The alpha value was preemptively set at 0.05, and all data was processed in R version 4.2.1 (R Core Team, 2022).

Results and Discussion

Are seaweeds used in local co-culture operations accumulating metals?

In our initial laboratory mesocosm experiment in which pieces of kelp were exposed to a range of metals concentrations, we found evidence of kelp hyperaccumulating metals. Metals concentrations in kelp increased linearly ($P \leq 0.01$; Table 1) with increasing metals concentrations in the media for Cd, Cr, and Zn, but not for Pb (Figure 2). Because there was no relationship between Pb concentrations in seawater and the kelp, BCFs were not calculated for Pb. Average BCFs (L/kg) for Cd, Cr, and Zn were 4.97, 7.32, and 49.5, respectively (Table 2). It is important to note that since we did not take samples over time, it is possible

that the experiments presented here did not reach equilibrium which could affect the BCFs. If the metals in the kelp did not reach equilibrium with the seawater, the concentrations in kelp tissue would be underestimates and therefore these BCFs would also be underestimates.

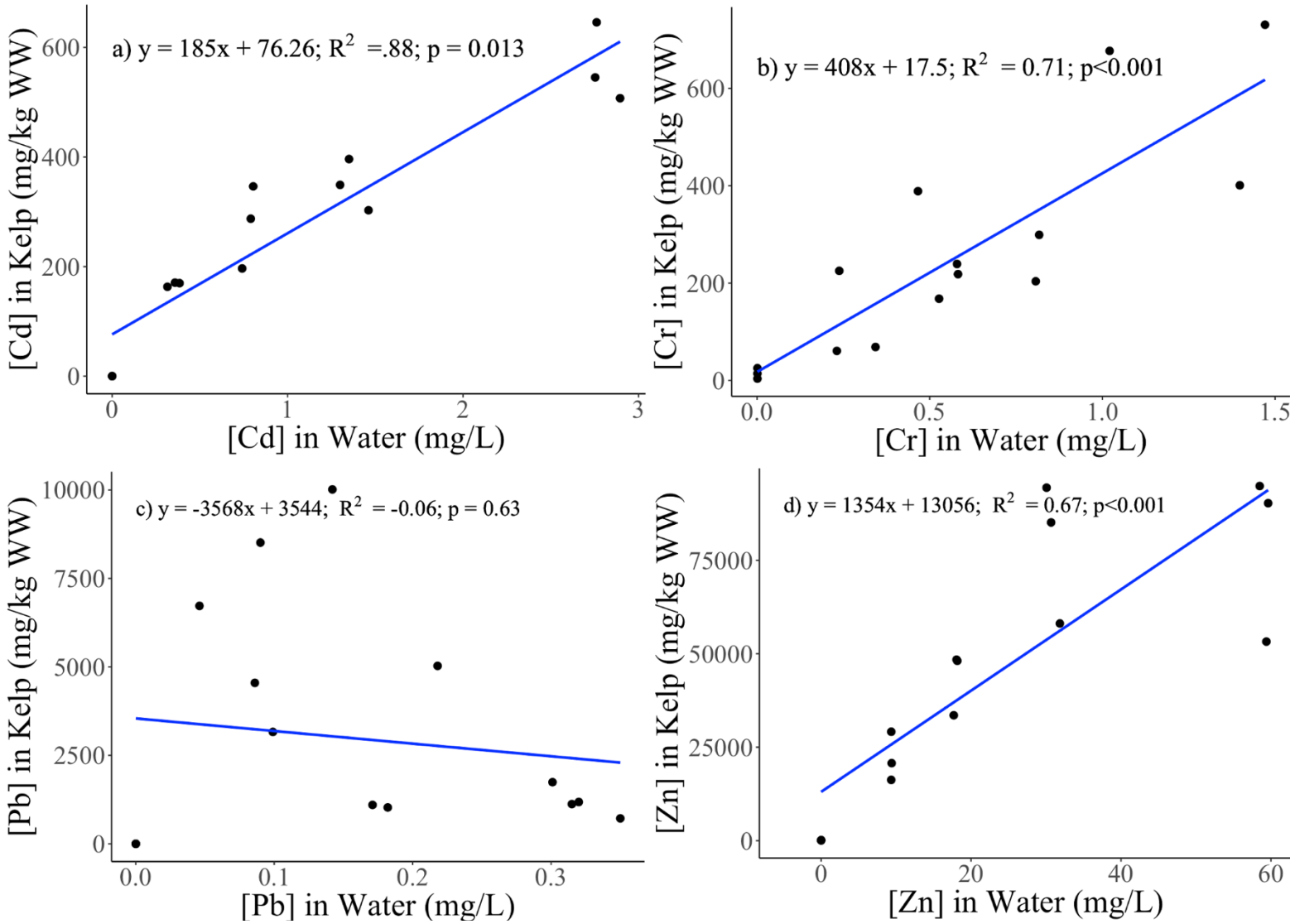

Figure 2 (a-d). Measured a) Cd, b) Cr, c) Pb, and d) Zn concentrations in kelp (*S. latissima*; (mg/kg wet weight of kelp) after 48 hours of exposure as a function of measured metals concentrations in the media (mg/L) in laboratory mesocosm experiments. Blue lines are linear regressions.

