THE EFFECTS OF DISSOLVED COPPER ON EARLY LIFE-STAGES OF BULL KELP IN PUGET SOUND, WASHINGTON.

by

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ABSTRACT

The effects of dissolved copper on early life-stages of bull kelp in Puget Sound, Washington.

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Bull kelp (*Nereocystis luetkeana*) is commonly found in coastal marine environments from Alaska to central California, and is the primary floating kelp found in Puget Sound, Washington. Recent observations suggest that kelp forests have declined significantly in Puget Sound over the last several decades, however the exact cause of the decline is unknown. A number of candidate stressors have been identified, one of which is toxic pollution. In this study, the toxicity of copper on the early life history stages of bull kelp was evaluated by performing a 48-hour toxicity assay. In brief, bull kelp spores were exposed to five different copper concentrations ranging from 50 to 1,000 parts per billion (ppb) for 48 hours. After this exposure duration, two toxicological response endpoints were observed: meiospore germination success; and length of the embryonic germination tube. Adverse impacts to kelp spores were observed starting at 100 ppb. The EC_{50} values for germination success and germ tube growth were 337 and 224 ppb, respectively. However, considering the influence of copper adsorption during the experiment, it is estimated that adverse impacts to bull kelp spores begins with copper concentrations as low as 50 ppb.

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Introduction

Bull kelp (*Nereocystis luetkeana*) is commonly found in coastal marine environments from Alaska to central California. Within the Puget Sound, this species can be found as far south as Squaxin Island (Mason County, WA), and is commonly encountered in areas with rocky substrate and fast-moving currents. Bull kelp is often referred to as foundational species due to its critical role in providing food and unique habitat for many fish, bird, and marine mammal species within Puget Sound (Mumford 2007).

Recent observations suggest that kelp forests have declined significantly in Puget Sound over the last several decades (Berry, Calloway, and Ledbetter 2019; Dunagan 2018), however the exact cause of the decline is unknown. A number of candidate stressors have been identified including: warmer temperatures (Schiltroth, Bisgrove, and Heath 2018); low nutrient availability (Hurd et al. 2014); increased sedimentation (Deiman, Iken, and Konar 2012); decreased light availability (Rubin et al. 2017); increased herbivory (Dobkowski 2017); changes to spore dispersal (Gaylord et al. 2002); and toxic pollution (Eklund and Kautsky 2003).

This study focuses on the role of toxic pollution on bull kelp. More specifically, this study evaluates the toxic effects of copper on early life stage development of this species. Copper is ubiquitous in the marine environment and has both natural and anthropogenic sources. Trace amounts of copper are essential to the growth and development of marine plants and algae, however at high enough concentrations it becomes highly toxic (Leal Sandoval 2016). The impact of excess copper has been evaluated for a number of brown macroalgae species, including eight species with the kelp order (Laminariales) (Leal, Hurd, Sander, Armstrong, et al. 2016). However, the author is unaware of any such study evaluating copper's effect on bull kelp.

The toxicity of copper on the early life history stages of bull kelp was evaluated by performing a 48-hour toxicity assay. In brief, bull kelp spores were exposed to five different copper concentrations ranging from 50 to 1,000 parts per billion (ppb) for 48 hours. After this exposure duration, two toxicological response endpoints were observed: meiospore germination success; and length of the embryonic germination tube. In the following sections I provide an overview of bull kelp and copper in the Puget Sound, I then describe the method and results of the copper toxicity assay, finally I discuss the results in the context of risk to bull kelp populations within Puget Sound.

Literature Review

This literature review examines available data relevant to the interaction of copper pollution and bull kelp in Puget Sound. First, I will introduce the subject - bull kelp - with a discussion of its life history, ecological and cultural significance, common stressors to its health, current trends in abundance, as well as some of the approaches used in kelp restoration. Next, I will provide background regarding the biological response to heavy metals generally and copper in particular. Finally, I will describe the distribution of bull kelp and copper within Puget Sound.

Bull Kelp Life History

Bull kelp, like other macroalgae, maintain a life history which is composed of both haploid and diploid phases. When fully grown, bull kelp is in the diploid phase. During this life history phase, bull kelp consists of a long, narrow stipe which is attached via a holdfast to the sea floor. Bull kelp maintain buoyancy via a hollow stipe as well as via a gas filled bulb (pneumatocyst) at the upper end of the kelp. Each plant has between 30 and 60 blades which can grow up to 4 meters in length each. Bull kelp continue to grow until the bulb and blades of the plant reach the sea surface; kelp holdfasts can be found in waters as deep as 20 meters (Springer et al. 2010). Bull kelp reach sexual maturity in the fall when they develop sori – patches on the blades which contains a high density of spores. Spores are then either released from the sori, or the sori break off and the entire spore package is transported through the system. Once settled, spores germinate into male and female gametophytes. After a period of development, the female gametophyte produces an egg and releases pheromones which attract sperm from the make gametophyte. If successful, the resulting sporophytes develop holdfasts and begin to develop their first blades. Bull kelp are generally considered annual species, however, mortality is usually

driven by dislodgement during storms or predation and individuals do sometimes survive to release spores again the following year (Springer et al. 2010).

Ecological and Cultural Significance

Along the west coast of north America two types of kelp dominate the rocky nearshore marine habitat: giant kelp *Macrocystis pyrifera*, and bull kelp *Nereocystis luetkeana* (Springer et al. 2006). Together, these kelps form dense forests complete with layered canopies and understory ecosystems. Early life stages of kelp (e.g. spores, gametophytes, and juvenile sporophytes) are food for various filter feeders, limpets, and chitons (Dayton 1985). For some of these species, kelp is an essential resource. For example, (Bustamante, Branch, and Eekhout 1995) found increased levels of mortality and decreased growth in certain limpet species when kelp resources were removed. More recently, the importance of kelp detritus to benthic filter feeders has been challenged. (Yorke et al. 2019) performed an experiment in which filter feeders were provided kelp particulates and – separately - phytoplankton. The filter feeders exposed to phytoplankton grew whereas those exposed to kelp did not. The story may, however, be more complex: other research has recently demonstrated that kelp-derived sea urchin feces are significantly more nutritious than kelp itself and may be an important dietary item for filter feeders (Dethier et al. 2019).

As they grow, kelp become food for other herbivorous grazers such as sea urchins (Rogers-Bennett and Catton 2019), abalone (Cox 1962), and kelp crabs (Dobkowski 2017; Berry, Calloway, and Ledbetter 2019). Sea urchins are well-known for their preference for kelp along the California and (more recently) Oregon coasts. There, sea urchin populations have boomed due to complex trophic dynamics involving starfish, sea otters, and ocean heat waves

(Rogers-Bennett and Catton 2019). Within the Puget Sound, sea urchins are less of a concern to kelp populations. However, there is some evidence that herbivory from kelp crabs may be contributing to declining kelp abundance (Dobkowski 2017). The contribution of kelp to the food web extends beyond the intertidal zone. For example, Kelp washes up on beaches along with other debris, collectively referred to as wrack. This wrack becomes food for a variety of terrestrial herbivores. (Lastra et al. 2008) estimated that sand fleas process over 50 percent of beached kelp on California coasts. The presence of macrophyte wrack has also been shown to correlate with the species richness and abundance of terrestrial invertebrates as well as shorebirds (Dugan et al. 2003).

Kelp that doesn't end up on beaches eventually sinks to the ocean floor, sometimes ending up in deep ocean canyons. (Vetter and Dayton 1999) used submarines and remote underwater vehicles to observe the presence of kelp detritus in deep (900 m) submarine canyons of the California coast. The authors observed increases in species richness and abundance in megafaunal invertebrates and fish associated with detritus. Another way to measure kelp's influence on the food web is through carbon and nitrogen isotope analyses. In this method, tissue is taken from higher-trophic level species and analyzed in order to characterize the relative contribution from different primary producers (e.g. kelp, phytoplankton) (Fry and Sherr 1989). Recently the approach was used to evaluate black rockfish and kelp greenling, two nearshore fish species (von Biela et al. 2016). The results indicate that as much as 65 and 89 percent of rockfish and greenling energy was derived from kelp.

Kelp are often referred to as foundational species for their ability to provide unique habitat, as well as influence features such as water flow, light availability, and nutrient load (Benes and Carpenter 2015). In regards to habitat, kelp forests provide necessary habitat for

species across a wide range of trophic positions, and each major part of kelps (holdfast, stipe, and blades) provide unique habitat. For example, (Christie, Norderhaug, and Fredriksen 2009) found that some kelp holdfasts contain as many as 10,000 macrofaunal individuals such as amphipods and gastropods. Evidence from carbon isotope analysis suggests that these holdfasts may represent mini-ecosystems, as organisms within these kelp holdfasts rarely leave and rely primarily on resources derived from within the system (Schaal, Riera, and Leroux 2012). Kelp stipes provide less habitat complexity than holdfasts but have been found to contain large numbers of sessile invertebrates. (Leclerc et al. 2015) found invertebrates representing 34 different taxa along the stipe of certain kelp species. Additionally, kelp stipes serve as habitat for a high diversity and abundance of epiphytic algae (Bartsch 2008) which serve as secondary habitat to dense macrofauna assemblages. (Christie, Norderhaug, and Fredriksen 2009) found macrofauna abundance as high as 55,000 individuals per kelp. Kelp blades are typically associated with lower densities of macrofauna than the stipe and holdfast, however blades often contain herbivorous grazers such as limpets, gastropods and kelp crabs (Dobkowski 2017). Additionally, kelp forests serve an important habitat to a number of fish species. Within the Puget Sound, juvenile salmon, surf smelt and rockfish (to name a few) have all been found associated with kelp forests (Shaffer 2004; NMFS 2017).

Bull kelp is an important cultural resource for tribes in the Puget Sound area. Although bull kelp is edible (Barta, Branen, and Leung 1981), the more common traditional uses were technological and ceremonial. For example, Bull kelp's long hollow stipes were historically used to make strong ropes used for fishing lines and nets, for anchoring boats, and as harpoon line (Turner 2001). The process for making kelp rope involves a year-long process of soaking the stipes alternatively in freshwater, saltwater, and dogfish oil (Pasco, Compton, and Hunt 1998).

Another technological use of bull kelp was as a steaming apparatus to help with shaping wooden halibut hooks and bow staves. In this application, yew-wood was placed inside of the hollow kelp stipes and bulbs and buried overnight next to a fire to steam; once steamed, the wood was able to be bent to make hooks and bows (Turner 2001). Bull kelp was also used in the creation of medicines. For example, The Nuu-Chah-Nulth of modern-day Vancouver Island used bull kelp bulbs in the making of skin salve. Here, cottonwood bulbs were infused into deer fat and then poured into the hollow bull kelp bulb. Once cool, the kelp could be peeled away to reveal the hardened salve (Turner and Loewen 1998). Bull kelp was also important for ceremonial use. For example the Kwakwaka'wakw peoples of modern-day British Columbia used kelp tubes theatrically in winter ceremonials by speaking through the tubes which would be buried under the ground and into the center of a fire (Turner 2001).

Bull kelp continues to be an important cultural resource for tribes in the Puget Sound area and tribes are actively involved in research and restoration. For example, the S'klallam Tribe has partnered with the Suquamish Tribe and the Puget Sound Restoration Fund to restore bull kelp forests in Port Gamble, Washington (Dunagan 2011). According to tribal elders of the Port Gamble S'klallam Tribe bull kelp beds were once abundant in the bay, along with herring; today few of either remain (Northwest Treaty Tribes 2011). Starting in 2011, restoration efforts in Port Gamble have consisted of securing kelp-seeded ropes in a grid-like patter to the bay floor in an attempt to increase the likelihood of bull kelp recruitment (Dunagan 2011). The Samish Tribe is also actively involved in active kelp research in Skagit and San Juan counties. Samish Tribe work in this area includes interviews with elders, underwater surveys with remote operated vehicles, and aerial surveys (Woodard and Palmer-McGee 2018). In additional to research and

restoration projects, numerous tribes have been involved with the drafting of the Puget Sound Kelp Conservation and Recovery plan.

Stressors to Kelp Recruitment and Growth

Recent observations suggest that kelp forests have declined significantly in Puget Sound over the last decade (Berry, Calloway, and Ledbetter 2019; Dunagan 2018), however the exact cause of the decline is unknown. A number of candidate stressors have been identified including: warmer temperatures (Schiltroth, Bisgrove, and Heath 2018); low nutrient availability (Hurd et al. 2014); increased sedimentation (Deiman, Iken, and Konar 2012); decreased light availability (Rubin et al. 2017); increased herbivory (Dobkowski 2017); changes to spore dispersal (Gaylord et al. 2002); and toxic pollution (Eklund and Kautsky 2003). The relative importance of each of these factors depend on life stage; for example, large scale hydrodynamics can determine the overall patterns of spore dispersal (Pineda 2000), whereas localized factors such as heavy metal concentration may limit germling growth rates (Leal et al. 2018).

Kelp reproduction is limited by light availability. Spatially, light availability sets limits on the depth distribution of different kelp species. In a study with bull kelp, (Vadas 1972) demonstrated that light availability of less than 161 lumen, in combination with shorter day periods (8 hours), resulted in decreased gametophyte maturation. Within the Puget Sound, benthic light availability is highly seasonal and is dependent on tides (timing and magnitude), turbidity. This is consistent with (Thom and Albright 1990) who observed increased in kelp biomass following the winter solstice. The inhibitory effect of sediment on light availability was made apparent in a study on kelp recruitment in the Elwha delta following large-scale dam removal (Rubin et al. 2017). For bull kelp in the Puget sound, light availability sets limits on the

depth at which kelp will grow, as well as the ability of juvenile kelp to establish within existing kelp forests (Vadas 1972).

