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Assessing the Use of the California Sea Cucumber (Apostichopus californicus) Within Integrated Multitrophic Aquaculture (IMTA)

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Assessing the Use of the California Sea Cucumber (*Apostichopus californicus*) Within Integrated Multitrophic Aquaculture (IMTA)

By

Casey Pruitt

Accepted in Partial Completion
of the Requirements for the Degree
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Master's Thesis

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Casey Pruitt

May 31, 2022

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A Thesis
Presented to
The Faculty of
Western Washington University

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Of the Requirements for the Degree
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Abstract

There has been recent interest in Washington State, USA in the culture of the California sea cucumber (*Apostichopus californicus*) for both wild-stock enhancement and as an aquaculture resource. The species is a good candidate for Integrated Multi-Trophic Aquaculture (IMTA), where the animals are supported entirely on the excess organic biodeposits from existing floating aquaculture operations (e.g., bivalves or finfish). In IMTA, excess nutrients and organic materials from higher-trophic-level organisms are taken up by lower-trophic-level species, providing both environmental benefits and secondary products for sale. Unfortunately, a reliable longterm tagging and tracking method is not currently known for *A. californicus*, which has negatively impacted management of the wild fishery and complicates IMTA efforts. A tagging method is needed for both entities to delineate wild individuals from cultured. To resolve this problem, I tested four tagging methods on large and small sea cucumbers: 1) a stainless-steel suture through the body wall, 2) a stainless-steel suture through the cloaca, 3) an 8-mm PIT tag inserted into a tentacle, and 4) a papilla clip. Tag retention time, water parameters, and stress indicators were monitored throughout the study. In a second study, controlled mesocosms were used to measure feeding of *A. californicus* on three diets that replicated the waste deposited under floating aquaculture – mussel biodeposits, biodeposits mixed with the algae *Palmeria mollis*, and *P. mollis* alone. I specifically examined whether *A. californicus* processed different quantities of the three waste types, and whether different size sea cucumbers removed different quantities of carbon and nitrogen from the waste. In a third study, *A. californicus* were deployed beneath existing *Mytilus galloprovincialis* aquaculture rafts to assess the effects of *A. californicus* density (2 or 4 individuals m^{-2} (top, bottom, and side cage area)) and location (directly under raft or 250 m away from raft) on survival, growth, and nitrogen and carbon

assimilation. Results indicate that sea cucumbers tagged with a body wall tag exhibited the longest median retention time (87 and 72 days for small and large individuals) with no additional stress than was seen in untagged control sea cucumbers. Feeding experiments revealed that sea cucumbers will more effectively assimilate nutrients from waste types that are higher in organic quality, and that larger sea cucumbers processed more of the *P. mollis* diet than they did the other diets. In the third study, sea cucumbers that were grown at a density of 2 individuals m^{-2} exhibited a higher growth rate than those that were grown at a density of 4 individuals m^{-2} under mussel aquaculture with maximum growth in late summer. Carbon and nitrogen present in cage biodeposits sediments were reduced in late summer when sea cucumber were present in cages. However, density had no effect. By the end of the study, the organic quality of mussel biodeposits had dropped to the point that there was no obvious effect of sea cucumber on total carbon or nitrogen in cage sediments. The three components of this study increase our understanding of A. californicus as potential subjects for multitrophic aquaculture. Specifically, I suggest options to improve tracking of *A. californicus* and inform resource managers of seasonally dependent nutrient assimilation when *A. californicus* are farmed under mussel aquaculture.

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Introduction

The Food and Agriculture Organization of the United Nations calculated an average annual increase in global fish consumption of 3.2% between 1961 and 2016. This figure exceeds both population growth (increasing 1.6% per year) and terrestrial animal consumption (increasing 2.8% per year) in the same period (United Nations 2018). This growth in fish consumption has put exceptional strain on capture fisheries around the globe. In 1974, the fraction of marine fish stocks fished within a biologically sustainable level was estimated at 90%. In 2015, this value had dropped to only 66.9% (United Nations 2018). Aquaculture – the breeding, rearing, and harvest of marine or freshwater fish, shellfish, algae, and other organisms – has grown to supplement this increasing demand for fish protein and has been increasing at an average rate of 7.5% per year since 1970 (United Nations 2020). The contribution of aquaculture to global fish production has risen substantially in the last few decades. From 2016-2018, aquaculture contributed 46% of worldwide fish production – up from 25.7% in 2000, with China, India, Vietnam, Bangladesh, Peru, Russia, United States, Japan, and Norway leading the way (United Nations 2020). In 2018, the worldwide annual production of finfish, mollusks, crustaceans, and other aquatic animals including sea cucumbers, sea urchins, turtles, frogs, and jellyfish net a total annual production of 80 million tons, valued at $243.5 billion (United Nations 2020).

The expansion of aquaculture has not occurred without challenges. Traditional aquaculture requires space for cultivation and equipment. The increasing demand for property on which to build aquaculture facilities has turned space into a limiting factor, and in some developing countries, has led to the destruction of coastal habitat. Bangladesh is one example of this, where commercial shrimp farming requires construction of shallow ponds that can be flooded with

seawater. Flooding these artificially created canals and ponds has led to chronic salinization of land and groundwater, decreasing productivity of nearby agricultural crops and destroying crucial mangrove habitat (Islam and Bhuiyan 2016). Other problems arising from high-density commercial aquaculture include heightened risk of spreading disease to resident species and high organic loading from uneaten food and accumulating waste (Buschmann et. al. 2008). The latter issue can lead to problems in both the water column and in the sediment underneath and surrounding aquaculture facilities. In the water column, excess nitrogen and phosphorus increase the likelihood of eutrophication. In the sediment, accumulated food and waste support high levels of benthic microbes, leading to enhanced respiration and anoxic conditions in the top 1-2 cm. sediment. These conditions decrease biodiversity in the benthos (Buschmann et. al. 2008). Re-mineralized nutrients like nitrogen can exacerbate eutrophication because, in anoxic conditions, NH_4 is released as a bioavailable compound, bypassing aerobic denitrification to N_2 (Buschmann et al. 2008). NH_4 that is resuspended from the benthos under these conditions can lead to additional bacterial activity and hypoxia, whereby gaseous N_2 is released from the system.