Algae utilize extracellular and intracellular metal binding methods, including physical adsorption, ion exchanges, and chelation, to mitigate the toxicity induced by metal ions but this also results in elevated levels of metals in the tissue. Through passive and active transport systems, these ions accumulate within cells after traversing cell membranes (Ankit et al., 2022). The cell wall constituents in brown algae, alginate and fucoidan, are responsible for binding metals (Davis et al., 2003). Higher BCFs for Zn could be attributed to its use as an essential element in algae. BCFs of *Ulva fenestrata* from a field study in China for Cd were comparable (5.87), but up to 37x lower for Zn (Qiu, 2015), and another field study from Egypt were again comparable to Cd (4.53) but up to 18x lower for Zn (Shams El-Din et al., 2014). In a more heavily polluted range of sites in another field study, the BCF for Cd was up to 3.5x higher and comparable for Zn (El-Mahrouk et al., 2023; Table 2). In a study using different concentrations of metals to measure accumulation in the green algae *Ulva lactuca*, rapid adsorption of higher-concentration Cd and Zn occurred within the first two hours followed by a linear increase, however, equilibrium was not reached in the 47-hour exposure time (Wang and Dei, 1999). This is an example of BCFs having high variability, even when limited to a small range of metal concentrations (McGreer et al., 2003). As there is no available literature to compare the bioconcentration of metals in *S. latissima* specifically, it is likely that variability in comparisons could be due to a difference in species. As green and red algae cell wall matrices are characterized by different polysaccharides (ulvans, xylans and galactans for green and carrageenans and agars for red; Hentati et al., 2020), their metal uptake mechanisms differ, and so would likely result in different bioaccumulation patterns. Another reason for this difference may be due to a difference between measurements of field-collected organisms as opposed to those under laboratory conditions (McGreer et al., 2003), since organisms in the field tend to have generally lower concentrations than intentionally-dosed laboratory organisms.

Table 1. Metals concentrations (mean ± 1SD; n=3) in kelp (*S. latissima*) in the bioaccumulation experiment. ND: below detection. Amounts of metals added to each treatment are given in Table A7.

Treatment Code	[Cr] (mg/kg)	[Cd] (mg/kg)	[Zn] (mg/kg)
Control	ND	ND	ND
1	0.27 ± 0.06	0.35 ± 0.03	9.38 ± 0.03
2	0.56 ± 0.03	0.78 ± 0.03	18 ± 0.25
3	0.7 ± 0.2	1.37 ± 0.08	30.9 ± 0.9
4	1.3 ± 0.24	2.8 ± 0.08	59.2 ± 0.6

Table 2. BCF (L/kg) from seaweeds in this study and the literature. Metals concentrations in kelp (*S. latissima* corresponding to Treatment Codes for this study are given in Table 1.

Species	**Location**	**Cd**	**Cr**	**Zn**	**Reference**
Saccharina latissima	Anacortes, WA, USA (Treatmt 1)	2.92	6.07	20.5	This study
	Anacortes, WA, USA (Treatmt 2)	3.65	5.04	43.0	
	Anacortes, WA, USA (Treatmt 3)	5.89	8.72	63.1	
	Anacortes, WA, USA (Treatmt 4)	7.44	9.43	71.4	
Ulva fasciata	Daya Bay, South China	5.87	---	1.91	Qiu, 2015
Ulva fasciata	Egyptian Mediterranean Coast, 2010	4.53	---	4.07	Shams El-Din et al., 2014
Ulva spp.	Alexandria, Egypt (Range across sites)	10-26.1	---	66.1-133	El-Mahrouk et al., 2023

Is there evidence for the transfer of these metals from seaweeds to shellfish, particularly in IMTA systems?

There was limited support for metals being transferred from kelp to shellfish in the IMTA system. Except for the first collection on 2/8/23, concentrations of metals in kelp were generally consistent (Figure 3). We hypothesize that if metals are being transferred from kelp to oysters, concentrations in oysters should become elevated when kelp are present and available as food (Figure 4). They should then decrease in the oysters after kelp are harvested. To evaluate this, samples were categorized into three time periods: "before kelp were present" (not growing or too small to be a reasonable contributor to diet; 1/20/23-2/8/23), "while kelp were present" (2/16/23-3/24/23), and "after kelp were present" (post- kelp harvest; 4/6/23-5/2/23). For Cu, Zn, and Cr, there was no significant difference in the "before" and "during" samples. There was a

significant decrease after the kelp were harvested compared to “during” and “before” for Cu and Cr and compared to “during” for Zn; the Zn “before” was not significantly different than “after” (Table A8).

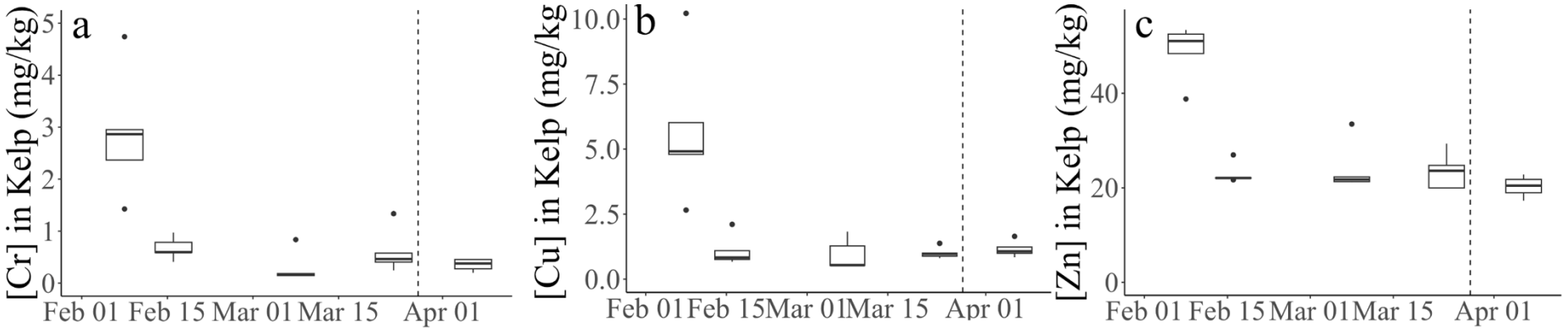

Figure 3(a-c). Metal concentrations (mg/kg) of a) Cr, b) Cu, and c) Zn in kelp (*S. latissima*) from the IMTA system obtained in 2023. Black dashed lines indicate when kelp was harvested. Black dots indicate outliers (points beyond 1.5x the inter-quartile range). Residual kelp remained for one sampling period after harvesting.