Kelp reproduction and growth rates are limited by water temperature. Temperature sets limits on the latitudinal distribution of different kelp species. (Vadas 1972) performed lab-based experiments on bull kelp and observed decreased gametophyte maturation at temperatures above 17 degrees Celsius. The growth rate of bull kelp stipes and blades have also been found to be temperature dependent, with the highest rates of blade growth occurring in summer (Maxell and Miller 1996). Upper temperature limits likely determine the southern range of bull kelp distribution. This is suggested by (Luning and Freshwater 1988) who tested the thermal range of 46 different algal species; they found the bull kelp survival range to span from 1.5 to 18 degrees Celsius which roughly corresponds to central California.

Nutrient levels influence timing and rate of growth. The nutrient requirements of kelp species is well described (Dayton 1985). Of particular importance to kelp is the availability of nitrogen. Kelp growth has been shown to be nitrogen limited during summer months, conversely, kelp growth is high in late winter and spring when inorganic nitrogen is available (Ahn, Petrell, and Harrison 1998). The importance of nitrogen as a limiting factor is further demonstrated by (Jackson 1977) who observed that kelp canopies deteriorated when nitrogen levels dipped below 1 micromolar. Some kelp species, such as giant kelp, have the ability to store nitrogen as free amino acids within the blades, however such reserves have been shown to only last up to 30-days (Zimmerman and Kremer 1986).

Herbivory is a major factor limiting the recruitment of kelp forests. A variety of organisms graze on algae including red and purple sea urchins, red abalone, kelp crabs, limits and snails (Springer et al. 2010). Perhaps the most well-known grazer interaction is that of the

sea urchin. Recently, sea urchins have been attributed with decimating kelp forests off the California and Oregon coast (Rogers-Bennett and Catton 2019). Puget Sound does have populations of both red and purple urchins, however, unlike in California and Oregon, the effect of urchin grazing has not yet been demonstrated to be causing kelp forest declines in Puget Sound (Berry, Calloway, and Ledbetter 2019). There is some evidence that kelp crab herbivory may be contributing to declines in Puget Sound but extensive surveys have not been conducted (Dobkowski 2017). Small sea snails may also play significant roles are grazers. (H. Chenelot and Konar 2007) observed that sea snail *Lacuna vincta* preferentially targeted juvenile bull kelp plants and thus exerted significant influence on local kelp forest dynamics. Although herbivory on microscopic and early life-stage kelp has been documented, to date no quantitative studies have been conducted (Springer et al. 2010). Another effect of grazing is that it increases kelps susceptibility to breakage during storm events. (Koehl and Wainwright 1977) conducted surveys and found that 90 percent of severed bull kelp individuals had some grazing damage at the point of breakage on the stipe.

Kelp Restoration in Puget Sound

Research currently underway address two parallel pathways to: 1) better understand the causes of kelp decline; and 2) reestablish (e.g. out-plant) degraded kelp forests (Dunagan 2018). In 2017 the National Oceanic Atmospheric Administration published a recovery plan for yelloweye rockfish and bocaccio, two species listed federally under the Endangered Species Act (NMFS 2017). The plan identifies kelp forests as important habitat for these species and lists kelp conservation as a high priority. The Puget Sound has seen a renewed interest in kelp forest restoration in response to both the decline in kelp forests, and also in response to the 2017

rockfish recovery plan. However kelp restoration is costly, and labor intensive (Springer et al. 2010).

In 2003, (Carney et al. 2005) tested several restoration techniques with bull kelp in the San Juan islands. The first technique involved out-planting lab-raised kelp in the gametophyte and microscopic sporophyte phase. These early-life stage kelps were then placed into petri dishes either directly on the rocky substrate or elevated above with PVC pipes. In the second technique, juvenile kelp was transplanted from other nearby kelp forests. The juveniles were tied to plastic clips and then glued to rocks with marine epoxy. None of the kelp survived under the first technique owing largely to sedimentation and herbivory. The authors note that the use of petri dishes may have actually served to trap sediment and herbivores. The second technique saw some success; nine of the 32 kelp juveniles survived and grew to release their own spores. The majority of mortality to this group was due to failure of the marine epoxy as well as some herbivory (Carney et al. 2005). The authors make several recommendations: 1) elevate kelp on seeded twine to avoid predation; 2) clear grazers from the site as often as once a week; 3) plant kelp densely and repeatedly for several years.

Campbell et al. (2014) transplanted adult crayweed *Phyllospora comosa* (a kelp species) in Sydney, Australia. In this experiment, divers carefully removed kelp holdfasts from the substrate and then re-attached them in the new locations using mesh-bags and zip-ties. Adult kelp was transplanted into two locations. The survival and growth rate were high at one site (80 and 70 percent of controls) but was low at the other (40 and 50 percent survival and growth respectively). The authors conclude that herbivory was likely the main stressor reducing survival and growth. They recommend increasing the number of kelp transplants as a way of minimizing loss due to grazers (A. H. Campbell et al. 2014).

Westermeier et al. (2014) explored new techniques for out-planting kelp *Macrocystis integrifolia* in Bahia Chasco, Chile. In this experiment two methods were tested. In the first, kelp was raised to the juvenile stage in the lab and then attached to different types of substrates (plastic grids, ceramic tiles, boulders) using either rubber bands or marine glues. In the second technique, kelp spore-producing tissue (sorus) were placed into gauze sleeves along with boulders. The gauze-wrapped boulders were then placed underwater at the restoration sites. The results of method one indicated that kelp growth rates do not differ depending on the substrate type (plastic, ceramic, or boulder). The results of method two were promising; the 70 percent of the boulders that were wrapped in kelp had recruitment after 3 months. Although the experiment with the boulders showed some success, the authors recommend method 1 (the transplanting of more mature kelp) because kelp at this life-stage are more resistant to herbivory and changes in conditions between the lab and the field (Westermeir et al. 2014).

Transplanting kelp from natural populations into restoration sites is reportedly the most common restoration technique (Verdura et al. 2018). One concern with this approach is that donor populations, which may themselves be unstable, can be depleted. In response to this concern, the authors performed kelp restoration using only recruitment enhancement techniques. The group employed two methods. In the first method, spore-bearing kelp tissue was placed inside of mesh bags which were then secured to posts underwater. Additional rocky substrate was filled in around the posts in order to promote settlement success. In the second method, kelp spores were germinated in a lab and allowed to settle and attach on boulders. Once the kelp had successfully established, the boulders themselves were transported to restoration sites. In this case, both techniques proved to be successful; after 6 years the kelp populations in these areas have returned to pre-disturbance levels (Verdura et al. 2018).

A recent review on kelp restoration (Wood et al. 2019) outlines the importance of "future-proofing" kelp restoration efforts. The authors suggest that climate-change related stressors (e.g. temperature, ocean acidification, storm intensity, etc.) may represent the greatest threat to macroalgae restoration. In response to this, the authors suggest that restoration efforts should consider whether the need is to restore species or functions and, if the latter, consider the possibility of introducing alternative species or artificial habitats. In regards to current/traditional kelp restoration strategies, the authors make a number of specific recommendations including: 1) taking caution to not deplete donor populations when undertaking transplant-based restoration; 2) taking advantage of the large amount of kelp that is naturally dislodged during storm events; and 3) advancing lab-based germination techniques so that larger quantities of kelp seed is available for large-scale restoration (Wood et al. 2019).

In 2011, the Puget Sound Restoration Fund partnered with the Port Gamble S'klallam Tribe and the Suquamish tribe to restore bull kelp forests near Port Gamble, Washington. Three general restoration methods were employed. In the first method, kelp gametophytes were seeded onto ropes which were then lowered to the seafloor in a 30-by-30ft grid. The second method consisted of placing spore-packed sorus tissue into mesh bags which were then secured to the substrate. In the third method, adult, detached bull kelp plants were anchored to the seafloor. Unfortunately, the restoration effort in Port Gamble was not a success, largely due to heavy predation by marine snails (https://restorationfund.org/programs/bullkelp/).

In 2018, the Puget Sound Restoration Fund partnered with the Port of Seattle to reestablish bull kelp forests at Smith Cove, Seattle as part of the Port's Blue Carbon Pilot Project. The group deployed a similar kelp grid; however, this time kelp gametophyte density was increased by seeding onto twine which could then be wrapped around the larger-diameter

anchor line. The group also tested with introducing 15 plots of transplanted juvenile bull kelp from nearby populations. The overall cost of the bull kelp restoration effort at Smith Cove was about 125,000 dollars. A kelp forest initially established following the restoration but did not return the following year. The Port is currently working with University of Washington on establishing a long-term monitoring program at the site (Sloan 2018).

The Puget Sound kelp restoration efforts showcase the difficult task of trying to restore kelp to areas that do not have an abundance of kelp spores available. Successful restoration in Puget Sound may require, as (Carney et al. 2005) has recommended, multiple years of dense kelp transplant and recruitment enhancement in order for forests to establish.

Chemical Exposure and Response

The toxicity of contaminants depends in large part upon their bioavailability (Di Toro et al. 1992). The term bioavailability refers to a chemical's ability to interact with an organism i.e. the ability to adsorb or absorb and influence cellular processes (P. G. Campbell et al. 2002). Mercury, for example, has been found to be highly bioavailable to marine bivalves in the form of methylmercury. In this form, bivalves such as mussels and clams can uptake and accumulate mercury at toxic levels. However, mercury in its inorganic form will bind tightly to sediments and will pass though bivalves, resulting in significantly less accumulation and toxicity (Gagnon and Fisher 1997). The bioavailability of a contaminant can change depending on the chemical properties and processes in the environment as well as the organism and route of exposure (Maarten De Jonge, Blust, and Bervoets 2010).

The bioavailability of toxicologically relevant metals, such as copper, lead, cadmium, and zinc, depends on both the location of the metal (e.g. dissolved in the water vs sorbed to

sediment), as well as the route of exposure (Hansen et al. 1996). Studies in the 1990s (Di Toro et al. 1992; Ankley 1996) focused heavily on the bioavailability of metals in the presence of acid volatile sulfates, which are a bi-product of anaerobic respiration. When exposed to anoxic conditions, certain benthic microorganisms are capable of switching to anaerobic respiration, at which point they rely on sulfate, rather than oxygen, to drive energy production (Diaz and Rosenberg 1995). When this occurs, microorganisms generate acid volatile sulfates (AVS) as a bi-product which are then able to concentrate in the benthic environment (Maarten De Jonge et al. 2009). Acid volatile sulfates readily bind with dissolved metals to form metal-sulfide complexes (Di Toro et al. 1992). The formation of these metal-sulfide complexes results in a net decrease in the concentration of dissolved (bioavailable) metal ions. The complexes themselves precipitate out of the water column and bind to sediment making them, in theory, less bioavailable. According to this theory, as long as the concentration of AVS is greater than the concentration of metal cations (in terms of molar equivalency) metals will be bound and thus no longer bioavailable via water (Di Toro et al. 1992).

In this way, low oxygen conditions will cause heavy metals to precipitate out of the water column and into the sediment where, depending on the species, they become more (or less) bioavailable. A number of studies have examined the effect of this interaction on invertebrate species. Swartz et al. (1986) found that amphipods (phylum: Arthropoda) were more sensitive to heavy metal exposure when the metal was located in the porewater, as opposed to the sediments. Similarly, Landrum (1989) found that amphipods exhibited greater levels of uptake of PAH when it was partitioned into the water column. A study involving the phylum Echinodermata found that brittle stars accumulated less heavy metal in low oxygen conditions. The authors concluded that the creation of AVS had caused metals to partition out of the water column and

thus they were less bioavailable to the brittle stars (Hylland et al. 1996). The same study found that oxygen level had no effect on the accumulation of metals in Annelida worms.

Taken as a whole, the literature indicates that the interactive effect involving AVS is taxa-dependent. In general, species which occupy the sediment-water interface (e.g. Arthropoda and Echinodermata) become less exposed to heavy metals in the presence of low dissolved oxygen because the metals have precipitated out of the water column. Conversely, those species which occupy the sediment (e.g. Annelida and Mollusca) potentially become more exposed as the metals partition into the sediments in which they burrow (M. De Jonge et al. 2012).

Relatively little has been published on the bioavailability and route of exposure in regards to kelp. However, the literature on benthic marine organisms as described above can provide insight as to the risk posed to marine algae species. The process of kelp germination occurs on the sediment-water interface, thus it can be expected that kelp will be at higher risk of exposure to heavy metal which is dissolved into the water column. Gaur and Rai (2001) have reviewed the available literature on heavy metal toxicity to algae and conclude with a general hierarchy of toxicity: mercury > copper > cadmium > silver > lead > zinc. Of these compounds copper is perhaps the most common contaminant in marine systems. Copper is, for example, the most prevalent pesticide used for marine antifouling boat paints (Johnston et al. 2011; Johnson 2007). Copper exposure to kelp has been demonstrated to result in negative effects on germination, germling growth, gametophyte production, and sexual differentiation (Leal et al. 2018).