With rapid worldwide growth of aquaculture, it is increasingly important to implement new sustainable systems that minimize harm to the environment and other aquatic species. Integrated Multi-Trophic Aquaculture (IMTA) is one such design that could promote more sustainable approaches. IMTA is the culture of tropically compatible species (ones that can be grown together to take advantage of inherent feeding characteristics) in close proximity to one another. In IMTA, a lower-trophic-level species feeds on excess food and excreted waste (particulate matter and inorganic nutrients) generated from the aquaculture of a higher-trophic- level species. This combined culture of different trophic levels is intended to bring the biological and chemical

processes in the system into balance (Chopin 2006). The lower-trophic-level species are supported partially or entirely from waste of the primary species, reducing the organic loading while producing a marketable product. Suspended aquaculture systems like mussel rafts or fish pens can easily be converted into an IMTA system by layering or suspending lower-trophic-level species directly beneath the rafts or pens (Palzat et al. 2008, Hannah et al. 2013). The UN Food and Agriculture Organization (2009) describes IMTA as the logical next step in the evolution of aquaculture as a method whereby "production can be intensified, diversified and yet remain environmentally responsible". Countries currently involved in commercial-scale IMTA include Canada, Chile, China, South Africa, the UK, and Northern Ireland (United Nations 2009).

Deposit feeders have been identified as more effective consumers of organic waste generated from floating aquaculture than are filter feeders (Filgueira et al. 2017). The majority of organic particulates from floating aquaculture falls in a vertical flux and placing deposit feeders directly underneath floating systems in IMTA maximizes the mitigation of organic waste that would otherwise accumulate on the seafloor (Filgueira et al. 2017). Of the species preferred for IMTA, several have been identified as of particular value in seafood markets. Sea cucumbers (Class Holothuroidea) are one such group.

Sea cucumbers are a valued commodity in some markets where they are consumed for their supposed healing and aphrodisiacal properties and are harvested and exported worldwide (Chen 2003). They are in particularly high demand in Asia, where they are considered a delicacy and consumed either dried whole or split and served fresh. Different species of sea cucumber are harvested in Japan, Canada, Indonesia, Russia, and the U.S., with a significant import market in China (Whitefield 2019). Certain species are particularly valued and this, without sufficient regulation of the fishery, has led to overfishing and collapse of some sea cucumber fisheries. For

example, harvesting of the tropical sand-fish sea cucumber (*Holothuria scabra*) bolstered the economy of Indo-Pacific coastal communities for many years, but local populations have declined throughout the Western Indian Ocean (Purcell et al. 2013). This large-scale decline has resulted in a switch from commercial harvest to aquaculture.

Globally, aquaculture now accounts for 80–85% of the sea cucumber market (Whitefield 2019). China is the leader in total sea cucumber production volume. Since 2002, all sea cucumber production in China has come from aquaculture exclusively. In 2014, China produced 200,969 tons of cultured *Apostichopus japonicus*, a shallow temperate-water species (Han et al. 2016). In China, "sea ranching" or bottom culture is the most prevalent method of sea cucumber aquaculture. This method costs less and, without the costs associated with shrimp ponds or suspended pens, produces higher returns (Chen 2003). Sea cucumber ranching involves the release of hatchery-raised juvenile sea cucumbers (larger than 2 mm) onto the seafloor where they mature into adults and are later collected at a harvestable size by divers. Han et al. (2016) note that, because of their potential movement, sea cucumber ranching works best in restricted natural environments such as enclosed bays with a single exit or entrance. With sea ranching, larger numbers of sea cucumbers can be kept at low densities without the added time and expense (e.g., cleaning fouled cages, feeding) that occurs with other methods.

A number of studies looking at growth and aquaculture-sourced waste consumption have found that sea cucumbers readily consume and benefit from waste produced by floating aquaculture. In New Zealand, the deposit-feeding sea cucumber *Australostichopus mollis* has been observed aggregating beneath commercial mussel farms, attracted there by mussel biodeposits (a mix of feces and pseudofeces, which is suspended particles that are sloughed in mucus from the feeding structures of bivalves and resemble feces) that end up on the seafloor (Zamora and Jeffs 2012).

The sediment under these farms can often have a total organic matter (TOM) content twice that of sediments on the adjacent seafloor (Zamora and Jeffs 2012). The ability of *A. mollis* to process biodeposits from floating farms caught the attention of the mussel aquaculture industry in New Zealand, leading to the development of *A. mollis* as a second crop beneath mussel farms (Zamora and Jeffs 2012).

Sea cucumbers beneath finfish farms can also assimilate carbon and nitrogen from fish wastes in IMTA. Hannah et al. (2013) observed that *Apostichopus californicus* beneath an IMTA farm with sablefish (*Anoplopoma fimbria*) off Vancouver Island, British Columbia (BC) assimilated 60.3% of the total organic carbon and 62.3% of the total nitrogen produced by the farm.

Floating aquaculture in British Columbia and Washington State could benefit immensely from IMTA with *A. californicus*. Regionally, Canada is the fourth largest producer of Atlantic salmon in the world, producing 105,608 tons per year at a net profit of $381 million (United Nations 2009). Similarly, in Washington State, commercial production of the Mediterranean mussel (*Mytilus galloprovincialis*) is a profitable and growing industry; approximately 155 acres in Washington's inland marine waterways are currently leased by commercial shellfish companies and dedicated to the cultivation of *M. galloprovincialis* (WADNR 2019). Mussels cultivated in floating aquaculture constitute 7% of the total shellfish production in Washington, generating 3.6 million pounds per year, valued at $7.9 million (Washington Sea Grant 2015).

In Alaska, Canada, and Washington, wild harvest of *A. californicus* has occurred since the early 1970s. The species has found favor in China, likely because it is morphologically similar to *A. japonicus* – both species being large and covered with spike-like papilla. Washington State has seen a rapid decline in wild populations of *A. californicus*, resulting from a period of unregulated

exploitation from 1971 to 1994. Strict quotas were imposed starting in 1994 with the Rafeedie court decision, reaffirming fishing rights to 15 Native American Tribes in Puget Sound and dividing harvest quotas equally between non-native and native commercial harvest. An industry driven license buy-back program in 2000 put further regulation on the commercial harvest of *A. californicus*, reducing the commercial fleet size from 49 to 25 harvest licenses (Carson et al. 2016).

Despite these efforts to more effectively manage the fishery in Washington, recent video surveys and anecdotal reports from tribal and non-tribal sea cucumber fishery divers suggest that *A. californicus* populations have not recovered to sustainable levels in harvest zones (Carson et al. 2016). To protect *A. californicus* populations from further over-exploitation, fisheries in Puget Sound and the San Juan Islands have simply been closed until definitive information shows that populations are at recovered levels (Carson et al. 2016, WDFW 2019).