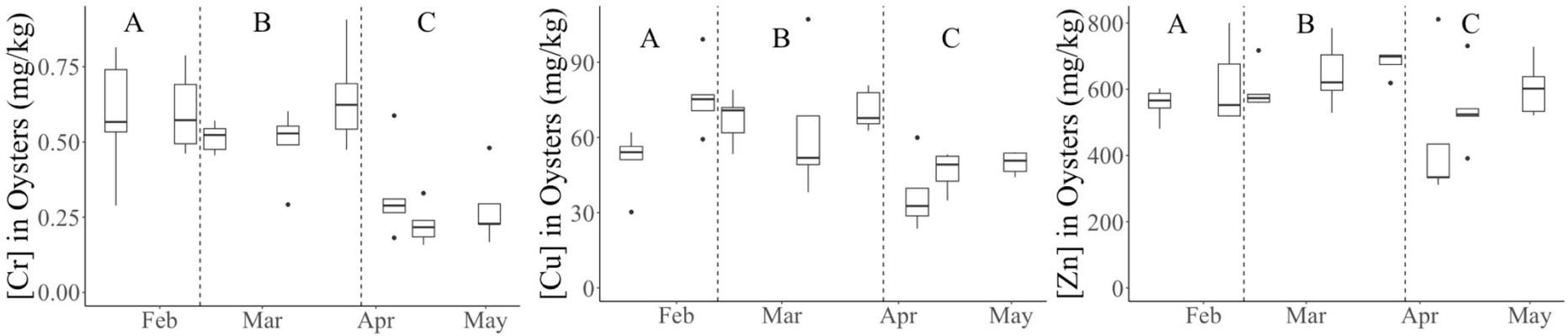

Figure 4. Metal concentrations (mg/kg) of Cd (left), Cu (center), and Zn (right) in oysters (*C. gigas*) from the IMTA system obtained in 2023. Kelp (*S. latissima*) was present within the black dashed lines. A is “before kelp were present”; B is “while kelp were present”; and C is “after kelp were present”.

Thus, the question of whether seaweed transfers metals to oysters cannot be conclusively resolved with the 2023 IMTA system samples alone. Several assumptions had to be made, including that kelp were sloughing during the growing period and consumed by the oysters. We did not collect data to evaluate whether this is occurring, but the IMTA workers harvest kelp before they start degrading to have kelp that is marketable for human consumption; the kelp, therefore, are not likely producing a lot of particulate matter.

The elevated metals in oysters “while kelp were present” compared to "after kelp were present” support that kelp was a source of metals to oysters, but the "before kelp were present” oysters indicate that another factor may better explain the results. For example, the tides in the Hood Canal gradually changed from daytime low tides around the third week of March, which was in between the “while kelp were present” and “after kelp were present” sample dates. The change in tides could cause a change in seawater chemistry and phytoplankton abundance, resulting in a change in available diet; both of these may explain the different oyster metal concentrations before and after kelp harvest. The ongoing work at Penn Cove will provide more evidence to evaluate the question of metal transfer from seaweed to shellfish. To date, samples have not been collected during the active growing season for sea lettuce. The summer 2023 collection dates are expected to provide important information to address this question.

Support for trophic transfer of metals from kelp to oysters in the laboratory experiment was also equivocal. Although we were able to produce kelp feed with a range of Cd, Cr, Pb, and Zn concentrations (Table A7), concentrations of Cd, Cr, and Zn in oysters did not differ significantly (Cd: ANOVA, $p = 0.14$, $F_{3,68} = 1.9$; Cr: Kruskal-Wallis, $p = 0.09$, $df = 3$; Zn: ANOVA, $p = 0.59$, $F_{3,68} = 0.64$) when these kelp were incorporated into the oysters’ feed. Pb concentrations in oysters were below detection for 97% of samples; therefore, we did not include these results in our analyses. Cd, Cr, and Zn concentrations in oysters did not differ when they were fed ambient kelp versus treated kelp, nor did they differ among oysters fed kelp treated with different concentrations of these metals (Figure 5). However, concentrations of Cd and Zn in oysters fed metals-treated kelp were significantly different from the baseline oysters (Cd: $F_{3,60} = 7.3$, $p = <0.001$, Zn: $F_{3,60} = 6$, $p = 0.001$).

The significant increase in Cd and decrease in Zn in baseline oysters compared to oysters fed metals-treated kelp is not easily explained. To some extent, shellfish can moderate metal concentrations by producing special proteins (metallothionein), that bind metals such as copper, zinc, and cadmium or can produce metal-rich granules that sequester the metals inside the shellfish. These natural processes can stop the metal from exerting toxicity on the organism even though the metal is retained in the body of the shellfish; with the metals retained, the expectation is that exposure to the Cd and Zn would cause both metals to increase in the body of the oysters (Liu and Wang, 2013).