Copper is a necessary micronutrient for many organisms, including kelp. For example, copper is a constituent of proteins and enzymes involved in processes such as mitochondrial respiration, metabolism, and hormone signaling (Sandmann and Boger 1983). Additionally, copper plays a primary role in photosynthesis as an electron doner in photosystem I (Gledhill et

al. 1997). However, at higher concentrations copper can have negative impacts. General adverse impacts observed in plants and macroalgae include: inhibition of photosystem II, interference with thylakoid membrane and chloroplasts, and oxidative stress, all of which can lead to sublethal impacts or even mortality (Gledhill et al. 1997).

There have been a number of studies on the impact of copper on algae seaweeds within the same order as bull kelp (order Laminariales) (Leal, Hurd, Sander, Kortner, et al. 2016). However, the author is unaware of any such study evaluating the impact to bull kelp itself. Within studies of other seaweeds (e.g. giant kelp, *Marcocystis pyrifera*) the most common response variables measured are germination and germ tube length (Leal, Hurd, Sander, Kortner, et al. 2016). The precise mechanisms of action causing these particular adverse responses is currently unresolved. However, it has been suggested that impediments to germination and germ tube length are perhaps the result of interruptions in calcium transport across cell membranes (B. Anderson et al. 1990; H. D. Nielsen, Brown, and Brownlee 2003) The copper concentration and exposure duration at which these adverse effects are observed varies. For germination, EC50 values (concentration at which a 50 percent impact is observed) range from 120 ppb to 330 ppb (Han et al. 2011; Burridge, Campbell, and Bidwell 1999). For comparison, the present study observed a germination EC50 of 337 ppb. For germ tube length, EC50 values range from 81 ppb to 480 ppb (Han et al. 2011; Burridge, Campbell, and Bidwell 1999). Again, the present study observed a germ tube length EC50 of 224 ppb. The EC50 concentrations are not always directly comparable across studies due to differences in study design and test species. For example, seven different species (all within the order Laminariales) are represented by these ranges, with exposure durations ranging from 24-hours to 9-days.

Distribution of Bull Kelp and Copper in Puget Sound

Along the west coast of north America two types of kelp dominate the rocky nearshore marine habitat: giant kelp *Macrocystis pyrifera*, and bull kelp *Nereocystis luetkeana* (Springer et al. 2006). Together, these kelps form dense forests complete with layered canopies and understory ecosystems. Within Puget Sound, bull kelp is the predominant floating kelp species. Bull kelp occurs throughout Puget Sound within aggregated kelp forests, which are located in established locations. These kelp forests range in size from large (many miles of shoreline) along the Strait of Juan de Fuca to small isolated forests (less than 1 mile) such as those in the South Sound (Berry, Calloway, and Ledbetter 2019). Locations of established bull kelp forests include: the northern coast, Strait of Juan de Fuca, San Juan islands, Admiralty Inlet along Whidbey Island, the Central Basin along the coast from Edmonds to Seattle, and in the South Sound, most notably within the Tacoma Narrows (see figure 1, panel B).

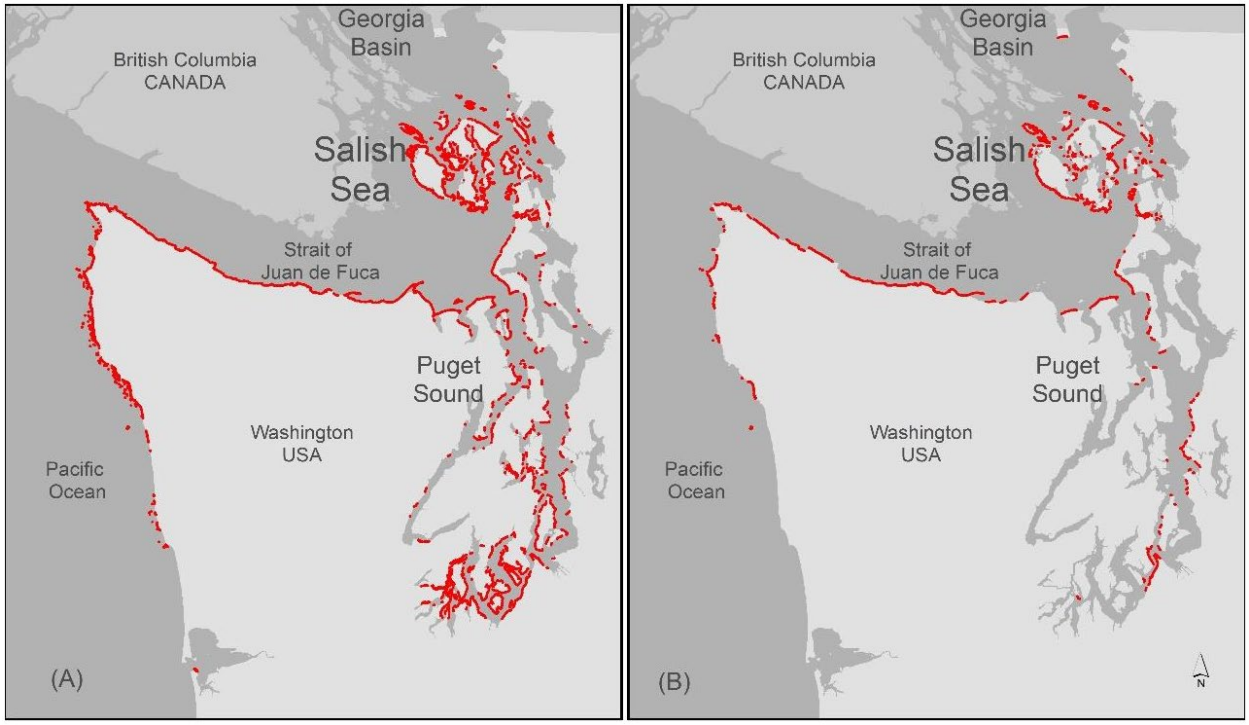

Figure 1. (A) understory and (B) floating kelp distribution in Washington State (figure and caption from Washington Department of Natural Resources 2001 as cited in (Berry, Calloway, and Ledbetter 2019)).

Recent observations suggest that kelp forests have declined significantly in Puget Sound over the last several decades (Berry, Calloway, and Ledbetter 2019; Dunagan 2018). However, the declining trends appear to be regional. Kelp forests along the coast and Strait of Juan de Fuca have been reported as stable (Pfister, Altabet, and Weigel 2019), whereas kelp forests within the South Puget Sound may have declined as much as 62 percent since the 1870s (Conservation and Fund 2020).

Copper is ubiquitous in the marine environment and has both natural and anthropogenic sources. Within the Puget Sound, copper has been identified as one of the seven toxic substances most likely to exceed the criteria concentration for aquatic organisms i.e. the regulatory concentration above which adverse effects are anticipated (County 2011). The chronic and acute aquatic life criteria for copper is 3.1 and 4.8 respectively (WAC 2006). The major sources of

anthropogenic copper include pesticides, industrial and household plumbing and roofing, brake pad wear, and marine antifouling paints (County 2011). Over eighty percent of the copper that enters Puget Sound does so via storm water runoff. Additional pathways include public treatment works, and atmospheric deposition (County 2011). (Hobbs et al. 2015) compared land use types to copper concentrations in surface water and sediment. The authors found that copper concentrations were highest in industrial areas, followed by commercial, low-density, and finally residential land uses. Dissolved copper concentrations reported ranged from 0.62 to 122 ppb and were present in nearly all samples taken (Hobbs et al. 2015). Agricultural areas were not sampled, however (County 2011) reported agricultural areas as having the highest total copper concentrations. The Nature Conservancy has developed a predictive model and educational app called Storm Water Heat Map (www.stormwaterheatmap.org) which is based, in part, on the data provided by (Hobbs et al. 2015). This model predicts copper concentrations up to about 40 ppb associated with all major urban areas in the Puget Sound, but especially within the Seattle/Tacoma area. Comparisons of the locations of kelp forests to areas with predicted higher copper concentrations reveals, as would be expected, that kelp forests are at greater risk to copper exposure within Central and South Puget Sound, and in close proximity to urbanized areas.

Taken together, the literature has shown that kelp in Puget Sound has both ecological and cultural significance, yet faces numerous stressors which have contributed to overall declines in the extent of kelp forests. To date, restoration and reintroduction efforts have been met with mixed success. Marine pollution, including copper, is one of the stressors that has been identified for kelp, however there have been few studies evaluating this factor. The relative impact of

copper on various life stages of bull kelp in largely unknown, which forms the basis for this study.

Methods

The toxicity of copper on the early life history stages of bull kelp was evaluated by performing a 48-hour toxicity assay. In brief, bull kelp spores were exposed to copper for 48 hours; during this time, the copper/sea-water test media was not renewed nor aerated. Bull kelp spores were exposed to nominal copper concentrations of 50, 100, 200, 500, and 1,000 ug copper/L filtered seawater. Each exposure concentration was replicated five times. Two toxicological response endpoints were observed: germination of the spores; and length of the embryonic germination tube. The test procedures used were based the Environmental Protection Agency (EPA) standard method for the National Pollutant Discharge System (NPDES) development and compliance program (Gary A. Chapman, Denton, and Lazorchak 1995), which in turn were adapted from (B. S. Anderson and Hunt 1988). The EPA standard method was further modified in regards to trace metal clean techniques, stabilization of the copper stock solution, and sample preparation (Charrier, Wichard, and Reddy 2018; Leal, Hurd, Sander, Armstrong, et al. 2016). The following is an overview of the process followed. See Appendix X for the complete procedure as was followed in the laboratory.

Collection and preparation of kelp spores

To obtain bull kelp spores, kelp tissue was collected on October 17, 2020 at North Beach County Park in Jefferson County, Washington. Kelp blades with sori (reproductive tissue) patches present were selected for harvest. Care was taken to cut the blade cleanly, and no closer than 24 inches from the bulb. Sorus material was then trimmed from the blades and gently cleaned with paper towels and filtered seawater before being layered in paper towels and placed within plastic bags in a cooler. Bull kelp tissue was then held for 24-hours at roughly 4 degrees

Celsius. To induce spore release in the laboratory, soral tissue was cut into roughly 2-inch squares and immersed in filtered seawater at 10 degrees Celsius. After about 15 minutes the seawater became noticeably cloudy, suggesting that soral tissue had released its spores. The soral tissue was then removed from the seawater into a separate holding bucket while the spore-density was estimated with a hemocytometer. The target spore density was 500,000 spores per ml, this density was selected as optimal for the observation of endpoints with a compound microscope at 40x without overcrowding of spores. In this case, the spore solution needed to be diluted with filtered seawater in order to achieve the target density. Note that the spore solution was diluted again by half during the copper exposure (described below), so that the actual observed density was roughly 250,000 spores per ml. The spore solution was held in a refrigerator at 10 degrees Celsius prior to being dispensed into individual chambers for copper exposure.

Preparation of copper stock solutions

Prior to the copper exposure test, all labware was washed with nonabrasive soap followed by deionized water. Labware was then soaked for 4 days in an acid bath of 1.5 Molar hydrochloric acid. Labware was subsequently rinsed three times with deionized water and air-dried before being sealed in sterile storage containers. Copper(II) chloride dihydrate (ACS, 99+%, CAS: 10125-13-0) was used to prepare five copper stock solutions via serial dilution in filtered seawater to nominal concentrations of: 0.100mg/L; 0.200mg/L; 0.400mg/L; 1.00mg/L; and 2.00mg/L. The seawater was collected at the site of the kelp collection (North Beach County Park), and was filtered twice through unbleached coffee filters prior to being held at 10 degrees Celsius.

48-hr exposure of kelp spores

Kelp spores were exposed to five different concentrations of copper: 50, 100, 200, 500, and 1,000 ug/L (copper/seawater). Each exposure concentration was replicated five times. Five control (no copper, yes spores) and five blanks (no copper, no spores) were prepared as well. Each individual exposure replicate was created by combining 75ml of spore solution with 75ml of the applicable copper stock solution, resulting in the target copper concentrations described above. Also, each individual replicate contained a glass microscope slide, which was later removed for the observation of endpoints. The 35 individual replicates were created in random order over the span of 20 minutes and placed into an environmental chamber at 10 degrees Celsius on a 12-hr:12hr light:dark schedule. The total duration of the exposure was 48-hours. During this time the copper/sea-water test media was not renewed nor aerated.

Collection and observation of endpoint

Two toxicological response endpoints were measured: germination of the spores; and length of the embryonic germination tube. Images were collected under 400x magnification with a compound microscope with attached Leica microscope camera. One at a time, exposure replicates were removed from the environmental chamber for observation. The microscope slide was removed from the solution and tipped slightly to allow excess seawater to drain away. A coverslip was placed on the center of the slide. Five images were captured for each slide: upper left corner, upper right corner, center, lower left corner, and lower right corner. Exposure replicates were chosen for observation in the same order in which the exposures began. After all images were collected, they were analyzed with the aid of ImageJ software. Kelp spores were

characterized as “germinated” when germination tube was present. The presence of germ tubes was further characterized as a protuberance at least one spore diameter (Gary A. Chapman, Denton, and Lazorchak 1995). Germination tube length was measured in 10 randomly selected germinated spores in each image. See Appendix A for more detailed guidelines for recording observations from the images.

Statistical Analysis

Logistic regression was used to create dose response curves for each of the endpoints using R, programming language statistical package. For each endpoint the EC_{50} (exposure concentration effecting 50 percent of the population) were calculated. Concentration-response relationships were fit using logistic regression (R, drc package, fct=LL.4). Data for both endpoints were further evaluated with an ANOVA and Tukey HSD post hoc tests.