Management of the *A. californicus* fishery has failed in part because we lack fundamental data on recruitment, growth rates, ages, population sizes, and mortality. These measurements require tracking individual animals over time, and there is no reliable long-term tagging method for these soft-bodied animals. Attempts to tag sea cucumbers using different techniques have revealed that sea cucumbers have a remarkable ability to expel their tags. Plastic t-bar tags, coded wire tags, cinch type tags, physical branding, and fluorochrome dyes are just some of the tagging techniques that have been trialed (Gianasi et. al 2015). For resource managers, tag retention on the order of years would be ideal for gathering basic information about sea cucumber population dynamics (Gianasi et al. 2015, Carson et al. 2016, Fujino et al. 2017). Unfortunately, of the tagging methods trialed, only two have yielded > 50% retention after a year. These are tags with the chemical fluorochrome, where sea cucumbers are dipped with

fluorescent dye (sp. *Holothuria scabra*), and those that are PIT (Passive Integrated Transponder) tagged in the oral tentacle (sp. *Cucamaria frondosa*) (Taylor 2016, Gianasi et. al. 2015). PIT tags inserted by this method have seen high retention rates compared to those that are simply inserted into the animal's body wall. This is because, when inserted in the oral tentacle, PIT tags were observed to migrate down those tentacles to become lodged in the pharangeal bulb (Gianasi et. al. 2015). Plastic and chemical tags have been tested for retention; however, stainless steel remains an untested material for use on sea cucumbers. Stainless steel is utilized as a non-absorbable suture material in medical practice due to its high tensile strength, minimal tissue reactivity, and biologically inert properties (Mercedes Scientific 2019).

In addition to understanding the basic life history parameters, additional information about *A. californicus* cultivation is needed for successful implementation on a commercial scale. Compared to other species of sea cucumbers that have been grown in aquaculture for decades (e.g., *A. japonicus*), *A. californicus* has been little studied. Recently, however, interest in cultivation for population enhancement and IMTA has spurred a number of researchers to examine the potential for successful *A. californicus* aquaculture in North America. Researchers have successfully produced this species in research hatcheries in Washington, Alaska, and BC (Azad et al. 2014, Whitfield 2019). Other studies, dedicated to assessing the growth of *A. californicus* within IMTA systems, have shown that the species grows well when placed under both Pacific oyster (*Crassostrea gigas*) (Paltzat et al. 2008) and sablefish (*A. fimbria*) aquaculture (Hannah et al. 2013). As an added benefit, *A. californicus* assimilates the organic waste of the primary aquaculture species at an efficiency of 50 to 60% (Paltzat et al. 2008, Hannah et al. 2013, Fortune 2013).

Although preliminary work indicates that *A. californicus* could be an effective IMTA species, many additional issues must be addressed before sea cucumber aquaculture is established at a commercial level in North America, particularly with regards to sea ranching, which evidence suggests may be more profitable and effective than caged aquaculture. A number of the challenges are rooted in our lack of knowledge of sea cucumber population biology. As Chinese producers have shown, sea ranching can be a low cost, high-return method for producing sea cucumbers. This, however, will not be possible in Washington State until we are confident we can differentiate cultured sea cucumbers from wild stock and ensure that the genetic lines of wild stocks are not compromised by hatchery-bred animals. This requires a method of tagging and tracking individuals over extended periods of time and additional life history information.

Fencing off areas below floating aquaculture is an option that could facilitate sea cucumber containment for sea ranching. Numerous fencing types and configurations have been tested. However, *A. californicus* is very capable of escaping these. Renewed efforts are being pursued in British Columbia by the North Island College to field test the most promising fence designs (Christopher Pearce, Fisheries and Oceans Canada, pers. comm. September 2019).

There is particular interest from state agencies that manage aquaculture leases in Washington in understanding the effectiveness of A. californicus in reducing organic loading through the assimilation of carbon and nitrogen, and in determining if certain density of sea cucumbers affects assimilation. Adaptive management through the implementation of stewardship measures like IMTA could allow the cultivation of certain species in locations that have been previously overlooked.

To understand the best approaches for sea cucumber IMTA in North America, I pursued the following research questions:

1. Can we effectively tag *A. californicus* for long-term tracking? Treatments included: 1) a veterinary-grade, stainless-steel suture through the body wall, 2) a veterinary-grade, stainless-steel suture through the cloacal opening, 3) an 8-mm PIT tag inserted into the pharyngeal bulb, and 4) a physical mark from clipping papilla from the body wall. Tagging treatments were chosen to build on existing work with inexpensive, successful, and easy to employ non-chemical tag types and to investigate the novel use of stainless steel in sea cucumber tagging.
2. How effectively does *A. californicus* process biodeposits from IMTA aquaculture? I measured this in 3 ways: *A. californicus* growth, the amount of mussel biodeposits consumed, and the total carbon and nitrogen assimilation from mussel biodeposits.
3. How does density of *A. californicus* within cages beneath mussel rafts affect their growth rate and biodeposit assimilation? To address this question, field experiments were run in cages beneath Taylor Shellfish *M. galloprovincialis* rafts in Totten Inlet, Washington, biweekly throughout the summer of 2020.

Materials and Methods

2.1 Sea Cucumber Tagging Study

One hundred and twenty *A. californicus* were collected from two sites in the San Juan Islands in August 2019 and acclimated for two weeks in a flow-through seawater system at the Shannon Point Marine Center in Anacortes, WA. Sea cucumbers were selected for tagging based on measured contracted length and wet weight mass. In North Puget Sound, the commercial

voluntary size limit for market size sea cucumber is 20 cm or greater (commercial mass from 135 – 1000g, average of 410 g) (Mueller 2016). Fifty healthy individuals ≥ 20 cm contracted length were selected for a large (harvestable) class, and fifty individuals < 20 cm contracted length were selected for a small size (non-harvestable) class.

Apostichopus californicus and other sea cucumbers lack hard structures that indicate the animal's age. Therefore, a calculated size index to estimate age and make size-based comparisons has been developed (Hannah et al. 2012, Mueller 2016). Each sea cucumber was measured and assigned a size index (SI) based on the formula developed by Yingst (1982) for the sea cucumber *A. parvimensis*:

$$SI = WL\ (cm) \times WW\ (cm) \times 0.01$$

where *WL* = animal contracted length out of water, *WW* = animal contracted width out of water.

Table 1 is a set of SI-to-year class comparisons developed for *A. californicus* by Fankboner and Cameron (1985), later modified to a scaling factor of .01 by Mueller (2016). While this method of calculating the age of *A. californicus* is imperfect, it can give us valuable insight into estimating the age structure of our sample individuals.

Table 1. Age class to body SI values developed by Fankboner and Cameron (1985) and later modified for the Yingst (1982) equation by Mueller (2016).