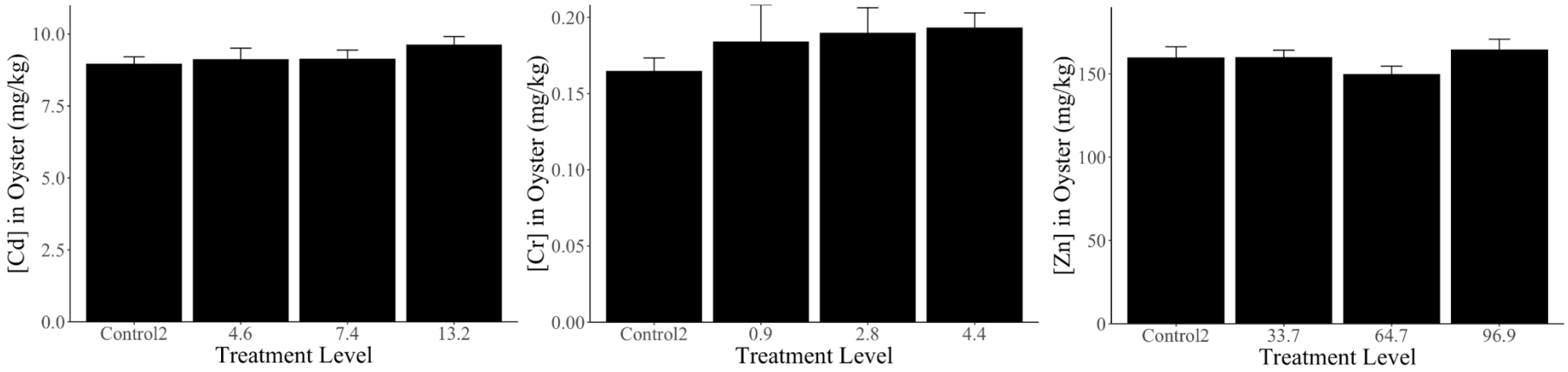

Figure 5. Concentration of Cd (left), Cr (center), and Zn (right) in oyster (*C. gigas*) tissue (mg/kg). Control2 = *Isochrysis* and ambient kelp.

The lack of differences in metals concentrations in oysters fed treated kelp could be explained by oysters not having access to the kelp because it may have settled in the containers during the experiment or because the oysters selectively avoided the kelp and fed preferentially on *Isochrysis.* Oysters can actively select particles based on size and food quality (Ward et al., 1998; Cognie et al., 2003). The feeding mechanism in oysters begins with cilia on the gills that generate a current to pull water and particles over the surface of the gills, which then becomes enshrouded in mucus. The cilia then route the mucus-covered particles either towards the mouth or a cavity inside the shell that houses the pseudofaeces. Further sorting can occur at the labial palps, based on size or nutritive value (Cognie et al., 2003).

Oysters not feeding on kelp in our mesocosm is supported by the analyses of stable carbon and nitrogen isotope ratios. This assumes, however, that the two-week experimental period was long enough to generate differences in stable isotope ratios, which it is possible that it was not. Plots of carbon versus nitrogen isotopes showed distinct *Isochrysis*, kelp, and oyster groupings (Figure 6). Carbon ratios, in particular, differed among food types. Carbon isotope ratios ($\delta^{13}C$) averaged –44.3 ‰, –14.5, and –19.2 ‰ for *Isochrysis,* kelp, and oysters, respectively. If oysters were consuming kelp, we would expect that stable isotope ratios in oysters fed a mix of *Isochrysis* and kelp would be more similar to the isotope ratios of kelp than oysters fed only *Isochrysis*. However, there was no significant difference in $\delta^{13}C$ between oysters that were fed just *Isochrysis* and oysters fed *Isochrysis/*kelp mixtures (ANOVA, $F_{2,42} = 0.3$, $p = 0.74$). There was a significant difference in $\delta^{13}C$ in oysters fed low versus medium metals-treated kelp (ANOVA, $p = 0.028$). However, despite this difference, we did not detect a consistent trend in $\delta^{13}C$ between oysters fed only *Isochrysis* versus oysters whose feed included kelp, and therefore, cannot conclude that oysters were ingesting the kelp.