Results

Exposure to copper for 48-hours resulted in concentration-dependent decreases in the percent of spores germinated as well as the length of germination embryonic tubes (germ-tubes). Copper treatment levels were 50, 100, 200, 500, and 1000 ug/L (nominal). Concentration-response relationships were fit using logistic regression (R, drc package, fct=LL.4). This statistical approach is common within the field of environmental toxicology, and allows for the calculation of a EC50 value (the exposure concentration associated with a 50 percent response level) and corresponding slope (Suter 2007). These descriptive statistics can then be compared, between endpoints, species, toxicants (e.g. comparisons of copper sensitivity between kelp species). Reduction in germ-tube length was a found to be a more sensitive endpoint than percent germination with EC50 values of 223 ppb and 337 ppb respectively.

Percent of spores germinated

Kelp spores were identified as either germinated or not germinated by the presence or absence of a conspicuous embryonic germination tube (Figure *2*). Within each concentration replicate (n=5), all spores were assessed, via compound microscope, at five different stage positions. The mean number of spores observed per replicate was consistent across copper treatment levels (mean of 660 spores per replicate), except for the highest treatment level in which a decrease in total spores was observed (mean of 440 spores per replicate).

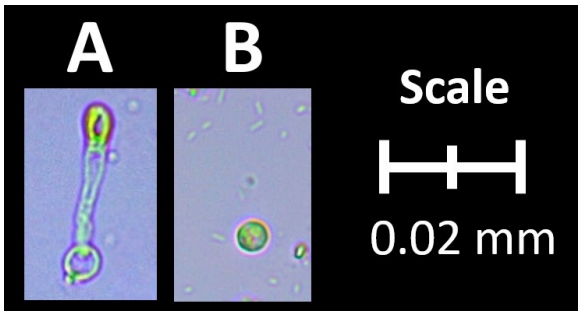

Figure 2. Bull kelp spores: germinated (A); not germinated (B). Image captured at 400x magnification.

The mean percent of spores germinated ranged from a high of 44 percent to a low of 0.5 percent at 50 and 1,000 ppb, respectively. The mean percent of spores germinated within the controls was 45 percent. A concentration-response relationship was fit using a four-parameter logistic regression (Figure 3). The upper parameter was set to the mean of the control (45 percent) and the lower parameter was set to zero. The regression produced an EC50 of 337 (SE = 15.0; 95% CI = 306.3, 367.8) with a slope of 2.8 (SE = 0.26). The percent of spores to successfully germinate varied significantly with different copper concentration levels ($F_{5,24}$ = 162.7, $p < 0.001$). Exposure to copper concentrations of 200 ppb and higher yielded significantly less germination success as compared to the control (post hoc comparison using Tukey's HSD, mean difference ± 95% CI = 7.9 ± 6.4, $p < 0.01$) (Table *1*).

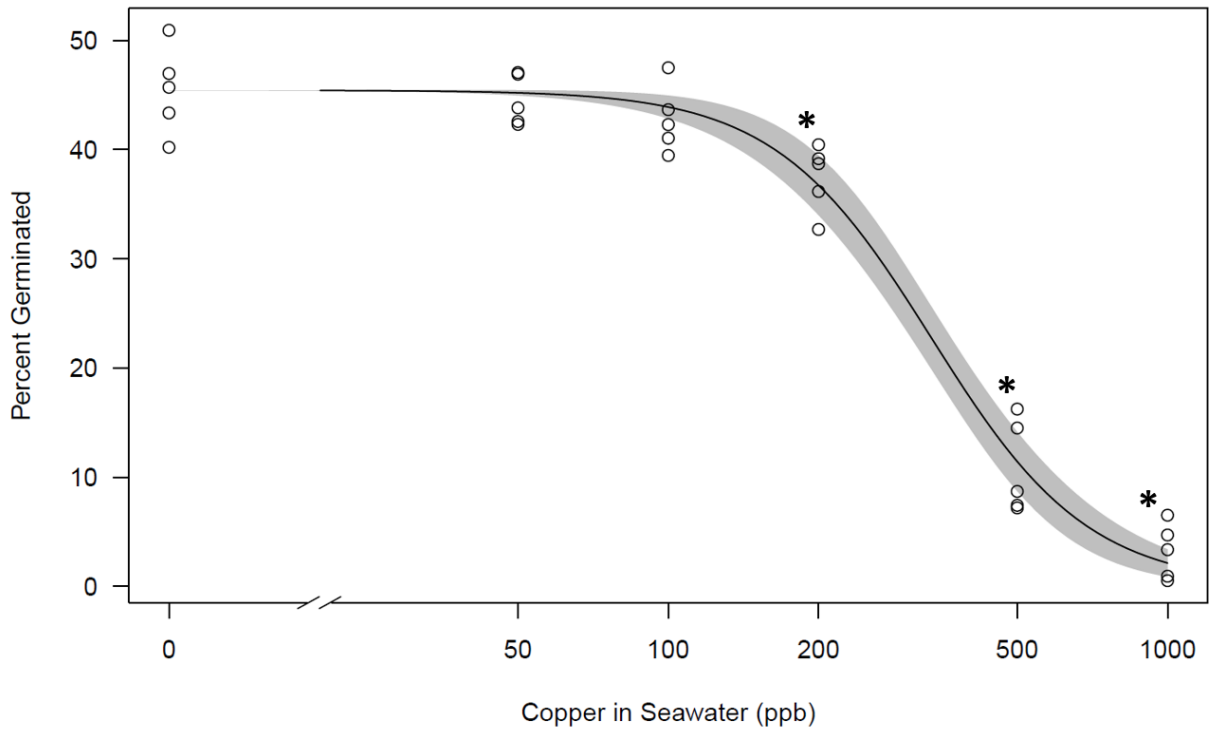

Figure 3. Percent germination of spores exposed to copper concentrations of 50, 100, 200, 500, and 1,000 ppb. Open circles represent replicates at each concentration (n=5). Fitted line is log logistic regression; shaded area is 95% confidence interval. Symbol (∗) signifies that the difference from control is statistically significant ($p < 0.01$).

Table 1. p-value matrix for endpoint: percent germinated. Copper treatments are in ug/L, control (ctr) equals 0.

	ctr	50	100	200	500	1000
ctr	**1.000**					
50	**0.998**	**1.000**				
100	**0.796**	**0.957**	**1.000**			
200	**0.009**	**0.024**	**0.141**	**1.000**		
500	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**1.000**	
1000	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.014**	**1.000**

Length of embryonic germination tube (germ-tube)

Germ tubes were measured from images captured under 400x magnification with a compound microscope with attached Leica microscope camera. Within each concentration replicate (n=5), 10 germinated spores were selected at random for measurement, except for the

highest treatment level in which fewer germinated spores were available for measurement (mean of 5.6 spores per replicate). Measurements were made with the aid of ImageJ (using the segmented line tool) which was calibrated with a stage micrometer (Figure *4*).

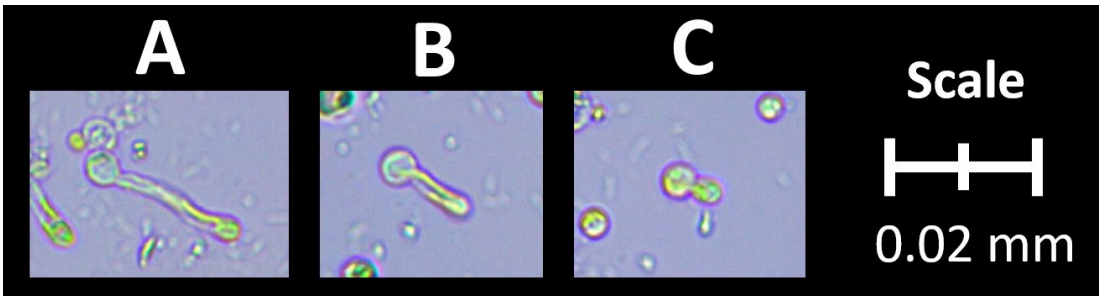

Figure 4. Embryonic germination tubes of varying lengths, 0.015mm (A); 0.008mm (B); and 0.002mm (C). Image captured at 400x magnification.

The mean germ-tube length ranged from a high of 0.013mm to a low of 0.001mm at 50 and 1,000 ppb respectively. The mean germ-tube length for the control was 0.013mm. A concentration-response relationship was fit using a four-parameter logistic regression (Figure 5). The upper parameter was set to the mean of the control (0.013) and the lower parameter was set to zero. The regression produced an EC50 of 223 (SE = 14.0; 95% CI = 195.0, 252.4) with a slope of 1.3 (SE = 0.11). The germ-tube length varied significantly with different copper concentration levels ($F_{5,24} = 124.1$, $p < 0.001$). Exposure to copper concentrations of 100 ppb and higher yielded significantly shorter germ-tubes as compared to the control (post hoc comparison using Tukey's HSD, mean difference ± 95% CI = 0.004 ± 0.002, $p < 0.001$) (Table *2*).

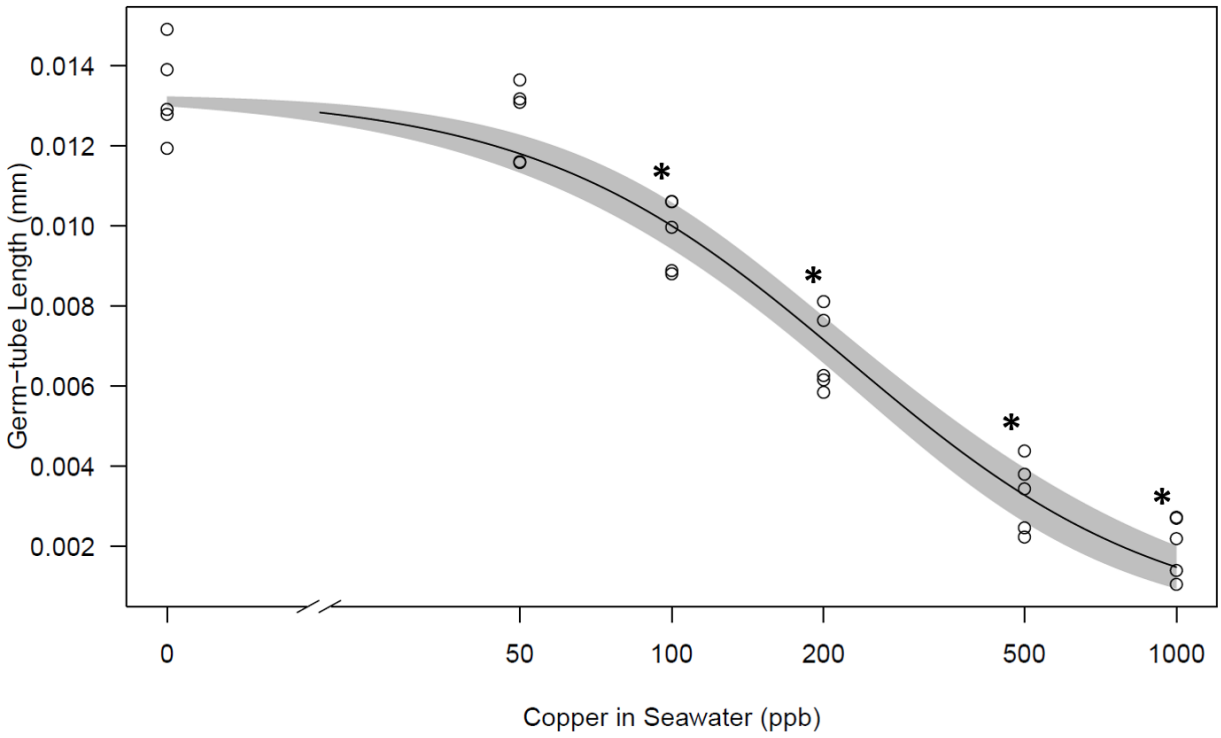

Figure 5. Length of embryonic germination tube of spores exposed to copper concentrations of 50, 100, 200, 500, and 1,000 ppb. Open circles represent replicates at each concentration (n=5). Fitted line is log logistic regression; shaded area is 95% confidence interval. Symbol (∗) signifies that the difference from control is statistically significant ($p < 0.01$).

Table 2. p-value matrix for endpoint: germ-tube length. Copper treatments are in ug/L, control (ctr) equals 0.

	ctr	50	100	200	500	1000
ctr	**1.000**					
50	**0.870**	**1.000**				
100	**< 0.001**	**0.001**	**1.000**			
200	**< 0.001**	**< 0.001**	**< 0.001**	**1.000**		
500	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**1.000**	
1000	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.332**	**1.000**

Discussion

Data quality guidelines for toxicological studies often evaluate the health of the test organism by reference to the controls. The method used for this study was based on the NPDES standard method developed for giant kelp (Gary A. Chapman, Denton, and Lazorchak 1995). According to that method, mean germ-tube length in the controls must be at least 0.010mm, and mean control germination must be at least 70 percent. The mean germ-tube length for the control in the present study was 0.013mm (acceptable). However, the mean control germination was only 45 percent (not acceptable). The NPDES method was developed for a different kelp species (*Macrocystis pyrifera*); it is possible that the seemingly low (i.e. 45 percent) germination success of bull kelp spores may be within the range of what is typical for this species, rather than a sign of poor fitness. Studies on bull kelp germination rate are limited, but several suggest that the lower germination rates may be typical, at least within the Puget Sound. (Harshman 2019) observed about 20 percent germination of kelp spores collected in the San Juan Islands. Researchers at The Puget Sound Restoration Fund (https://restorationfund.org/) have observed germination rates of around 50 percent (B. Allen, personal communication, April 2022). Interestingly, Bull kelp studies in Alaska have observed higher germination rates while using similar methods. Spores produced from Kelp collected in Cook Inlet, AK have germination rates over 80 percent (Lind and Konar 2017; H. A. C. Chenelot 2003). Variability in the control between studies may also be attributed to biological differences between populations, test design, poor fitness of the test individuals, or other unintentional lab-introduced stressors (e.g. contaminants).