Age (year)	Mean SI	Range SI
1	0.10	0 – 0.25
2	0.44	0.25 - 0.75

3	0.89	0.75 – 1.15
4	1.34	1.15 – 1.45
5 +	> 1.34	> 1.45

Tagging treatments included: 1) an 8-mm PIT tag inserted into the pharyngeal bulb, 2) a veterinary-grade, stainless-steel suture through the body wall, 3) a veterinary-grade, stainless-steel suture through the cloacal opening, and 4) a physical marker created by clipping papilla from the body wall.

For the first treatment, sea cucumbers were tagged in the pharyngeal bulb with an 8.4-mm PIT tag. To do so, a small quantity of mussel biodeposits was first placed in the holding tanks to stimulate sea cucumber feeding. Once a sea cucumber had begun to feed, a single oral tentacle was grasped with tweezers, and the cucumber was removed from the holding tank. A PIT tag (previously sterilized in ethanol) was then inserted into the base of the tentacle using a Biomark brand Biotherm pre-loaded syringe and needle.

A second treatment group was tagged with a veterinary grade, stainless-steel suture looped through the body wall and into the animal's coelom. Sutures for this treatment were placed (as best discerned from visible exterior skin tension) around longitudinal muscles running from the posterior to anterior ends of the animal. Both insertion site and dissection tools were sterilized in ethanol and the tools were autoclaved prior to the procedure. The suture was completely looped through the body wall, penetrating the coelom, and was tied off on the outside with an overhand knot. The site of insertion was selected midline along the dorsal body wall halfway between

posterior and anterior ends of the animal. A 5 x 10-mm unique aluminum identifier tag with rounded edges was attached to the outside of this loop.

A third group of cucumbers was tagged with the same stainless-steel suture, but at the dorsal edge of the cloacal opening. The cloacal opening is surrounded by a large sphincter muscle that provides some structure, which makes the region a good candidate for tag retention. Veterinary grade stainless-steel wire was inserted through the side of the cloacal opening and tied off to complete a full loop. A unique aluminum identifier tag was attached to the outside of this loop before joining both ends. Loops for the cloacal opening and for the body wall were similar in diameter.

The fourth group of sea cucumbers was marked by cutting body wall papillae off in a recognizable pattern. Three haphazardly chosen papillae from each cucumber were clipped at their base without penetrating the coelom. Surgical instruments were disinfected with ethanol before use. While this method provides the least specificity, it is fast and easy and may provide an obvious marker. Anecdotal evidence suggests that papilla clipped off at the base from *A. californicus* were replaced with two new papillae (C. Whitefield, University of Alaska Fairbanks, pers. comm., Dec. 2019). However, this has not been documented in the literature. Branding and physical cuts on the surface of tropical species of sea cucumber have been tested as marking techniques but are not effective due to the rapid healing ability of the animals (Shiell 2006).

2.1.1 Water Parameters

Temperature (°C), salinity (ppt), and flow rate (ml/sec) were measured prior to tagging on September 28th, 2019, and at every day of tag retention assessment until the end of the study. A

handheld YSI model P2030 was used to collect temperature, and salinity. Flow rates were collected by measuring the volume of water coming into the sea tables at ten-second intervals with a 1000 ml graduated cylinder.

2.1.2 Tag Retention

Retention of PIT tags over time was non-invasively assessed by external scanning with a Biomark brand PIT tag reader twice per week. Cloaca, body wall, and clipped papillae tags were also assessed for retention visually twice per week. All four marking treatments were monitored for as long as tags remained attached or visible in any animal or until the end of the experiment on June 9th, 2020.

2.1.3 Measuring effects of tagging

Sea cucumbers were tagged on September 29, 2019. Twenty sea cucumbers were tagged (10 small and 10 large) with the body wall, cloaca, and papillae clip treatments. Only ten individuals (5 small and 5 large) were tagged with the PIT tag treatment type due to the difficulty of inserting PIT tags into the feeding tentacle (the sea cucumbers retracted their feeding tentacles when disturbed).

After tagging, sea cucumbers were placed into ten 152 cm x 68 cm x 15 cm sea tables that had each been sectioned into 5 compartments using 1 cm mesh dividers. Four of the five compartments of a sea table each held a large and small sea cucumber of the four tag types. The fifth compartment held two control sea cucumber (a large and small) that had not been tagged.

Sea cucumbers were monitored for indicators of tagging stress. Changes in feeding, respiration rates, the occurrence of skin lesions, irritation around the tagging site, and internal organ evisceration are all stress indicators that were monitored in this experiment.

Sea cucumbers were monitored for feeding during the experiment to ensure that the PIT tag treatment, which was inserted into an oral tentacle, would not impair feeding. Mussel biodeposits used to monitor feeding behavior after tagging were collected in August 2019 from beneath floating mussel aquaculture rafts in Totten Inlet, Washington. Twelve 18.9-liter buckets were hung beneath lines of mature *M. galloprovincialis* mussels where they collected mussel biodeposits that precipitated. After 30 days, buckets were lifted to the surface and excess water was poured off the top. The remaining material was sifted for shell material and other large debris then distributed into freezer bags and frozen at -18º C until they were used in feeding trials.

Feeding activity of the sea cucumbers was measured 1 day prior to tagging, 1 day after tagging, and every 1 to 5 days thereafter for the first 20 days. To initiate feeding, 50 grams of defrosted mussel biodeposits were mixed with 1000 ml of seawater and 100 ml of that solution was distributed into each tank section. The presence or absence of feeding was recorded over the course of 1 hour. Animals that were actively moving particles into their mouths were marked as feeding. Those that were not, were marked as non–feeding. Feeding occurrence was analyzed among the tagging treatments with a generalized linear mixed model with a binomial distribution and random intercepts per cucumber (*lme4* package in the software package R).

Changes in respiration have been used as an indicator of stress in sea cucumbers, with increased respiration related to higher level stress (Gianasi et al. 2015). Sea cucumber respiration was

measured for all tagged individuals 1 day prior to tagging, immediately after tagging, and at 3–5-day intervals thereafter by counting cloacal opening rates in 1-min intervals. In the event an animal's orientation obscured the cloaca, the animal was moved to the bottom of the sea table, left for 30 min, then observed.

Sea cucumbers have the remarkable ability to expel their organs (eviscerate) or to absorb them as part of a natural cycle (aestivation) and remain living. They may do so as part of a normal annual regenerative cycle, but also may do so when disturbed by predators or when under stress (Gianasi et. al. 2015). To test the possibility that tagging induced evisceration, sea cucumbers were monitored twice weekly after tagging until all tags had been shed. Any eviscera in the tank were observed and immediately removed.

2.2 Sea Cucumber Laboratory Feeding Behavior

Small juvenile sea cucumbers were collected from Taylor Shellfish floating mussel aquaculture rafts in Dabob Bay in August of 2018. Larger sea cucumbers were repurposed from the sea cucumber tagging study.