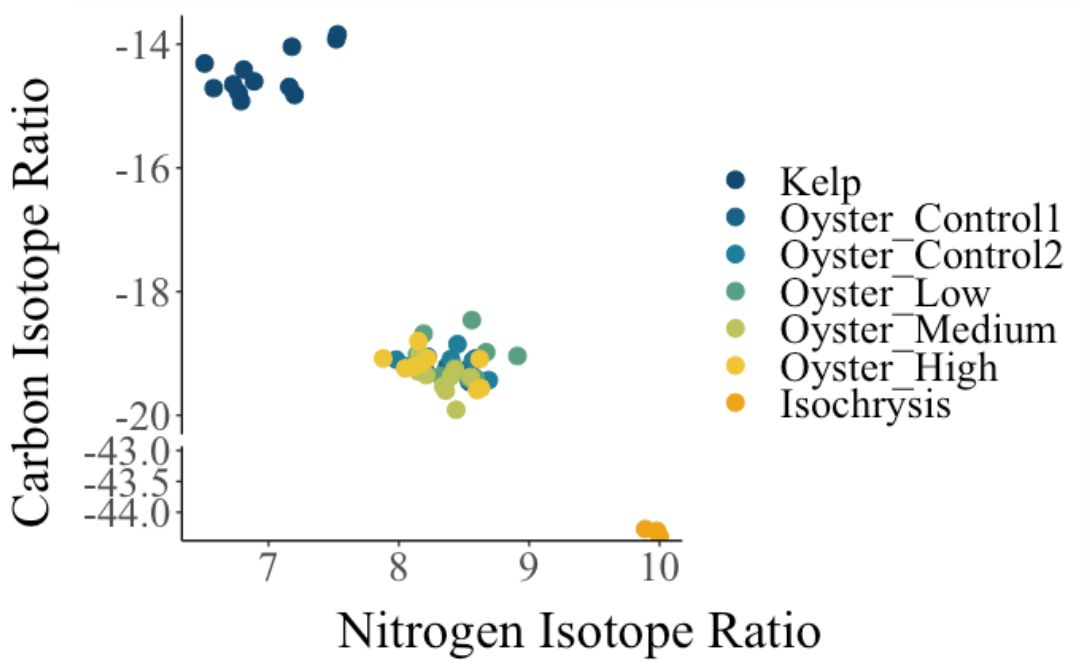

Figure 6. Carbon and nitrogen isotope ratios of *Isochrysis*, kelp (*S. latissima*), and oysters (*C. gigas*) fed Ambient kelp control and Isochrysis only controls and metals-treated kelp. Control1 = *Isochrysis* only, Control2 = Ambient Kelp Control. Kelp represents ambient, low, medium, and high metals treatments.

Because it is possible that size-based selective feeding was occurring, with the small (10 µm) *Isochrysis* fed upon by the oysters and the larger (150 µm) kelp particulates avoided or rejected in pseudofeces, summer 2023 experiments are designed to advance this work and eliminate size as a factor. Planned work includes experimentally determining the optimal size of the kelp particulates to use for trophic transfer experiments. This will potentially be followed by another trophic transfer experiment using the optimally size kelp particulates and informed by our work (e.g., the BCF to determine metals concentrations in water for target kelp tissue concentrations and data from the field experiments to refine which metals to use in the mesocosms).

Which metals in seaweed and shellfish are high enough to pose human health risks?

Metals Concentrations in Seaweed and Shellfish

Summaries of metals concentrations in kelp, oysters, and mussels collected at the IMTA system in 2022 and 2023 are given in Tables 3 and 4, respectively; metals concentrations in sea lettuce and mussels

from Penn Cove are in Table 5. Except for Be and Se, which were below LOQs, metals concentrations were generally above LOQs (Tables 3, 4, 5; see Table A9 for LOQs).

Metal concentrations of *S. latissima* from Scotland are comparable to those in this study for Cr, Mn, and Ni, while the highest values in our study for Ba, Cu, and Zn are about 2x higher (Schiener et al., 2015). Median metals concentrations for *S. latissima* were also compared to *N. luetkeana* in the Salish Sea (Hahn et al., 2022); some fell within the range of reported values (Ba, Be, Cr), while others were higher from our study (Cr, Cu, Mn, Ni, Se, and Zn), and still others were lower (Cd and V). Finally, *Ulva* spp. near Alexandria, Egypt, showed metals concentrations (mg/kg) in polluted waters of Cd (4.6-12.8), Cu (7.7-26.7), Ni (23.9-35.8), and Zn (14.2-19.5) over various sampling locations (El-Mahrouk et al., 2023), which are all higher than values reported in our work except for Zn, which ranges from 10.2-30.7 mg/kg. *Ulva fenestrata* metal concentrations were comparable to those in *Ulva* spp. in Ireland (Mn and Zn), while Ba was 2x as high in our study (Wan et al., 2017). In Spain, concentrations of Cu and Zn in *Ulva* spp. were comparable, while Mn and Ni were 3x higher in our study (Villares et al., 2001).

In general, concentrations of metals in shellfish measured in this study are comparable to concentrations measured elsewhere. The Stormwater Action Monitoring (SAM) program monitors mussels in the Puget Sound on a regular basis, and the most recent report (Langness et al., 2022) gives mean values of 2.45 mg/kg for Cd, 4.49 mg/kg for Cu, and 88.1 mg/kg for Zn in mussels collected at Penn Cove. Mean values from our study for the same metals were 3.27, 7.96, and 107 mg/kg, respectively. While slightly higher, concentrations from SAM fell within the range of concentrations found at Penn Cove in our study (except for Cu, for which the minimum value in our work is slightly higher than the mean from SAM).

Oysters sampled in China in the spring of 2017 contained higher levels of Cr (1.78 mg/kg) and lower levels of Cd (2.38 mg/kg), Cu (15.3 mg/kg), and Zn (68.2 mg/kg) than are reported in our study, with very little variation between the fall and the summer (Liu et al., 2021). Bendell, (2009) found that Cd levels in oysters in many retail outlets in British Columbia harvested from various locations within the Pacific Northwest exceeded safety guidelines. These values ranged from 1.5 to 3.56 mg/kg wet weight, so to compare to values in our study, they were converted to a dry weight basis using a predetermined moisture

content of 75%, which gives a new range of 6-14.2 mg/kg dry weight, only slightly lower than the mean value of 15.9 mg/kg of our study, and well within the range. Measurements reported for this study as well as Bendell, (2009) are higher than those reported around the world for Cd in *C. gigas*, including two locations in France that reported measurements 24-400x lower (Amiard et al., 2008). The high Cd concentrations may in part be due to the hydrology and geology of the region, as upwelling may bring Cd-rich water to the surface where it can then be taken up by the local biota (Apeti et al., 2009).

Human Health Implications

In kelp, sea lettuce, and mussels, concentrations never exceeded RfD-based SLs at either the IMTA system or Penn Cove. In oysters, concentrations only exceeded SLs for Cd and Cu at the IMTA system in 2022 and 2023 and did so for all samples.