In the present study, copper was found to inhibit early life stages of bull kelp at concentrations as low as 100 ppb. The EC50s for decreased germ tube length and germination

success was 224 and 337 ppb, respectively. However, these reported concentrations are likely higher than the actual concentrations at which these effects would be observed. This is because the reported concentrations are nominal, not actual. In other words, analytical chemistry was not conducted to determine the actual exposure concentrations. This is important to note because it is expected that a portion of the copper in the exposure media will sorb to the sides of the test containers, thus lowering the actual exposure concentration. In experiments using similar equipment and chemicals, dissolved copper concentrations have been shown to decrease as much as 50 percent due to sorption with test container walls (Leal, Hurd, Sander, Kortner, et al. 2016). Therefore, when comparing copper concentrations in this study to those found within the Puget Sound, concentrations as low as 50 ppb may be considered to be relevant to the biological endpoints.

Within the marine environment, copper exists in one of several different chemical forms. First, copper exists either in particulate form or dissolved. If the copper is dissolved, then it will exist either as free ions or bound to other organic or inorganic molecules (i.e. ligands). This is important because the bioavailability of copper depends on its form. In general, copper is most bioavailable while in the free ion form; copper can also be bioavailable when weakly-bound to ligands (Millero et al. 2009). Thus, the degree to which dissolved copper will be able to exert a toxic effect will depend, in large part, on the portion of the total dissolved copper that exists in the free ion state. The factors that influence the form (or "speciation") of copper include pH, temperature, and the concentration of organic and inorganic ligands. In seawater, copper is stable in the dissolved form at concentrations up to around 500 ppb. At higher concentrations the copper will precipitate out of the dissolved form and into the less-bioavailable particulate form. However, higher concentrations of dissolved copper can exist for shorter time periods (days or

weeks) depending on other factors (Angel et al. 2020). In the present experiment, the highest copper treatment level was 1000 ppb. No precipitates were observed in the test solution; however, it is possible that some portion of the copper in this treatment level existed in particulate form. The speciation of copper introduces an additional level of uncertainty when comparing copper concentrations reported in the environment to the exposure concentrations of the present study. However, it should be noted that these experiments were conducted using Puget Sound water, not synthetic seawater, so ligands and other compounds present in the environment would have also been present in the experiment.

Within the Puget Sound, water monitoring for heavy metals is most often done inland, and in the context of stormwater runoff. For example, (Hobbs et al. 2015) compared land use types to copper concentrations in surface water and sediment. Dissolved copper concentrations reported ranged from 0.62 to 122 ppb (median = 3.9) and were present in nearly all samples taken (Hobbs et al. 2015). The National Pollutant Discharge Elimination System (NPDES) sets limits on certain water quality parameters, including dissolved copper. The daily maximum limit for copper varies by permit, but are generally in the range of 10 to 60 ppb. For example the 2016 NPDES permit for SeaTac airport has daily outfall concentration limits for several rivers ranging from 25.6 to 59.2 ppb (WDOE 2016). The most recent stormwater monitoring report for SeaTac found outfall concentrations ranging from 1.0 to 35.0 ppb (mean = 7.0 ppb) (Port of Seattle 2021). Fewer studies have evaluated water samples for copper within the Puget Sound's marine environment. Water column sampling in the South Sound and Admiralty inlet in 2019 reported dissolved copper concentrations ranging from 0.006 ppb to 0.130 ppb (Bergman 2019). In 2017, Washington Department of Ecology monitored heavy metal concentrations associated with five marinas throughout the Puget Sound. Dissolved copper concentrations outside the marinas

ranged from about 0.2 to 1.0 ppb. Concentrations within the marinas were found to be higher, with several samples exceeding 5 ppb (Hobbs, McCall, and Lanksbury 2018). Together, these data comport with the expectation that copper concentrations within the marine environment will be significantly lower than concentrations observed in the stormwater runoff studies.

The biological response endpoints in the present study (germination success and germ tube length) have been observed for several other kelp species (e.g. giant kelp, *Macrocystis pyrifera*) (Leal, Hurd, Sander, Kortner, et al. 2016). For germination, EC50 values range from 120 ppb to 330 ppb (Han et al. 2011; Burridge, Campbell, and Bidwell 1999). For comparison, the present study observed a germination EC50 of 337 ppb. For germ tube length, EC50 values range from 81 ppb to 480 ppb (Han et al. 2011; Burridge, Campbell, and Bidwell 1999). Again, the present study observed a germ tube length EC50 of 224 ppb. Comparisons between studies is complicated by differences in experimental design. However, the results of this study suggest that bull kelp has a similar sensitivity to copper as compared to other kelp species. Early life stage endpoints, like the ones observed in this study, are biologically meaningful because these life stages are often more sensitive to stressors than later life stages (S. L. Nielsen, Nielsen, and Pedersen 2014). Additionally, adverse impacts to these life stages can magnify throughout the subsequent life stage development. For example, in an experiment with a different kelp species (Giant kelp), copper exposure resulted in a 20 percent decrease in germination, however of the surviving germlings, growth and sexual differentiation was inhibited by nearly 70 percent (Leal et al. 2018). A similar theme was observed in the present study: at the 100 ppb treatment level the percent of spores to germinate had decreased by only about 2.5 percent (not statistically significant). However, the surviving spores exhibited a 26 percent decrease in germ-tube length

(statistically significant; $p < 0.001$). This suggests that kelp spores may experience decreases in fitness, even if they are able to survive copper exposures.

Conclusion

In this study, the toxicity of copper on the early life history stages of bull kelp was evaluated by performing a 48-hour toxicity assay. Bull kelp spores were exposed to five different copper concentrations ranging from 50 to 1,000 ppb for 48 hours. After this exposure, two toxicological response endpoints were observed: meiospore germination success, and length of the embryonic germination tube. Adverse impacts to kelp spores were observed starting at 100 ppb. The EC50s for germination success and germ tube growth were 337 and 224 ppb, respectively. When considering the influence of copper adsorption during the experiment (Leal, Hurd, Sander, Kortner, et al. 2016), it is estimated that adverse impacts to bull kelp spores begins with copper concentrations as low as 50 ppb. These effect levels are comparable to those seen in other kelp species (e.g. giant kelp), suggesting that bull kelp has a similar sensitivity to copper as other marine macroalgae species.

Water quality monitoring throughout the Puget Sound has demonstrated that copper is ubiquitous in the aquatic environment. However, copper concentrations observed in the marine environment are typically lower than those at which adverse impacts are observed. The highest copper concentrations observed are found in upland waterbodies, for example streams and rivers adjacent to agricultural or industrial land uses. Concentrations in these locations range from 0.62 to 122 ppb (County 2011). Although these sources often drain directly into the Puget Sound, dissolved copper concentrations within the marine environment are typically observed at less than 1 ppb (Bergman 2019; Hobbs, McCall, and Lanksbury 2018).

The risk of copper exposure is anticipated to increase as the Puget Sound becomes more acidic i.e. via climate change and ocean acidification (Millero et al. 2009). This is because low pH causes copper to transition to the more-bioavailable free ionic form. In other words, the

existing copper in Puget Sound is anticipated to become more toxic as the pH decreases. (Millero et al. 2009) estimated that, by this mechanism alone, ionic copper concentrations may increase by nearly 20 percent by 2100.

Overall, this study has demonstrated that copper exposure can adversely impact bull kelp by causing direct mortality to early life-stages. Further, this study has demonstrated that growth (germ tube length) can be decreased with exposure to copper. For both endpoints, the magnitude of the response increased with higher copper concentrations. The literature suggests that the risk of these impacts depends on both the concentration of copper as well as the form the copper is in (e.g. particulate vs dissolved). Although water quality monitoring occurs frequently within the Puget Sound, marine waters are seldom assessed for heavy metals. Kelp forests in shallow habitats in proximity to urban or agricultural areas are perhaps most at risk of copper exposure. In these areas is it possible that discharge events (e.g. those following heavy rains) could temporarily reach harmful levels. Research has shown that adverse impacts to early-life stages can magnify throughout the subsequent life stage development (CITE). In this way, sub-lethal impacts (e.g. decreased germ tube growth) may translate into decreased in individual fitness, or perhaps even lead to population-level impacts. Further research is needed on the long-term impact of non-lethal copper concentrations on bull kelp. This is especially important as the threat copper posed to kelp is likely to increase in the future, due to the continued development of watersheds and the further acidification of marine environments.

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Appendix A: Laboratory Procedures for Toxicity Assay

Brief Overview

The toxicity of copper(II)chloride ($CuCl_2$) on the early life history stages of bull kelp will be evaluated by performing a 48-hour toxicity assay. In brief, bull kelp spores will be exposed to copper for 48 hours; during this time, the copper/sea-water test media will not be renewed nor aerated. Bull kelp spores will be exposed to nominal copper concentrations of 50, 100, 200, 500, and 1,000 ug copper/L filtered seawater. Each exposure concentration will be replicated five times. Two endpoints will be measured: 1) germination of the spores and: 2) length of the embryonic germination tube. The test procedures used are based the Environmental Protection Agency (EPA) standard method for the National Pollutant Discharge System (NPDES) development and compliance program (Gary A. Chapman, Denton, and Lazorchak 1995), which in turn are adapted from (B. S. Anderson and Hunt 1988). The EPA standard method was further modified in regards to trace metal clean techniques, stabilization of the copper stock solution, and sample preparation (Charrier, Wichard, and Reddy 2018; Leal, Hurd, Sander, Armstrong, et al. 2016).

Note: this lab method includes sections regarding analytical chemistry for obtaining dissolved copper concentrations (see sections on ICP-MS). Although this procedure was developed and tested, it did not end up being used due to time constraints. The dissolved copper concentrations reported in the results section are nominal, not actual.

Trace metal clean procedures

All labware was washed with nonabrasive soap followed by deionized water. Labware was then soaked for [4 days (Oct 8 – Oct 12)] in an acid bath of 1.5 Molar hydrochloric acid. Labware was subsequently rinsed three times with deionized water and air-dried before being sealed in sterile storage containers.

1. **Prepare a fresh HCl acid bath**
    a. **Dispose old acid bath**
        1. Don PPE: lab coat, goggles, double-dip gloves
        2. Set up work station in/near hood and collect materials.
        3. Collect ice in large beaker and leave in hood, about 1 Liter.
        4. Use clear plastic bucket to transfer 1 Liter of acid bath solution into large blue bucket
        5. Transfer blue bucket into the fume hood.
        6. With the hood sash pulled down, while stirring with plastic spoon, slowly and gradually add 40 g of KOH
            a. check pH regularly
            b. add ice if needed
        7. If needed, add additional KOH (~20 g), stir with plastic spoon, check pH
            a. If pH is close to 5 (5-6), add bicarb until pH is close to 7 (6.5-7.5).
        8. Once neutral, remove blue bucket from hood and dispose in lab sink, while running cold water.
        9. Repeat steps 4-8, increasing the volume if comfortable with the procedure.

10. Once the acid bath is empty, rinse with DI water (no soap).
11. Fill out neutralization log.

Mass of KOH to add
The acid bath is currently 1.5M HCL. KOH and HCl have a 1:1 molar ratio, thus I will need to add 1.5 moles of KOH to 1 Liter to neutralize the existing HCl solution.

$$1.5\ \frac{mole\ KOH}{L} * \frac{56.1\ g}{mole} = 84.2 \frac{g\ KOH}{L}$$

Begin with adding about ½ (40g), then add ¼ (20g). The existing acid bath is 30 L, so the maximum mass of KOH theoretically needed will be about 2.5 kg.

b. Prepare new acid bath

1. Use dilution equation to determine the amount of acid to add to the water (always add acid).
2. Place empty acid bath tub inside flume hood.
3. Add HCl to DI water in a 1:7 ratio.
    a. Use beaker to add 7 liters of DI H20
    b. Then use separate beaker to add 1 liter of HCI
    c. Repeat four times
4. Mix with clean and well washed plastic spoon.
5. Place lid on acid bath
6. Label with chemical, concentration, date, and name
7. Contact Jenna to have acid bath moved to counter top.

Volume (and ratio) of HCl to add
The target volume in the acid bath is 32 liters. The target molarity in the acid bath is 1.5M. The HCL stock bottle is 37% HCL and 12M.
M1V1 = M2V2
(12M)(V1) = (1.5M)(32L)
V1 = 4 L HCl
4:28 = 1:7 ratio of HCl to DI water.

2. Clean labware

a. Don PPE
b. Pre-wash all labware with non-abrasive soap followed by a deionized water rinse; remove all tape and labels.
c. Prepare tub with DI water in front of acid bath for dipping gloves; paper towels
d. Immerse labware in acid bath for 24 hours to one week.
e. Fill a separate rinse-tub with deionized water
f. Transfer labware from acid bath into rinse tub
g. Rinse labware an additional 3 times in deionized water
h. Allow labware to dry inverted on drying rack
i. Place clean labware in designated lab drawers.