To determine how *A. californicus* size affects the amount of mussel biodeposits they consume, and how effectively they process carbon and nitrogen in those biodeposits, three diets were fed to each sea cucumber in three separate 24-hour trials. These foods, which represented different kinds of diets the sea cucumbers might receive in an aquaculture situation, included 1) mussel biodeposits, 2) the alga Pacific dulse (*Palmaria mollis*), and 3) a 50:50 mixture by mass of mussel biodeposits and *P. mollis*.

Figure 1. Flow thorough sea water system setup for feeding trial experiment with totes (n=24). 18 different sea cucumbers were placed into 18 totes and fed a randomized diet of either mussel biodeposits, dulse, or a 50:50 mix of the two diets. 6 totes were used as controls. After 48 hours, bins and sea cucumbers were rearranged and a new food treatment was fed to the same sea cucumbers. All treatments shared water supplied from the same inlet.

For these trials 24 (100 cm (L) x 60 cm (W) x 30 cm (H)) plastic totes were set up in a filtered flow through seawater system (Figure 1). Constant seawater flow (0.2 l/sec) was supplied to each bin through a small hose and exited through a 2-cm diameter opening near the top at the other

end of the tote. Sea cucumbers were chosen from a range of sizes to also include small post-hatchery individuals that might reasonably be used in IMTA grow-out (9.8 – 509 g wet weight). Prior to the start of the feeding experiments, the sea cucumbers were starved for 1 week to ensure they had empty guts. At the start of all three trials, one sea cucumber was placed in each of eighteen bins. Diet rations were measured out and placed on pieces of tin foil, which were gently placed at the bottom of the bins that held sea cucumber as well as 6 bins without sea cucumbers in them that were used as controls to understand food loss through the outflow in the totes. Sea cucumbers were then allowed to feed for 24 hours. At the end of 24 hours, both sea cucumber feces and uneaten food were collected by siphon.

2.2.1 Water Parameters

Temperature, dissolved oxygen (DO), and flow rate were measured during each feeding trial. A handheld YSI model P2030 was used to measure temperature and DO. Flow rate was determined by measuring the volume of water coming into each tank over a ten-second interval with a 1000 ml graduated cylinder.

2.2.2 Preparation of Diets

Mussel biodeposits were processed after collection from below the commercial mussel rafts by vacuum filtering away excess water using a vacuum pump and a 25-µm pore size VWR glass microfiber filter. The samples were filtered until water stopped dripping from them. The filter paper with the retained material was then wrapped in tin foil and frozen at -18º C until it could be used in the feeding trials.

A second diet was prepared by allowing freshly collected *P. mollis* to age in seawater at room temperature for one week. The decomposing material was then removed from the seawater,

drained, and minced with a blender into a sludge. This dulse sludge was then vacuum filtered and frozen as described above. A mixed diet was prepared by mixing the previously filtered mussel biodeposits and filtered dulse.

To standardize the estimated dry mass of each diet fed to the sea cucumbers and to account for changes in water content of the filtered samples during the time they were held in the freezer, wet-to-dry-weight relationships were established for the filtered mussel biodeposits and the filtered dulse prior to the beginning of every trial. To do this, frozen mussel biodeposits were thawed then dried to a constant weight at 100° C for 24 hours. These samples were then reweighed and the difference in mass between the recently thawed and the dried forms of each was used to calculate their individual water content (Table 2). Wet form of the three diets were fed to the sea cucumbers to retain any nutrients that would be lost in the drying process. The values in Table 2 allowed us to calculate an expected dried mass of the wet form fed in each trial. Because the processed dulse had a higher water content than the mussel biodeposits, it was necessary to mix different amounts of each to create a true 50:50 dry weight ratio of the two for the mixed diet treatment. It was also necessary to feed different amounts of the recently thawed material to the sea cucumbers to achieve equal dry weight equivalents in the dulse and mussel biodeposits. In all cases, enough of the recently thawed dulse or mussel biodeposits was given to equal 5 g of dried material. The mixed diet included enough of the filtered mussel biodeposits and dulse to equal 2.5 g dry weight of each, producing the 50:50 mixed diet. The intent of the mixed diet was to simulate the combination of decomposing *M. galloprovincialis* biodeposits and macroalga detritus that might be found under an IMTA farm.

Table 2. Water content of mussel biodeposits and dulse diets used in three feeding trials (n=30). Standard errors are shown. Differences in water content, the result of changes to the material

while it was held in the freezer, were corrected by adjusting the amounts fed to the sea cucumbers in the three trials.

	Trial A	Trial B	Trial C
Mussel biodeposits	62.7 ± 0.003%	62.9 ± 0.001%	63.6 ± 0.001%
Dulse	88.5 ± 0.001%	88.6 ± 0.001%	87.6 ± 0.001%

Over a period of 2 weeks, every sea cucumber in the experiment was sequentially fed each of the three diets, offered in random order for each of three 24-hour trials. A 48-hour interval between feeding trials was included to allow the sea cucumbers to evacuate their guts. For each trial, enough of a specified diet was added to each of 18 totes containing a single sea cucumber to equate to 5 grams of dried diet material. Totes were randomized so that sea cucumbers were in a different one for every trial. To confirm that conditions were relatively consistent across trials and totes, salinity, oxygen, flow rate, and temperature were measured in every tote at the start of each feeding trial. After 24 hours, food that had not been eaten was easily discerned from sea cucumbers feces, and both this unconsumed food as well as the sea cucumber feces were carefully suctioned from the tote. They were then vacuum filtered onto 25-μm filter paper, dried at 100 °C for 24 hours, and frozen in scintillation vials for later analysis of total carbon (TC) and total nitrogen (TN).

Consumption (C) for each diet was calculated as the difference of the mass of food fed to each sea cucumber subtracted from what was collected after allowing the individual to graze for 24 hours with a correction factor for the average loss from control totes during that trial:

$$C = (Expected\ Food\ Provided\ (g) - Control\ Loss\ (g)) - Food\ Remaining\ (g)$$

For TC and TN analysis, dried samples were thawed, weighed, scraped from the filter paper, and ground into a powder that was processed on a Flash™ 1112 Series Elemental Analyzer at 480 PSI oxygen and 1350 PSI helium to yield TC and TN.