Screening level exceedances in oyster samples suggest that consumption of these oysters at an average rate of 27.5g DW per day (110 g WW), the assumed portion size based on a body weight of 80 kg, could be associated with risks to human health. It should be noted, however, that these SLs are conservative and account for the uncertainty in extrapolating from animal toxicity tests to humans. Additionally, they only take into account the negative effects of the metals and do not balance these with the positive nutritional benefits of consuming shellfish. Furthermore, these risks can be mitigated by reducing consumption rates. To be protective of human health, a new consumption rate was calculated based on these exceedances using the methods of Hahn et al., (2022). Based on the average Cd concentrations in all oysters from the 2022 IMTA system samples, a recommended consumption rate would be 6.4 g DW, equivalent to 25.6 g WW per day (or 179 g WW per week). Based on the concentrations from the 2023 samples, new consumption rates would be 5 g DW (20 g WW) per day or 140 g WW per week based on mean Cd concentrations. Oysters in this study were roughly 10 g WW, so the average-sized person can still consume around 14 mid-sized oysters without worrying about deleterious health effects.

Table 3. Means, medians and ranges of metals concentrations from composited samples of kelp (*S. latissima*), oysters (*C. gigas*), and mussels (*Mytilus* sp.) collected at an IMTA farm. Data are summaries of four measurements, each collected at different dates from Feb to Apr 2022. Because these were composited samples, only a single replicate was obtained for each species on each date.

	Kelp				Oysters				Mussels			
	Mean (SD)	Median	Range	SL_{RFD}	Mean (SD)	Median	Range	SL_{RFD}	Mean (SD)	Median	Range	SL_{RFD}
Ba	17.1 (3.11)	16.8	14.0-20.8	3200	0.63 (0.37)	0.54	0.32-1.13	582	1.59 (1.47)	0.99	0.64-3.75	1616
Be	<LOQ[a]	-----	-----	32	<LOQ[a]	-----	-----	5.82	<LOQ[a]	-----	-----	16.2
Cd	1.52 (0.16)	1.54	1.31-1.68	16	**13.0** (2.8)	**12.80**	**10.1-16.2**	**2.91**	4.55 (1.73)	3.82	3.43-7.13	8.08
Cr	0.52 (0.26)	0.40	0.37-0.91	48	0.50 (0.13)	0.53	0.32-0.64	8.73	0.87 (0.21)	0.94	0.64-1.04	24.2
Cu	10.5 (17.7)	1.82	1.40-37.0	80	**76.8 (37.8)**	**60.05**	**54.1-133**	**14.5**	6.33 (0.37)	6.45	5.80-6.60	40.4
Ni	1.10 (0.52)	1.17	0.47-1.58	320	0.41 (0.10)	0.39	0.32-0.54	58.2	0.94 (0.55)	0.68	0.63-1.76	162
Mn	14.1 (5.6)	12.7	9.05-21.9	2240	12.32 (2.13)	12.35	9.68-14.9	407	14.5 (10.3)	9.66	8.62-29.9	1131
Se	2.68 (0.27)	2.57	2.50-3.08	80	3.92 (0.45)	3.80	3.53-4.56	14.5	5.94 (0.68)	5.64	5.54-6.95	40.4
V	1.48 (0.40)	1.60	0.93-1.81	144	0.45 (0.15)	0.40	0.32-0.67	26.2	0.90 (0.47)	0.68	0.63-1.60	72.7
Zn	29.9 (6.9)	30.6	20.9-37.4	4800	661 (132)	602	582-858	873	102 (22.6)	93.1	86.9-136	2424

[a] 100% of measurements were <LOQ

Table 4. Metal concentrations of kelp (*S. latissima*) and oysters (*C. gigas*; mg/kg DW) collected from the IMTA system from Jan to May 2023. Bold indicates concentrations above screening levels. Replicates are the median of the samples collected from each date.

	Kelp				Oysters			
Analyte	Mean (SD)	Median	Range	SL_{RfD}	Mean (SD)	Median	Range	SL_{RfD}
Ba	15.0 (2.78)	13.1	12.9-18.3	3200	1.79 (1.39)	1.29	1.08-5.20	582
Be	0.01 (0.004)[a]	0.01	0.01-0.02	32	0.01 (0.002)[b]	0.01	0.01	5.8
Cd	1.67 (0.34)	1.86	1.21-1.95	16	**15.8** (2.71)	**15.8**	**11.3-20.9**	2.9
Cr	0.89 (1.11)	0.46	0.17-2.87	48	0.44 (0.17)	0.53	0.22-0.62	8.7
Cu	1.66 (1.83)	0.97	0.54-4.91	80	**56.5** (14.0)	**53.0**	**32.7-75.2**	14.5
Mn	14.0 (8.03)	10.3	7.16-26.8	2240	14.6 (3.69)	16.3	8.28-18.2	58.2
Ni	0.97 (0.80)	0.71	0.21-2.28	320	0.46 (0.19)	0.46	0.24-0.75	407
Se	0.54 (0.23)[c]	0.46	0.30-0.78	80	3.33 (0.72)	3.30	2.62-4.70	14.5
V	1.01 (0.66)	0.97	0.24-2.05	144	0.67 (0.17)	0.75	0.38-0.87	26.2
Zn	27.8 (13.1)	22.1	20.5-51.1	4800	559 (105)	570	334-699	873

[a] 75% of measurements were <LOQ
[b] 90% of measurements were <LOQ
[c] 42% of measurements were <LOQ
*Measurements below LOQ were estimated as LOQ/2

Table 5. Metal concentrations of sea lettuce (*U. fenestrata*) and mussels (*Mytilus* sp.; mg/kg DW) collected from Penn Cove site from Oct 2022 to Apr 2023. Bold indicates concentrations above screening levels. Replicates are the median of the samples collected from each date.