Preparation of copper stock solution

The copper stock solution was prepared in glass volumetric flasks by dissolving $CuCl_2$ in filtered seawater; resulting solutions are stored in 1-liter PP plastic bottles. The objective is to have five copper/seawater stock solutions of the following concentrations: 0.100mg/L; 0.200mg/L; 0.400mg/L; 1.00mg/L; and 2.00mg/L (mg copper/L seawater). These stock solutions will later be diluted with seawater/spore suspension to achieve target test concentrations.

1. **Prepare 1 liter seawater solution of 2.00 mg/L copper**
   a. Use analytical scale to weigh out 0.542 grams of copper(II)chloride dihydrate ($CuCl_2 \bullet 2H_2O$; 99+%; FW = 170.48) into plastic weigh boat.

$$0.200g\ Cu \times \frac{170.48g\ CuCl_2 \bullet 2H_20}{63.546g\ Cu} \times \frac{1g\ CuCl_2 \bullet 2H_20\ (sample)}{0.99\ CuCl_2\ (pure)} = 0.542g\ CuCl_2 \bullet 2H_20\ sample$$

   b. Use scoopula to transfer 0.542 $CuCl_2 \bullet 2H_2O$ sample from weigh boat into 1-liter volumetric flask. Use seawater squirt bottle to rinse weigh boat into the volumetric flask. Use funnel to fill volumetric flask halfway with filtered seawater.
   c. Allow for the CuCl2 to dissolve completely.
   d. Use funnel to fill volumetric flask to the neck with seawater. Then use volumetric pipette to carefully fill the flask to the 1-liter mark. The resulting solution is 200mg copper/L seawater.
   e. Use a 10ml volumetric pipette to withdraw 10ml of this solution and dispense into an empty 1-liter volumetric flask. Use funnel to fill volumetric flask to the neck with seawater. Then use volumetric pipette to carefully fill the flask to the 1-liter mark. The resulting solution is 2.00mg copper/L seawater.

$$C_1V_1 = C_2V_2$$
$$(200mg/L)(0.01L) = (C_2)(1.00L)$$
$$C_2 = 2.00mg/L$$

   f. Pour the remaining 990ml of the 200mg/L solution into a waste container, fill out a waste tag and set aside. Note: copper solution must be less than 0.5mg/L to pour down the drain.

2. **Perform a serial dilution to obtain the copper solutions of the following concentrations: 2.00mg/L; 1.00mg/L; 0.400mg/L; 0.200mg/L; and 0.100mg/L (mg copper/L seawater).**

   a. ***2.00mg/L solution (see previous step)***

   b. ***1.00mg/L solution***
   Measure out 500ml of 2.00mg solution into a 500ml volumetric flask. Use funnel to pour 500 ml into a 1-liter volumetric flask. Use seawater squirt bottle to rinse all copper solution from the 500ml flask and funnel. Fill volumetric flask to the

neck with seawater. Then use 100ml volumetric pipette to carefully fill the flask to the 1-liter mark. The resulting solution is 1.00mg copper/L seawater.

$C_1V_1 = C_2V_2$
$(2.00mg/L)(0.50L) = (C_2)(1.00L)$
$C_2 = 1.00mg/L$

c. ***0.400mg/L solution***
Measure out 400ml (two 200ml volumetric flasks) of 1.00mg solution into a 1-liter volumetric flask. Use funnel, seawater squirt bottle, volumetric pipette (as described in step 2b above) to carefully fill to the 1-liter line. The resulting solution is 0.400mg copper/L seawater.

$C_1V_1 = C_2V_2$
$(1.00mg/L)(0.40L) = (C_2)(1.00L)$
$C_2 = 0.400mg/L$

d. ***0.200mg/L solution***
Measure out 500ml of 0.400mg solution into a 1-liter volumetric flask. Use funnel, seawater squirt bottle, volumetric pipette (as described in step 2b above) to carefully fill to the 1-liter line. The resulting solution is 0.200mg copper/L seawater.

$C_1V_1 = C_2V_2$
$(0.400mg/L)(0.50L) = (C_2)(1.00L)$
$C_2 = 0.200mg/L$

e. ***0.100mg/L solution***
Measure out 500ml of 0.200mg solution into a 1-liter volumetric flask. Use funnel, seawater squirt bottle, volumetric pipette (as described in step 2b above) to carefully fill to the 1-liter line. The resulting solution is 0.100mg copper/L seawater.

$C_1V_1 = C_2V_2$
$(0.200mg/L)(0.50L) = (C_2)(1.00L)$
$C_2 = 0.100mg/L$

f. The resulting five copper stock solutions as well as control (500ml seawater) and blank (500ml seawater) are placed in polyethylene plastic bottles and transferred into a 10 degree Celsius refrigerator for storage.
g. Working from lowest concentration to highest concentration, use a 75ml volumetric pipette to dispense 75 ml of copper stock solution into each of the 30 pre-labeled test containers. Rinse the volumetric pipette between stock solutions Add one glass microscope slide to each test container.

Preparation of kelp material

1. Collection and desiccation of kelp sorus

a. Kelp tissue will be collected at North Beach county park, Port Townsend, Washington. Kelp blades with sori (reproductive tissue) patches present will be selected for harvest and severed from the plant.
b. Cut the blade cleanly, and no closer than 24 inches from the bulb.
c. Trim sorus material from the blades and gently clean with tissue and filtered seawater.
d. Layer sorus material in paper towels and place in a cooler for transport to the laboratory.
e. Place the wrapped sorus material into a 10 degree refrigerator for 14-24 hours prior to sporulation.

2. Kelp Sporulation

a. The experiment requires a minimum of 2.25 liters of seawater spore suspension of uniform spore density (75ml x 30 test containers). 4 liters will be prepared.
b. Fill blue bucket with 4.0 liters of 10 degree Celsius filtered seawater.
c. Immerse soral tissue into seawater and place the seawater/kelp mixture into the refrigerator.
d. Observe sporulation; may complete within 15 minutes or take up to one hour.
e. Once water becomes cloudy, pour off 2 liters of spore suspension into white 2 liter paint bucket. Do not allow large sorus material to transfer into the white bucket. Place the remaining material back into the refrigerator to be used to increase density if needed.
f. Measure and adjust spore density.

3. Measure and adjust spore density

a. Use two 2-liter white buckets to mix the spore suspension by pouring back and forth.
b. Use a micropipette to withdraw a small sample of spore solution for measurement with the hemocytometer.
c. Place coverslip on over hemocytometer
d. Use a micropipette to fill chamber.
e. Use a compound microscope to count the number of spore cells in each of the four corners and the center
f. Average the number of cells per square; multiply by 10,000 to get the number of cells/ml
g. Goal is 500,000 cells per ml (50 cells per major hemocytometer square).
h. If too low, reintroduce/add spore material
i. If too high dilute according to V1C1=V2C2
j. Place spore suspension into refrigerator at 10 degrees Celsius.

Exposure to copper

Kelp spores are exposed to five different concentrations of copper: 50, 100, 200, 500, and 1,000 ug copper/L seawater. Each exposure concentration is replicated five times. Five control (no copper, yes spores), five blanks (no copper, no spores), and one

temperature/pH (no copper, yes spores) containers are prepared as well. This equals a total of 36 test containers prepared. All 36 test containers are labeled according to their intended contents. All 36 test containers are also given a unique number to facilitate random ordering in treatment and random placement in environmental chamber (refrigerator).

a. Use a graduated cylinder to measure out and dispense 75ml of spore solution into each of the test containers. Note: at this point the test containers will already have 75 ml of copper stock solution in each. The order in which test containers receive spore solution will be random and recorded.
b. The 75ml spore solutions are thus diluted into the 75ml copper solutions to obtain the target concentrations according to the following:

1,000ug/L solution
$C_1V_1 = C_2V_2$
$(2.00mg/L)(0.075L) = (C_2)(0.150L)$
$C_2 = 1.00mg/L$

500ug/L solution
$C_1V_1 = C_2V_2$
$(1.00mg/L)(0.075L) = (C_2)(0.150L)$
$C_2 = 0.500mg/L$

200ug/L solution
$C_1V_1 = C_2V_2$
$(0.400mg/L)(0.075L) = (C_2)(0.150L)$
$C_2 = 0.200mg/L$

100ug/L solution
$C_1V_1 = C_2V_2$
$(0.200mg/L)(0.075L) = (C_2)(0.150L)$
$C_2 = 0.100mg/L$

50ug/L solution
$C_1V_1 = C_2V_2$
$(0.100mg/L)(0.075L) = (C_2)(0.150L)$
$C_2 = 0.050mg/L$

c. The order in which test containers are treated will be recorded as will the time of treatment. 48-hr observations will be taken in the same order. A temperature and pH probe will be secured into the "extra" control container. Temperature and pH data will be logged throughout the 48-hour exposure with a LabQuest data logger. Lights within the refrigerator will be set on a 12/12 light/dark schedule.
d. ICP sample collection (beginning-of-exposure samples). Use acid-washed 10ml volumetric pipette to withdraw 10ml of solution from each of the test containers and dispense into pre-labeled 15ml plastic ICP tube. Work from lowest

concentration to highest, rinse pipette between samples. Cap the ICP tubes and store in the freezer.

Observation of endpoints

1. **ICP sample collection (end-of-exposure samples)**
   a. Prior to image capture, use acid-washed 10ml volumetric pipette to withdraw 10ml of solution from each of the test containers and dispense into pre-labeled 15ml plastic ICP tube. Work from lowest concentration to highest, rinse pipette between samples. Cap the ICP tubes and store in the freezer.

2. **Image Capture**
   a. Observations were made under 400x magnification with a compound microscope with attached Leica microscope camera.
   b. Each test container contains a microscope slide. One at a time (following the same order in which exposure was initiated) containers are removed from the refrigerator for observation.
   c. Use a gloved hand to remove the microscope slide from the container. Allow seawater to drain from the slide.
   d. Use paper towels to wick water from the sides and bottom of the slide. Once the majority of the water is gone, place a coverslip on the center of the slide. It is important to replicate this procedure in as similar a major as possible among each of the slides.
   e. Place slide on microscope stage and obtain six images from each slide in the following order: one 10x image in center of coverslip; five 40x images (upper left corner, upper right corner, center, lower left corner, and lower right corner).
   f. Once images are captured, move to the next container.

ICP-MS: Pretreatment, Preservation, and Storage

Samples will be taken for ICP-MS analysis from each of the 35 test containers (all containers except the temperature/pH probe container) at both the beginning and end of the 48-hour exposure duration.

a. A 10ml volumetric pipette will be used to transfer 10 ml of sample from each test container to an acid-washed, labeled 15ml falcon tube. Samples will be collected working from lowest concentration to highest. The volumetric pipette will be rinsed between samples with the subsequent sample.
b. Falcon tubes containing samples will be placed into the freezer prior to sample processing.

ICP-MS: Sample Processing (incl. method blank)

1. **Filter samples**. Bull kelp spores are typically 6-8um in diameter (Hubbard et al. 2004), thus filtration is necessary. Use 0.45 micron mixed cellulose esters membrane filters (Weltje, den Hollander, and Wolterbeek 2003). Work from lowest

concentration to highest. Use new syringe and filter holder for each concentration series. Change filter paper between samples. Rinse syringe and filter holder between samples with subsequent sample. Note that method blanks will also be created during this stage. The blank will be processed as if it were the first sample in a given concentration series (see #2 Method Blank).

a. Remove frozen ICP tubes from the refrigerator and allow to thaw. Once the sample is thawed invert the tube to mix the solution prior to filtering.
b. Assemble syringe/filter apparatus: place a new filter paper into the filter holder and attach to the syringe with the plunger removed.
c. To rinse the syringe apparatus with the sample: pour a small volume (~ 2 or 3 ml) of sample into the syringe, insert the plunger into the backend, swirl to rinse the syringe body and then use plunger to press through the filter.
d. Remove filter holder, remove plunger, reconnect filter holder, pour in remaining sample.
e. Push the sample through the filter into a new (acid washed and labeled) falcon tube.
f. Remove the filter holder, remove plunger, place new filter into the holder, reconnect the filter holder and then pour in a small volume of next sample, and press through filter paper (to rinse). Then repeat steps d, e and f for each of the five samples within the same measured concentration range.
g. When moving to a new concentration range (e.g. 50ug/L to 100ug/L), switch to a new acid-washed syringe and filter holder.

2. **Method Blank.** Create method blanks for each syringe/filter holder apparatus. The syringe/filter holder will be changed out at the beginning of each concentration series as well as for the control and experimental blank. A total of 14 method blanks will be created.
    a. Use 10ml volumetric pipette to add 10 ml of DI water to each of 14 acid-washed falcon tubes.
    b. Filter and acidify along with other samples. Process the blank as if it were the first "sample" in a given concentration series (see steps above).

3. **Acidify samples**
    a. Use a graduated cylinder and 125ml polypropylene bottle to make 10ml of 50% trace metal grade nitric acid. Add 5ml of nitric acid to 5ml of DI water. Work within the hood with PPE.
    b. Use disposable plastic pipette to adjust each of the samples to pH 2: add one drop of acidic solution and use pH test papers to confirm pH equals roughly 2.
    c. Place ICP samples in the refrigerator for storage once they have been acidified.