A fecal production rate (FPR) was also calculated as follows where *c* is the dry mass of feces (g) collected at the end of the feeding trial, *mass* is the wet weight of each sea cucumber (g), and *time* is the time of each measurement trial (24 hours).

$$FPR = \frac{c}{(mass * time)}$$

2.3 In-situ *A. californicus* feeding

To study how holding *A. californicus in situ* beneath active mussel rafts affects growth rates and biodeposit assimilation, *A. californicus* (same individuals from experiments 1 and 2) were placed inside plastic enclosures below a commercial mussel raft in Totten Inlet, WA and were monitored for 154 days from mid-summer to late fall. Low- and high-density treatments with 2 or 4 cucumbers/cage were tested to replicate densities of 6 or 12 cucumbers per m² bottom surface area or 2 and 4 sea cucumbers per m² top, bottom and sides surface area. The cages had a 0.31 m² bottom surface area and a total top, bottom, and sides surface area of 1 m². These densities were chosen to compare growth of sea cucumbers at extremely low stocking densities to those that have been tested in other studies. Hannah et al (2016) found that sea cucumber grew

better at a density of 12 individuals m² when compared to 17 m² and 21 m² treatments under sablefish aquaculture. Our aim was to investigate whether this trend found under sablefish would persist at even lower numbers under mussel aquaculture.

An existing mussel raft had mature *M. galloprovincialis* mussels hung from the raft on 6-meter lengths of polypropylene line. The raft remained undisturbed by aquaculture operations during the time sea cucumbers were grown beneath it. At this mussel raft site, approximately 600,000 mussels were growing on 800 hanging lines.

Cages measured 56 cm (L) × 56 cm (W) × 18 cm (H) and included 1 cm^2 opening mesh on all 4 sides, the floor, and the lid. Shallow Tyvek walls and floor were installed inside the cages, with walls rising 8 cm from the bottom of the enclosures to prevent biodeposits from escaping as the cages were hoisted to the surface for processing. Four replicate cages of each density treatment as well as four identical empty cages were hung from the mussel raft with 8 -meter-long lengths of polypropylene line. In this configuration, the cages were 2 meters below the bottom of the vertically hanging mussel lines. Cages were arranged so they were at least four meters away from any other cage and 2 meters within any outside edge of the rectangular raft.

In addition to those under the raft, twelve cages were also placed 250 m away from a raft with no mussels present. These cages included 4 cages of 2 sea cucumbers, 4 cages of 4 sea cucumbers, and 4 empty cages deployed in the same manner. These were hung from lines at the same depth as those at the raft site, but without mussels above them.

Figure 2. Enclosure design used for in situ experiment. Tyvek walls extended 8 cm from the bottom of cages to capture sediments.

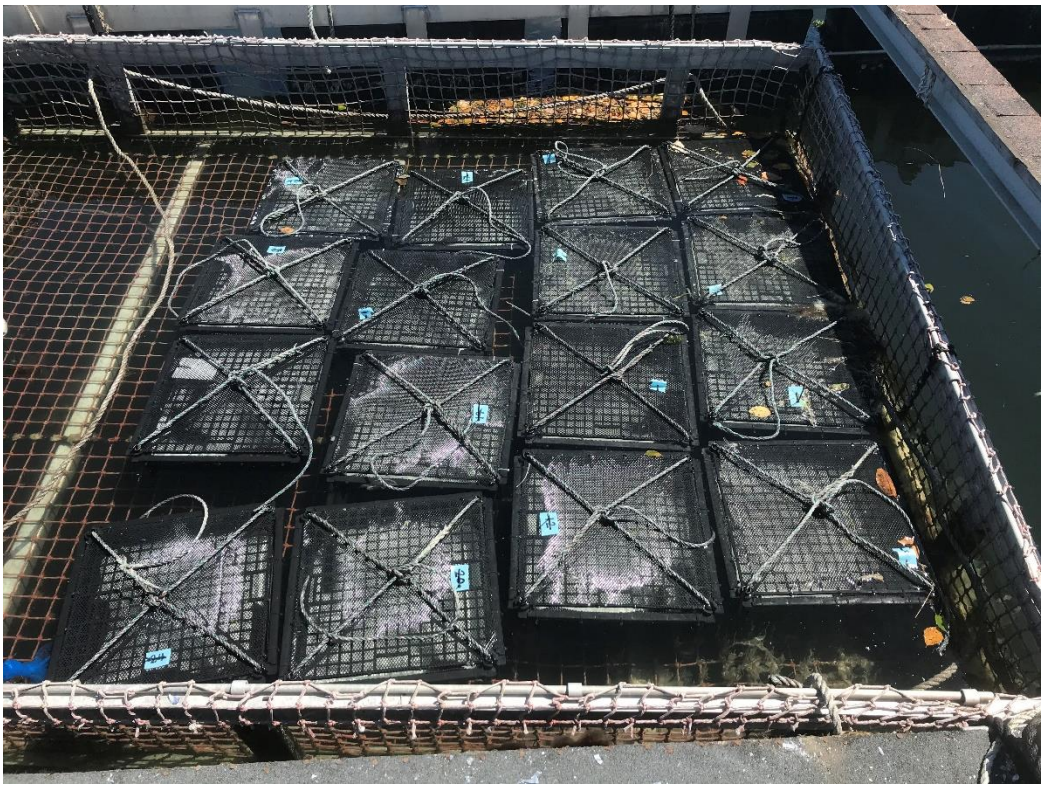

Figure 3. Twelve enclosures ready for deployment underneath the raft site.

2.3.1 Water Parameters

Temperature and dissolved oxygen were continuously logged at both the control and raft sites using ONSET HOBO Dissolved Oxygen Data Loggers (U26-001). Loggers were set to collect measurements every ten minutes. Sensors were placed inside PVC holders and hung from the middle of the raft with polypropylene line and in the middle of the control site deployed at the same depth as the treatment cages (8-meters).

To measure flow velocity at the sites, 16 cm^3 dissolution blocks were attached by zip tie on the side of every treatment cage. The dissolution blocks were created by mixing 1-part DAP brand plastic resin glue with 10.5 parts DAP brand drywall compound powder in 5.1 parts water, filling an ice cube tray with 16 cm^3 blocks, and leaving them to dry at 100° C for 48 hours. The blocks were then glued to wooden bases, weighed, and secured to an outside wall of each cage. After 2 weeks, blocks were recovered, gently rinsed of adhering material, dried for 48 hours at 100° C, and re-weighed. The difference in block mass before and after field deployment was assumed to be dissolved mass lost. This loss is a relative measure of the water velocity or flow influencing the cages at either site.

2.3.2 Sea Cucumber Growth

Sea cucumber morphology for all sea cucumbers was measured on the day of outplant, then every two weeks afterwards for a total of 15 weeks. To collect measurements, cages were carefully raised one at a time to the surface where their lids were removed and sea cucumbers (if there were any in that cage) were taken out. The sea cucumbers were identified via photo id, and measured for total wet weight (g), contracted length (cm), and contracted width (cm) following methods outlined by Yingst (1982).