	Sea lettuce				Mussels			
Analyte	Mean (SD)	Median	Range	SL_{RfD}	Mean (SD)	Median	Range	SL_{RfD}
Ba	12.1 (8.76)	10.5	3.03-25.1	3200	3.31 (0.78)	3.13	2.55-4.40	1616
Be	0.01 (0.02)[a]	0.004	0.004-0.05	32	0.01 (0.01)[b]	0.004	0.004-0.02	16.2
Cd	0.49 (0.17)	0.48	0.32-0.79	16	3.51 (1.13)	3.20	2.30-5.39	8.08
Cr	5.83 (4.24)	6.28	1.09-13.0	48	2.68 (1.88)	1.77	1.19-5.39	24.2
Cu	7.77 (3.14)	7.89	3.67-11.6	80	8.30 (1.88)	7.50	6.32-11.7	40.4
Mn	149 (141)	107	31.1-451	2240	14.2 (5.47)	14.7	7.32-22.0	1131
Ni	9.26 (3.96)	8.61	4.57-14.7	320	2.96 (0.93)	2.89	1.62-4.11	162
Se	0.95 (0.17)[c]	1.09	0.78-1.09	80	2.22 (1.48)[d]	1.09	1.09-4.42	40.4
V	1.91 (2.15)	1.03	0.45-6.53	144	1.37 (0.92)	1.50	0.38-2.33	72.7
Zn	18.7 (5.61)	18.9	13.2-27.9	4800	113 (19.1)	108	90.4-144	2424

[a] 69% of measurements were <LOQ
[b] 78% of measurements were <LOQ
[c] 100% of measurements were <LOQ
[d] 61% of measurements were <LOQ

Conclusions

This study has shown that several metals of concern to human health can be hyperaccumulated by Salish Sea *S. latissima*; however, evidence that these metals are being transferred to shellfish in the types of co-culture operations that currently exist in Washington State, at this point, is not well substantiated. While field measurements from the IMTA system in 2023 showed intriguing decreases in metals in oysters following kelp harvest, concentrations prior to harvest did not show patterns expected if the shellfish were taking up metals from kelp particulates. The expected patterns of accumulation include important assumptions for which data do not yet exist. Namely, we assume that oysters are consuming kelp particulates in proportion to the biomass of kelp available at the farm and that kelp are sloughing substantial amounts of particulate matter during their growing season. Our study did not include measurements of kelp size or abundance during the growing season. Therefore, we cannot estimate the biomass available over the growing season, although it is reasonable to expect that it increased from the January seeding until harvest in late March. The second assumption may be incorrect because kelp at the IMTA system are harvested before they become fouled and senescent. Consequently, they may not be producing large quantities of particulate matter prior to harvest. It is also not known whether shellfish are consuming kelp particulates. In our laboratory feeding experiment, metals concentrations in oysters fed contaminated kelp did not differ from those with ambient kelp, nor was there evidence of kelp ingestion from stable isotope measurements. It is possible that the small (approximately 10 μm diameter) *Isochrysis* were selectively fed upon by the oysters and the larger (150 μm) kelp particulates were avoided or rejected in pseudofeces. To investigate whether the large size of the kelp particulates prevented feeding on them, we plan to conduct a follow-up experiment this summer to determine the optimal size of the kelp particulates to use in these types of experiments. Then, if warranted, we will conduct another trophic transfer experiment using optimally sized kelp particulates. Altogether, these data should provide a better understanding of the uptake of kelp particulates by oysters and whether this could result in a transfer of metals.

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Appendix A

Table A1. Collection dates, locations, species, and number of species collected for field sampling at the IMTA system and Penn Cove.

Date	Location	Species (n)	Notes
2/15/2022	IMTA	*Crassostrea gigas* (10), *Mytilus sp.* (10-20), *Saccharina latissima* (10)	
3/11/2022	IMTA	*Crassostrea gigas* (10), *Mytilus sp.* (10-20), *Saccharina latissima* (10)	
3/22/2022	IMTA	*Crassostrea gigas* (10), *Mytilus sp.* (10-20), *Saccharina latissima* (10)	
4/14/2022	IMTA	*Crassostrea gigas* (10), *Mytilus sp.* (10-20), *Saccharina latissima* (10)	
10/07/2022	Penn Cove	*Ulva sp.* (5), *Mytilus sp.* (10-20)	
11/04/2022	Penn Cove	*Ulva sp.* (5), *Mytilus sp.* (10-20)	
12/05/2022	Penn Cove	*Ulva sp.* (5), *Mytilus sp.* (10-20)	
1/16/2023	Penn Cove	*Ulva sp.* (5), *Mytilus sp.* (10-20)	
1/20/2023	IMTA	*Crassostrea gigas* (10), *Mytilus sp.* (10-20)	
2/8/2023	IMTA	*Crassostrea gigas* (10), *Mytilus sp.* (10), *Saccharina latissima* (5)	
2/16/2023	IMTA	*Crassostrea gigas* (10), *Saccharina latissima* (5)	
2/17/2023	Penn Cove	*Ulva sp.* (5), *Mytilus sp.* (10-20)	
3/8/2023	IMTA	*Crassostrea gigas* (10), *Saccharina latissima* (5)	
3/20/2023	Penn Cove	*Ulva sp.* (5), *Mytilus sp.* (10-20)	
3/24/2023	IMTA	*Crassostrea gigas* (10), *Saccharina latissima* (5)	Kelp was harvested on 3/28
4/6/2023	IMTA	*Crassostrea gigas* (10), *Saccharina latissima* (5)	Kelp remaining on lines was collected
4/14/2023	IMTA	*Crassostrea gigas* (10),	
4/19/2023	Penn Cove	*Ulva sp.* (5), *Mytilus sp.* (10-20)	
5/2/2023	IMTA	*Crassostrea gigas* (10)	

Table A2. Chemicals used for dosing treatments in bioaccumulation and oyster feeding experiments.