ICP-MS: Working Standards and Blank Prep [day-of ICP-MS Analysis]

1. **Prepare 50:50 nitric solution** to acidify standards and samples to one percent nitric. A total of 33ml of 50:50 (nitric:DI water) is needed; 50ml will be prepared.

a. Use a graduated cylinder and 125ml polypropylene bottle to make 50ml of 50% trace metal grade nitric acid. Add 25ml of nitric acid to 25ml of DI water. Work within the hood with PPE.
b. Add the nitric solution to standards and samples as described below. Store within the hood in a secondary container.

2. **Internal Standard.** Gallium (Ga) was selected for the internal standard as it is within 20 amu of copper and is not expected to contribute to isobaric interferences with copper. Internal standard will be added to all standards, blanks, and samples with an initial target concentration of 50 ppb. Desired cps is 400,000 to 1,000,000. A total of 79.5 ml of the intermediate internal standard solution (1.0mg/L gallium) will be needed; 100ml will be prepared.
   a. Obtain a small volume (< 1ml) of gallium reagent (1,000 mg/L; TMG) in an acid-washed falcon tube from SSC.
   b. Use micropipette to add 2ml of 50:50 nitric solution into an acid-washed 100ml volumetric flask.
   c. Use calibrated micropipette to transfer 100ul of gallium solution.
   d. Use squirt bottle to carefully fill the flask to the mark. The resulting is an intermediate internal standard solution of 1.0mg/L gallium (see below).
   e. Pour gallium intermediate internal standard solution into a 125 ml acid-washed polypropylene bottle for storage prior to dispensing into samples, standards, blanks.

1.0mg/L gallium solution
$C_1V_1 = C_2V_2$
$(1000mg/L)(0.0001L) = (C_2)(0.10L)$
$C_2 = 1.0mg/L$

   f. Use a micropipette to add the 1.0mg/L gallium intermediate internal standard solution to all samples, standards and blanks in order to achieve a concentration of 50 ppb, as described in the section below

3. **External Standard.** A single-element external standard will be made using copper. The available copper reagent is 1000mg/L trace metal grade. Goal: six 50ml copper solutions of concentrations: 0.2, 1, 2, 10, 20, 100 ug/L (copper/DI water), each with 50 ppb gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric. These six external standards will be used to generate two separate calibration curves. Note: for the test run on the ICP-MS a single 6-point calibration curve will be attempted.
   a. **Concentration Range**. My samples will have initial nominal concentrations ranging from 50 ppb to 1,000 ppb. However, these will need to be diluted to a range of 3.5 ppb to 70 ppb, respectively, to account for TDS (see dilution method under Final Sample Prep section, below). Additionally, dissolved copper concentrations have been shown to decrease as much as 50 percent due to sorption with test container walls (Leal, Hurd, Sander, Kortner, et al. 2016). Therefore, I will decrease the lower end of my targeted concentration to 0.2

ppb and create an external standard concentration series to cover the range 0.2 to 100.0 ppb copper.

Highest nominal copper concentration
$C_1 V_{1\ (0.0007L\ from\ TDS\ dilution\ step)} = C_2V_2$
$(1{,}000ug/L)(0.0007L) = (C_2)(0.010\ L)$
$C_2 = 70ug/L$

Lowest nominal copper concentration
$C_1 V_{1\ (0.0007L\ from\ TDS\ dilution\ step)} = C_2V_2$
$(50ug/L)(0.0007L) = (C_2)(0.010\ L)$
$C_2 = 3.5ug/L$

b. **Preparation of external standard**: create six 50ml copper solutions of concentrations: 0.2, 1, 2, 10, 20, 100 ug/L (copper/DI water), each with 50 ppb gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric. The available copper reagent is 1000mg/L trace metal grade.

i. 100 ug/L

Obtain small volume (<2ml) of copper reagent from in an acid-washed falcon tube from SSC.

a) Create 10mg/L intermediate copper solution. Record mass of empty 100ml volumetric flask. Remove flask and use micropipette to transfer 1.0ml copper reagent into the flask. Record mass. Carefully fill volumetric flask to the mark; the resulting intermediate concentration is 10mg/L.

$C_1V_1 = C_2V_2$
$(1000mg\ copper/L)(0.001L) = (C_2)(0.100L)$
$C_2 = 10mg\ copper/L$

b) Use micropipette to add 2ml of 50:50 nitric solution into a separate 100ml volumetric flask.
c) Use volumetric pipette to add 5ml of the intermediate internal standard gallium solution (1.0mg/L gallium).
d) Record mass of flask (with acid and internal). Remove flask, use a micropipette to add 1.0ml of the 10mg/L intermediate copper solution. Record mass again.
e) Use analytical scale to weigh out 0.200 grams of sodium chloride (NaCl) into plastic weigh boat. Use scoopula and funnel to transfer NaCl sample from weigh boat into the volumetric flask.
f) Use DI squirt bottle to rinse weigh boat into the volumetric flask. Use funnel to fill volumetric flask halfway with DI water. Allow for the NaCl to dissolve completely.

g) Use squirt bottle to carefully add DI to the 100ml line. The resulting solution is 100ug/L copper, 50ug/L gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric (see table below). Pour 50ml of the solution into an acid-washed and labeled 50ml falcon tube; pour the remaining solution into a pp bottle for back-up storage.

Target	**C_1**	**V_1**	**C_2**	**V_2**
100 ug/L Copper	10 mg/L	0.001 L	0.10 mg/L	0.10 L
50 ug/L Gallium	1.0 mg/L	0.005 L	0.05 mg/L	0.10 L
1% Nitric Acid	0.5 L/L	0.002 L	0.01 L/L	0.10 L
0.2 % Sodium Chloride	0.2% (m/v) = 0.2 g NaCl/100 ml solution			

ii. 20 ug/L

a) Use micropipette to add 2ml of 50:50 nitric solution into a new 100ml volumetric flask.
b) Use volumetric pipette to add 5ml of the intermediate internal standard gallium solution (1.0mg/L gallium).
c) Record mass of flask (with acid and internal). Remove flask, use a micropipette to 200ul of the 10mg/L copper intermediate solution. Record mass again.
d) Use analytical scale to weigh out 0.200 grams of sodium chloride (NaCl) into plastic weigh boat. Use scoopula and funnel to transfer NaCl sample from weigh boat into the volumetric flask.
e) Use DI squirt bottle to rinse weigh boat into the volumetric flask. Use funnel to fill volumetric flask halfway with DI water. Allow for the NaCl to dissolve completely.
f) Use squirt bottle to carefully add DI to the 100ml line. The resulting solution is 20ug/L copper, 50ug/L gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric (see table below). Pour 50ml of the solution into an acid-washed and labeled 50ml falcon tube; pour the remaining solution into a pp bottle for back-up storage.

Target	**C_1**	**V_1**	**C_2**	**V_2**
20 ug/L Copper	10 mg/L	0.0002 L	0.02 mg/L	0.10 L
50 ug/L Gallium	1.0 mg/L	0.005 L	0.05 mg/L	0.10 L
1% Nitric Acid	0.5 L/L	0.002 L	0.01 L/L	0.10 L
0.2 % Sodium Chloride	0.2% (m/v) = 0.2 g NaCl/100 ml solution			

iii. 10 ug/L

a) Use micropipette to add 2ml of 50:50 nitric solution into a new 100ml volumetric flask.
b) Use volumetric pipette to add 5ml of the intermediate internal standard gallium solution (1.0mg/L gallium).
c) Record mass of flask (with acid and internal). Remove flask, use a micropipette to 100ul of the 10mg/L copper intermediate solution. Record mass again.

d) Use analytical scale to weigh out 0.200 grams of sodium chloride (NaCl) into plastic weigh boat. Use scoopula and funnel to transfer NaCl sample from weigh boat into the volumetric flask.
e) Use DI squirt bottle to rinse weigh boat into the volumetric flask. Use funnel to fill volumetric flask halfway with DI water. Allow for the NaCl to dissolve completely.
f) Use squirt bottle to carefully add DI to the 100ml line. The resulting solution is 10ug/L copper, 50ug/L gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric (see table below). Pour 50ml of the solution into an acid-washed and labeled 50ml falcon tube; pour the remaining solution into a pp bottle for back-up storage.

Target	**C_1**	**V_1**	**C_2**	**V_2**
10 ug/L Copper	10 mg/L	0.0001 L	0.01 mg/L	0.10 L
50 ug/L Gallium	1.0 mg/L	0.005 L	0.05 mg/L	0.10 L
1% Nitric Acid	0.5 L/L	0.002 L	0.01 L/L	0.10 L
0.2 % Sodium Chloride	0.2% (m/v) = 0.2 g NaCl/100 ml solution			

iv. 2 ug/L

a) Create 1.0mg/L intermediate copper solution. Record mass of empty 100ml volumetric flask. Remove flask, use volumetric pipette to transfer 10.0ml of the 10mg/L intermediate copper solution into the flask. Record mass again. Carefully fill volumetric flask to the mark; the resulting intermediate concentration is 1.0 mg/L.

$$C_1V_1 = C_2V_2$$
$$(10.0\text{mg copper/L})(0.01\text{L}) = (C_2)(0.100\text{L})$$
$$C_2 = 1.0 \text{ mg copper/L}$$

b) Use micropipette to add 2ml of 50:50 nitric solution into a separate 100ml volumetric flask.
c) Use volumetric pipette to add 5ml of the intermediate internal standard gallium solution (1.0mg/L gallium).
d) Record mass of flask (with acid and internal). Remove flask, use a micropipette to 200ul of the 1.0mg/L copper intermediate solution. Record mass again.
e) Use analytical scale to weigh out 0.200 grams of sodium chloride (NaCl) into plastic weigh boat. Use scoopula and funnel to transfer NaCl sample from weigh boat into the volumetric flask.
f) Use DI squirt bottle to rinse weigh boat into the volumetric flask. Use funnel to fill volumetric flask halfway with DI water. Allow for the NaCl to dissolve completely.
g) Use squirt bottle to carefully add DI to the 100ml line. The resulting solution is 2ug/L copper, 50ug/L gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric (see table below). Pour 50ml of the solution into an acid-washed and labeled 50ml falcon tube; pour the remaining solution into a pp bottle for back-up storage.

Target	C_1	V_1	C_2	V_2
2 ug/L Copper	1.0 mg/L	0.0002 L	0.002 mg/L	0.10 L
50 ug/L Gallium	1.0 mg/L	0.005 L	0.05 mg/L	0.10 L
1% Nitric Acid	0.5 L/L	0.002 L	0.01 L/L	0.10 L
0.2 % Sodium Chloride	0.2% (m/v) = 0.2 g NaCl/100 ml solution			

v. 1 ug/L

a) Use micropipette to add 2ml of 50:50 nitric solution into a new 100ml volumetric flask.
b) Use volumetric pipette to add 5ml of the intermediate internal standard gallium solution (1.0mg/L gallium).
c) Record mass of flask (with acid and internal). Remove flask, use a micropipette to 100ul of the 1.0mg/L copper intermediate solution. Record mass again.
d) Use analytical scale to weigh out 0.200 grams of sodium chloride (NaCl) into plastic weigh boat. Use scoopula and funnel to transfer NaCl sample from weigh boat into the volumetric flask.
e) Use DI squirt bottle to rinse weigh boat into the volumetric flask. Use funnel to fill volumetric flask halfway with DI water. Allow for the NaCl to dissolve completely.
f) Use squirt bottle to carefully add DI to the 100ml line. The resulting solution is 1.0ug/L copper, 50ug/L gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric (see table below). Pour 50ml of the solution into an acid-washed and labeled 50ml falcon tube; pour the remaining solution into a pp bottle for back-up storage.

Target	C_1	V_1	C_2	V_2
1 ug/L Copper	1.0 mg/L	0.0001 L	0.001 mg/L	0.10 L
50 ug/L Gallium	1.0 mg/L	0.005 L	0.05 mg/L	0.10 L
1% Nitric Acid	0.5 L/L	0.002 L	0.01 L/L	0.10 L
0.2 % Sodium Chloride	0.2% (m/v) = 0.2 g NaCl/100 ml solution			

vi. 0.2 ug/L

a) Create 0.1mg/L intermediate copper solution. Record mass of empty 100ml volumetric flask. Remove flask, use volumetric pipette to transfer 10.0ml of the 1.0mg/L intermediate copper solution into the flask. Record mass again. Carefully fill volumetric flask to the mark; the resulting intermediate concentration is 0.1 mg/L.

$$C_1V_1 = C_2V_2$$
$$(1.0\text{mg copper/L})(0.01\text{L}) = (C_2)(0.100\text{L})$$
$$C_2 = 0.10 \text{ mg copper/L}$$

b) Use micropipette to add 2ml of 50:50 nitric solution into a separate 100ml volumetric flask.

c) Use volumetric pipette to add 5ml of the intermediate internal standard gallium solution (1.0mg/L gallium).
d) Record mass of flask (with acid and internal). Remove flask, use a micropipette to 200ul of the 0.10mg/L copper intermediate solution. Record mass again.
e) Use analytical scale to weigh out 0.200 grams of sodium chloride (NaCl) into plastic weigh boat. Use scoopula and funnel to transfer NaCl sample from weigh boat into the volumetric flask.
f) Use DI squirt bottle to rinse weigh boat into the volumetric flask. Use funnel to fill volumetric flask halfway with DI water. Allow for the NaCl to dissolve completely.
g) Use squirt bottle to carefully add DI to the 100ml line. The resulting solution is 2ug/L copper, 50ug/L gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric (see table below). Pour 50ml of the solution into an acid-washed and labeled 50ml falcon tube; pour the remaining solution into a pp bottle for back-up storage.