2.3.3 Collection of Accumulated Sediments

Enclosure sediment (a mix of sea cucumber feces and mussel biodeposits) was collected and weighed starting 2 weeks after initial deployment. To collect the sediment that had accumulated in the cages, the recovered cages were slowly decanted by pouring excess water off the top, leaving settled biodeposits. Sediment was then poured from the bottom of the cage into a tared container and weighed with an electronic field scale. After weighing, samples of sediment from each cage were collected for later determination of 1) a wet weight to dry weight relationship, 2) total carbon (TC), total nitrogen (TN), and total organic matter (TOM).

Cages were then cleaned and the sides scrubbed of all fouling organisms. Sea cucumbers were returned to cages and each cage was attached to one of the 12 haphazardly chosen ropes from its treatment site (raft or control) to be lowered into position. This process rearranged the cage positions every 2 weeks, which was an attempt to homogenize effects of position within the treatments.

2.3.4 Wet-to-Dry Sediment Analysis

Samples collected to determine a wet-to-dry-weight relationship were first mixed to suspend any settled sediments into a slurry. 50 ml of this solution was then poured into a tared graduated cylinder, which was then weighed. After weighing, the slurry was poured into a funnel with a pre-weighed 25-μm pore size VWR glass microfiber filter on the bottom which was placed on a large Erlenmeyer flask attached via hose to a GE motors vacuum pump. The slurry was vacuumed onto the filters until water ceased to drip into the flask. Filters were removed from the funnel, placed on aluminum foil, and dried for 24 hours at 100° C in a drying oven. After 24 hours the filters were removed from the drying oven and immediately weighed.

2.3.5 Total Carbon, Total Nitrogen, and Total Organic Matter

To determine TC, TN, and TOM of dried biodeposits, material was scraped from the filters, ground into a fine powder, and placed into individually labeled scintillation vials. These samples were then processed on a Flash™ 1112 Series Elemental Analyzer at 480 PSI oxygen and 1350 PSI helium for percent TC and TN.

The TOM of samples was established in a loss on ignition (LOI) analysis with a Thermo Fischer Thermolyne FB1315M muffle furnace. Crucibles for LOI were cleaned with DI water and pre-combusted at 1000° C for two hours to sterilize them. Different quantities of sample were available after filtering, and varying amounts of dried sample (mean = 2.24 ± .009 g SE) were weighed out into sterilized crucibles that were combusted for 4 hours at 550° C. Percent TOM was calculated by subtracting the dry weight of samples after combustion (DW_{550}) from their pre-combusted mass (DW_{100}), and dividing by DW_{100}.

$$LOI = \left(\frac{(DW_{100} - DW_{550})}{DW_{100}}\right) * 100$$

2.4 Data Analysis

Due to repeated sampling of the same individuals and the lack of independence in resulting measurements, generalized linear mixed models (GLMMs) were fit to data from all three experiments and used as the primary method of analysis. The general form of these models was:

$$Y_i \sim \beta_0 + \beta_1 x_1 + \ldots \beta_n x_n + \varepsilon$$

Where Yi is the dependent variable, and β terms are fixed predictor variables (interactions included), and the variance within predictors for the error term (ε) was assumed to be equal across levels.

We used the *nlme* package to predict our measurement of interest and included a random variable for slope, intercept, or both based on the best model. All possible combinations of random terms were tested to arrive at the best fit model. These could include sea cucumber, enclosure, trial, or size index based on the experiment. All models were fit using restricted maximum likelihood (REML) in the *nlme* package in R. All fixed predictors were included in the first iteration of model fitting, and they were paired down to the best fit model by visually assessing residual plots and comparing Akaike's Information Criterion (AIC) values for competing models. A $\Delta\,\mathrm{AIC} > 2$ points was chosen to indicate a better model. If model AICs were equal, the simpler model was selected. Before models were run, collinearity was assessed using pairwise plots. Only 1 of 2 fixed predictors that were highly correlated ($r > 0.6$) was included, saving the predictor with the better explanatory power. For some models, I addressed patterning in the residuals by adjusting the variance structure of predictor variables with the *varIdent* function in the *nlme* package. Coefficients for models resulting from binomial (e.g., feeding observations) and Poisson (e.g., days of tag retention) families were exponentiated to display interpretable ratios. Significance of $p < 0.05$ was used to determine whether interaction terms were retained in the final models.

In a second analysis of the tagging study, survival curves were fit for tag retention across all treatments. Kaplan Meier log-rank estimates were fit using R *survival* and *survminer* packages, with days retained being tested as a function of the tagging treatment. Retention probability *S* at time t_i was calculated with the equation

$$S(t_i) = S(t_{i-1})\left(1 - \frac{d_i}{n_i}\right)$$

where $S(t_{i-1})$ is the probability of retaining a tag at t_{i-1}, n_i is the number of sea cucumbers retaining a tag just before t_i, and d_i is the number of tags dropped at t_i.

Two-way ANOVAs were also used to confirm that sea cucumber size was not different between treatments at the onset of the tagging experiment. Normality and homogeneousness of variance in the treatments was determined by plotting the data and looking for patterns.

Results

3.1 Sea Cucumber Tagging Study

3.1.1 Water Temperature, Salinity, and Flow Rate

Water temperature decreased in the sea tables over the course of the tagging experiment with an overall mean high temperature of 11.6 °C on September 28 and an average low of 9.1 °C on November 26 (Figure 4A). The mean temperature over this period was 10.7 ± 0.6 °C (SE).

Due to differences in sediment accumulation in different seawater supply lines, flow rate varied between sea tables and across time. Average flow was 156.8 ± 4.4 ml/sec , and never dropped below 102.1 ± 6.1 ml/sec throughout the study (Figure 4B). Average sea table water turnover time was 16.5 minutes. Salinity (Figure 4C) remained relatively stable, averaging 30.0 ± 0.03 (SE) parts per thousand (ppt) over the course of the experiment. Sea table water measurements relative to each other were relatively consistent, with similar variance over the study.