Chemical	Manufacturer	CAS Number
Cadmium Chloride ($CdCl_2$)	Thermo Scientific	7790-78-5
Potassium Chromate (K_2CrO_4)	Spectrum	7789-00-6
Lead (II) perchlorate trihydrate ($Pb(ClO_4)_2 \cdot 3H_2O$)	Thermo Scientific	13453-62-8
Zinc Chloride ($ZnCl_2$)	Thermo Scientific	7646-85-7

Table A3. Percent changes in metals (average $\pm$ 1 SD) in kelp from the beginning to the end of the bioaccumulation experiment (% change) were negligible. ND indicates that the change was below the instrument calculated detection limit.

Treatment	Cd	Cr	Pb	Zn
Control	ND	ND	ND	ND
1	0.62 ± 0.73	-0.07 ± 1.29	-46.2 ± 10.6	6.35 ± 2.76
2	2.17 ± 1.78	-2.18 ± 2.04	-47.2 ± 14.7	7.99 ± 2.22
3	2.55 ± 2.74	2.19 ±1.01	27.2 ± 58.4	1.32 ± 9.78
4	1.83 ± 2.19	5.77 ±1.76	20.9 ± 43.2	13.3 ± 5.46

Table A4. Wet weight, shell length, body length, and dry weight measurements of oysters sacrificed after feeding experiment.

	Wet Weight (g)	Dry Weight (g)	Shell Length (cm)	Body Length (cm)
Mean	9.92	2.49	6.36	4.61
Median (min-max)	10.14 (5.37-17.68)	2.51 (1.3-4.49)	6.40 (5.25-8)	4.60 (3.8-6)

Table A5. Protocols for tissue digestion for seaweed and shellfish.

Sample Type	Protocol Name	Digestion Components	Description
Seaweed	Beech Leaves (BCR-100)	1. 8 mL TMG HNO_3 2. 2 mL 30% H_2O_2 3. 0.2-0.25 g seaweed tissue	1. 5 minutes at 1000 W and 180 ºC. 2. 10 minutes at 1000 W and 180 ºC. The temperature slowly ramped up to 180 ºC.
Shellfish	Fish	1. 9 mL TMG HNO_3 2. 1 mL 30% H_2O_2 3. 0.2-0.25 g shellfish tissue	1. 2 minutes at 1000 W and 85 ºC. 2. 5 minutes at 1000 W and 145 ºC. 3. 3 minutes at 1000 W and 210 ºC. 4. 10 minutes at 1000 W and 210 ºC.

Table A6. Percent recovery of certified reference material. DOLT-3 dogfish liver was used for shellfish and Bladderwrack was used for algae.

	Shellfish Percent Recovery			Seaweed Percent Recovery	
Analyte	IMTA 2022 Oysters	IMTA 2023 Oysters	Penn Cove Mussels	Bioaccumulation Experiment Kelp	IMTA 2023 Kelp
As	---	---	---	106	64.3
Cd	14.4	1.74	1.99	117	73.1
Cu	13.6	57.5	62.8	152	61.9
Fe	22.5	0.00	22.8	---	---
Pb	0.00	69.1	163	72.8	32.9
Ni	40.9	56.9	65.2	---	---
Se	60.7	47.3	56.3	2323	151
Zn	69.5	64.7	72.7	123	68.0

Table A7. Concentrations of metals (Cr, Cd, Zn, and Pb in mg/kg) in kelp tissue used for oyster feeding experiment for each treatment.

Type	[Cd]	[Cr]	[Pb]	[Zn]
Ambient	0	0	0	17.2
Low	4.55	0.91	0	33.7
Medium	7.39	2.77	2.77	64.7
High	13.2	4.4	7.05	96.9

Table A8. t and p values for each comparison from a Tukey's HSD post-hoc test following 1-way ANOVAs of before, during, and after kelp harvest.

Comparisons	Cr	Cu	Zn
Before-During	t = -0.67, p = 0.78	t = 0.54, p = 0.85	t = 1.2, p = 0.44
During-After	t = 5.2, p <0.001***	t = 3.7, p = 0.002**	t = 2.5, p = 0.04*
Before-After	t = 5.4, p <0.001***	t = 2.8, p <0.02*	t = 1.1, p = 0.55

Table A9. Limits of quantitation (LOQs) for each analyte, calculated as 3.33x the instrument measured detection limit. Ranges across multiple ICP-MS runs.

	Min	Max		Min	Max
Ag	2.37E-03	2.84E-02	K	4.62E-03	3.68E-01
Al	4.50E-02	2.75E+01	Mg	6.48E-03	4.89E-01
As	4.14E-02	2.33E+00	Mn	1.59E-01	1.38E+00
Ba	5.22E-02	1.83E+00	Na	6.44E-03	4.91E-02
Be	1.61E+00	6.37E+01	Ni	3.57E-01	5.11E+00
Ca	2.59E+00	3.79E+01	Pb	1.26E-03	6.77E-02
Cd	2.18E-03	3.50E-02	Pb	9.84E-04	7.09E-02
Co	7.07E-03	4.54E-01	Sb	1.35E-03	1.11E-02
Cr	6.33E-03	3.97E+00	Se	2.50E-03	5.29E-01
Cu	4.44E-02	3.83E+01	V	4.64E-04	1.34E-02
Fe	2.40E-01	2.03E+01	Zn	2.74E-04	7.70E-03
Fe	5.94E-04	2.44E-02			