Target	**C_1**	**V_1**	**C_2**	**V_2**
0.2 ug/L Copper	0.10 mg/L	0.0002 L	0.0002 mg/L	0.10 L
50 ug/L Gallium	1.0 mg/L	0.005 L	0.05 mg/L	0.10 L
1% Nitric Acid	0.5 L/L	0.002 L	0.01 L/L	0.10 L
0.2 % Sodium Chloride	0.2% (m/v) = 0.2 g NaCl/100 ml solution			

4. **Quality Control Standard**. Copper solution of known concentration and accuracy. The available copper reagent is 1000mg/L trace metal grade. Use different stock bottle from the one used for the external standards. Two calibration curves will be generated; therefore, two QC standards will be made at concentrations 1.0 and 20.0 ug copper/L.

a. Obtain small volume (<1ml) of copper reagent in an acid-washed falcon tube from the SSC. Record mass of empty 100ml volumetric flask. Remove flask, use volumetric pipette to transfer 100ul of the 1,000mg/L copper solution into the flask. Record mass again. Carefully fill volumetric flask to the mark; the resulting concentration is 1.0mg/L. Add 2ml of 50:50 nitric solution AFTER transferring solution out per step "b" below.

Target Copper Concentration	**C_1**	**V_1**	**C_2**	**V_2**
1.0 mg/L (intermediate)	1000 mg/L	0.0001 L	1.0 mg/L	0.10 L
1.0 ug/L (QC # 1)	1.0 mg/L	0.0001 L	0.001 mg/L	0.10 L
20 ug/L (QC # 2)	1.0 mg/L	0.002 L	0.02 mg/L	0.10 L

b. To each of two volumetric flasks, add 2ml of 50:50 nitric solution, 5ml of gallium intermediate internal standard, copper solution: record mass of flask (with acid and internal). Remove flask, use a micropipette to add copper solution (100ul of the 1.0mg/L copper solution into one, and 2.0 ml of the 1.0 mg/L copper solution into the other) Record mass again. Then add 0.20 grams of sodium chloride (NaCl) (see table below). Use clean DI squirt bottle to halfway fill the 100ml

flask and allow sodium chloride to dissolve. Use squirt bottle to carefully add DI to the 100ml line. The resulting solutions are 1.0ug/L & 20.0ug/L copper, 50ug/L gallium, 2g/L NaCl (0.2 percent TDS), and one percent nitric. Transfer 50ml of each solution to an acid-washed and labeled 50ml falcon tube. Pour the remaining solutions into separate pp bottles for back-up storage.

Target	**C_1**	**V_1**	**C_2**	**V_2**
50 ug/L Gallium	1.0 mg/L	0.005 L	0.05 mg/L	0.10 L
1% Nitric Acid	0.5 L/L	0.002 L	0.01 L/L	0.10 L
0.2 % Sodium Chloride	0.2% (m/v) = 0.2 g NaCl/100 ml solution			

5. **Matrix Blank.** Matrix to match samples. Samples are seawater diluted with DI to 0.2 percent TDS, also with 50 ppb gallium and one percent nitric.
    a. Use micropipette to add 2ml of 50:50 nitric solution to a new 100ml volumetric flask.
    b. Use micropipette to add 5ml of gallium internal standard.
    c. Use analytical scale to weigh out 0.200 grams of sodium chloride (NaCl) into plastic weigh boat. Use scoopula and funnel to transfer NaCl sample from weigh boat into the volumetric flask. Use DI squirt bottle to rinse weigh boat into the volumetric flask. Use clean DI squirt bottle to halfway fill the 100ml flask; allow for the NaCl to dissolve completely.
    d. Use squirt bottle to carefully add DI to the 100ml line. The resulting solution is 2g/L NaCl (0.2 percent TDS), 50ug/L gallium, and one percent nitric. Transfer 50ml of this solution to an acid-washed and labeled 50ml falcon tube. Pour the remainder in a 125ml pp bottle for back-up storage.

ICP-MS: Final Preparation of Sample for Instrument.

Use a new set of acid-washed, labeled 15ml falcon tubes. Sample quantities added will be recorded by mass. Each prepared sample will be diluted to 0.2 percent TDS, will have 50 ppb gallium, and one percent acid. Mass will be recorded at four points for each sample (see figure 1).

1. Record the mass (#1) of each empty tube (with label, no cap).
2. Add acid and internal standard to all tubes.
    a. Acidify to one percent (v/v) nitric acid. Use micropipette to dispense 200ul of the 50:50 nitric acid solution into each of the falcon tubes.
    b. Add Internal Standard. Use a micropipette to add 500ul gallium intermediate solution (1.0mg/L) into each of the falcon tubes.
3. Zero scale. Record mass (#2) of selected tube (with acid and internal standard added).
4. Remove tube, add sample corresponding to label. Use micropipette to transfer 0.650ml of sample (after inverting to mix) to the falcon tube.
5. Zero scale. Record mass (#3) of tube (with acid, internal standard, and sample).
6. Remove tube. Use volumetric pipette to add 10ml of DI water.

7. Zero scale. Record mass (#4) of tube (with acid, internal standard, sample, and water).
8. Repeat steps 3-7 for each sample. Possible to do step 6 for all samples at once. Place cap on sample and store in refrigerator.

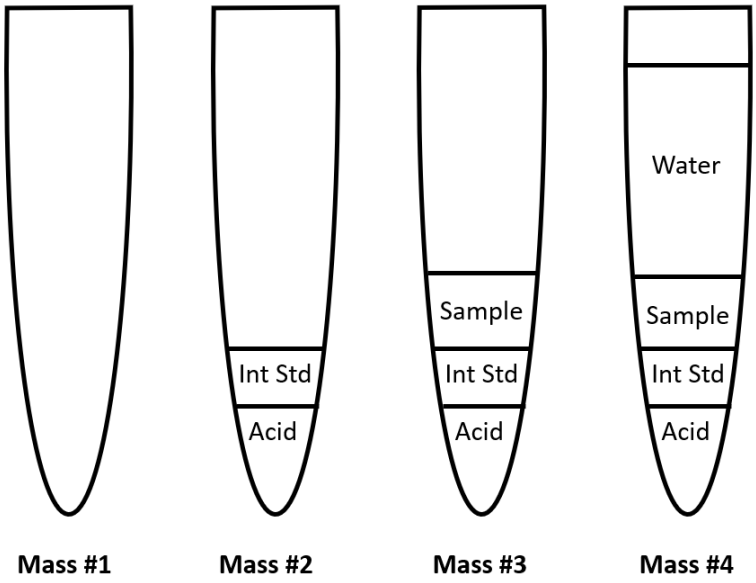

Figure 6. Record mass at four steps during the preparation of each sample.

Approximate Target	C_1	V_1	C_2	V_2
50 ug/L Gallium	1.0 mg/L	0.0005 L	0.05 mg/L	0.01 L
1% Nitric Acid	0.5 L/L	0.0002 L	0.01 L/L	0.01 L
Sample TDS @ 0.2%	30.5 g/L	0.00065 L	2.0 g/L	0.01 L

Dilution of samples to 0.2 TDS. Prior to this stage, all sample have been filtered with 0.45 micron mixed cellulose esters membrane filters. There are three remaining sources of dissolved solids: 1) the sodium chloride in the seawater, estimated to be about 3.0 – 3.1% (Moore et al. 2008); 2) the copper added during the experiment; and, 3) the internal standard (gallium). The highest concentration of copper chloride added to the solution amounts to 0.03%; gallium concentrations are in the ppb range, thus neither copper nor gallium were considered in the TDS dilution calculation. The target TDS for ICP analysis is 0.2 percent.

Test-run of samples on ICP-MS

Prior to running all samples, I will perform a test-run on the ICP-MS with a subset of samples. Below is a schematic of all available samples. Note that there are an additional 11 samples that were taken as part of a filter test. These sample will be analyzed during the full run. Of the 84 primary samples, 18 will be used for the test run (random

selection); these samples are highlighted in yellow below. “A”, “B”, “C”, “D”, and “E” represent 50, 100, 200, 500, and 1,000 ug/L copper respectively, nominal concentrations, prior to dilution for TDS; “F” is the control; “blk” is the blank; “Meth” is the method blank.

Beginning of 48-hour Exposure						**End of 48-hour Exposure**					
A-1	A-2	A-3	A-4	A-5	Meth-Blk A	A-1	A-2	A-3	A-4	A-5	Meth-Blk A
B-1	B-2	B-3	B-4	B-5	Meth-Blk B	B-1	B-2	B-3	B-4	B-5	Meth-Blk B
C-1	C-2	C-3	C-4	C-5	Meth-Blk C	C-1	C-2	C-3	C-4	C-5	Meth-Blk C
D-1	D-2	D-3	D-4	D-5	Meth-Blk D	D-1	D-2	D-3	D-4	D-5	Meth-Blk D
E-1	E-2	E-3	E-4	E-5	Meth-Blk E	E-1	E-2	E-3	E-4	E-5	Meth-Blk E
F-1	F-2	F-3	F-4	F-5	Meth-Blk F	F-1	F-2	F-3	F-4	F-5	Meth-Blk F
Blk1	Blk2	Blk3	Blk4	Blk5	Meth-Blk blk	Blk1	Blk2	Blk3	Blk4	Blk5	Meth-Blk blk

Filter Test (alternative method)				
				A-5
		B-3		
		C-3		
D-1				
E-1		E-3 (x5)		E-5

Data analysis

1. **Endpoint – Germination**. Kelp spores are characterized as “germinated” when germination tube is present. The presence of germ tubes was further characterized as a protuberance at least one spore diameter (Gary A. Chapman, Denton, and Lazorchak 1995). For each replicate, a minimum of 350 individuals were evaluated by this metric from five, randomly selected, visual fields.
   a. ***ImageJ method:***
      i. Create a stack with all images (image names randomly changed)
      ii. Set grid
      iii. Use multi-point tool to identify spores as germinated (category 1) or not germinated (category 2).
      iv. Save image stack
      v. Export excel output (alt+y)
   b. ***Image analysis rules:***
      i. Spores that are partially outside image border
         - Ungerminated spores must be entirely within the image in order to be counted

- Germinated spores may be counted as long as a length of germ tube equal to or greater than the diameter of an ungerminated spore is within the image.

ii. Spore clumps will be treated as a "border" i.e. spores entirely within the clump will not be counted and those that are partially within will be treated as described above.

iii. Spores that are outside the focal plane will not be counted. Spores that are in the "grey area" will be counted.

2. **Endpoint – Germ tube length.** Germination tube length was measured in each germinated spore.
   a. ImageJ method:
      i. Create a stack with all images (image names randomly changed)
      ii. Set scale (still need to obtain microscope image with scale)
      iii. Set grid
      iv. Use segmented tool to measure
      v. Record tube lengths with ROI manager
      vi. Save ROI output.
   b. Image analysis rules:
      i. The germ tube will be measured from the "base" where the germ tube leaves the spore, to the end of the germ tube.
      ii. The segment line must be drawn/segmented in order to stay within the borders of the germ tube. Same line width will be used throughout. Start point is centered on the "base"; endpoint is centered on the tip.
      iii. Additional rules from above still apply

3. **Statistical Analysis.** Logistic regression will be used to create dose response curves for each of the endpoints using R, programming language statistical package. For each endpoint the EC50 (exposure concentration effecting 50 percent of the population) will also be calculated.

Appendix B: Data Table

Bull kelp spores were exposed to nominal copper concentrations of 50, 100, 200, 500, and 1,000 ug copper/L filtered seawater. Each exposure concentration, and control, were replicated five times. Five images were collected (compound microscope at 400x) from each of the five replicates. Two endpoints were then measured from the images: 1) germination of the spores and: 2) length of the embryonic germination tube. To avoid pseudoreplication, the observations were then summarized at the replicate level (Table *3*).

For the germination endpoint, all spores in each image were identified as either germinated or not germinated. The counts in each image were then summed at the replicate level, and the percent germinated was calculated.

For the germ-tube length endpoint, two spores from each image were randomly selected using a 10x10 grid and a random number generator. In this way, 10 germ-tubes were measured at each treatment replicate. The average of these 10 lengths was then calculated at the replicate level.

Table 3. Observations summarized at the level of treatment replicate (experimental unit).

Copper Treatment (Concentration in ppb - replicate ID)	Percent Germinated	Germ-tube Length (mm)
50-a	42.30	0.0131
50-b	46.90	0.0136
50-c	47.05	0.0116
50-d	43.81	0.0132
50-e	42.56	0.0116
100-a	42.28	0.0106
100-b	39.45	0.0100
100-c	43.65	0.0088
100-d	47.48	0.0089
100-e	41.04	0.0106
200-a	36.16	0.0076
200-b	32.68	0.0058
200-c	39.16	0.0081
200-d	38.70	0.0063
200-e	40.44	0.0062
500-a	16.24	0.0038
500-b	7.42	0.0025
500-c	7.19	0.0022
500-d	14.50	0.0044
500-e	8.70	0.0034
1000-a	0.93	0.0011
1000-b	3.36	0.0027
1000-c	4.71	0.0022

1000-d	0.53	0.0014
1000-e	6.52	0.0027
Control-a	40.20	0.0119
Control-b	43.35	0.0128
Control-c	45.69	0.0149
Control-d	46.96	0.0139
Control-e	50.91	0.0129