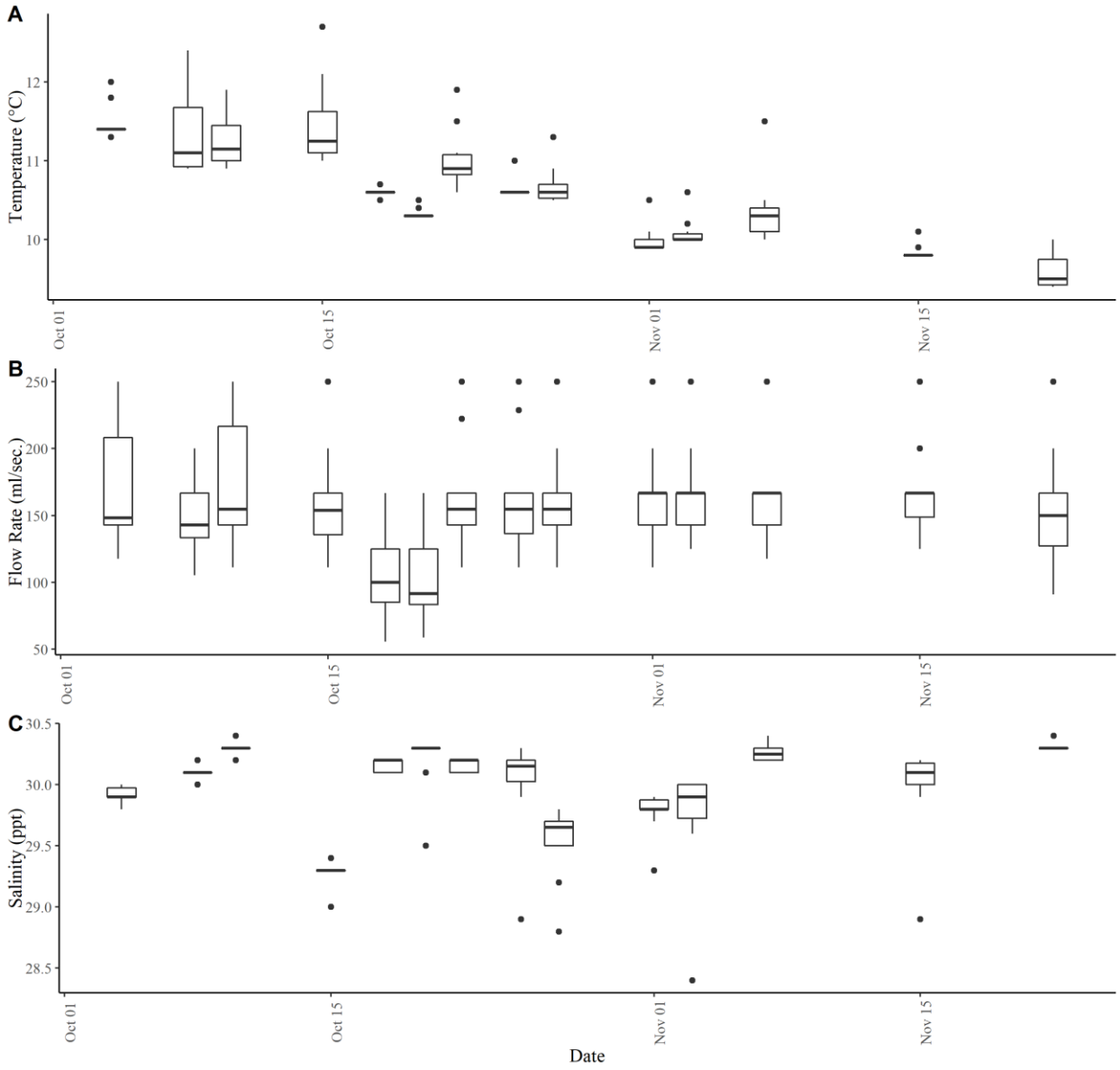

Figure 4. Seawater temperature (A), flow rate (B), and salinity (C) in tanks (n = 10) used for A. californicus *tagging trials from 4 October to 22 November 2019.*

3.1.2 Tagged Sea Cucumber Sizes

Sea cucumber used for this study averaged 20.98 cm ± 0.44 (SE) contracted length, and 423.3 g ± 23.8 (SE) wet weight (Figure 5). A 2-way ANOVA showed that size index was not significantly different among the tagging treatments, indicating a good distribution of sizes across these groups.

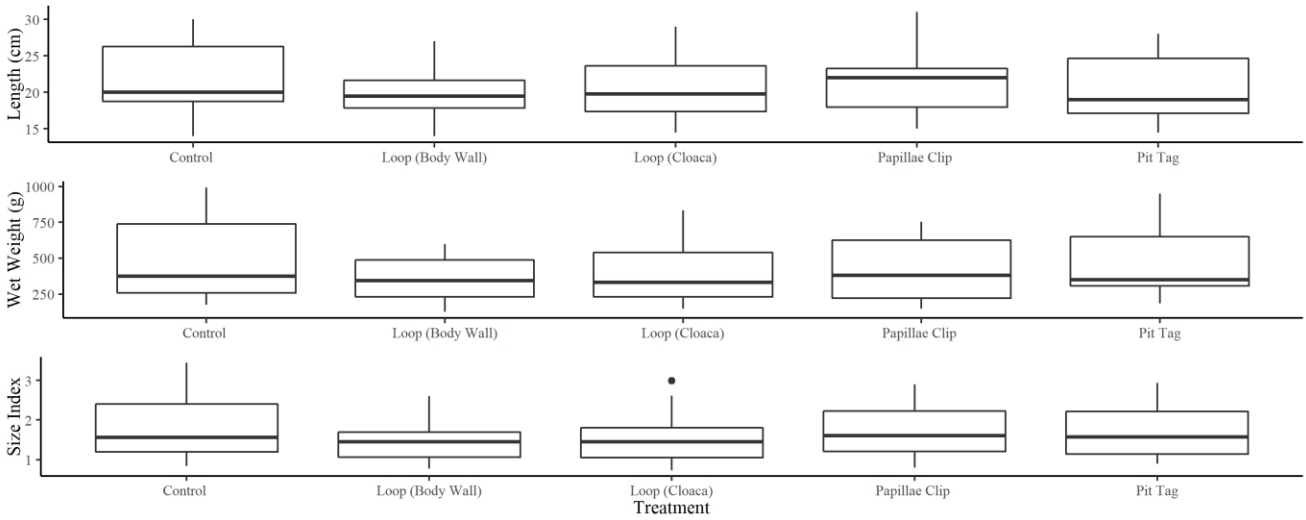

Figure 5. Sea cucumbers length, wet weight, and size index measured the day before tagging.

Table 3. ANOVA results for sea cucumber size index as a function of tagging treatment measured the day before tagging.

	DF	SS	MS	f value	p
Treatment	4	1.45	0.36	0.89	0.47
Residuals	85	34.37	0.40		

All individuals in the four tagging treatments healed quickly after tagging, and no sea cucumber died over the five-month observation period. While we saw no mortality, we did often see a film develop on the flesh surrounding the penetration site on some body wall and cloaca tags (Figure 6). This film did not spread to other portions of the sea cucumber body and for most individuals eventually disappeared. All physical tags applied in this study were eventually dropped by the sea cucumbers, and the papilla clips became indiscernible as the cucumbers quickly healed (Figure 7).

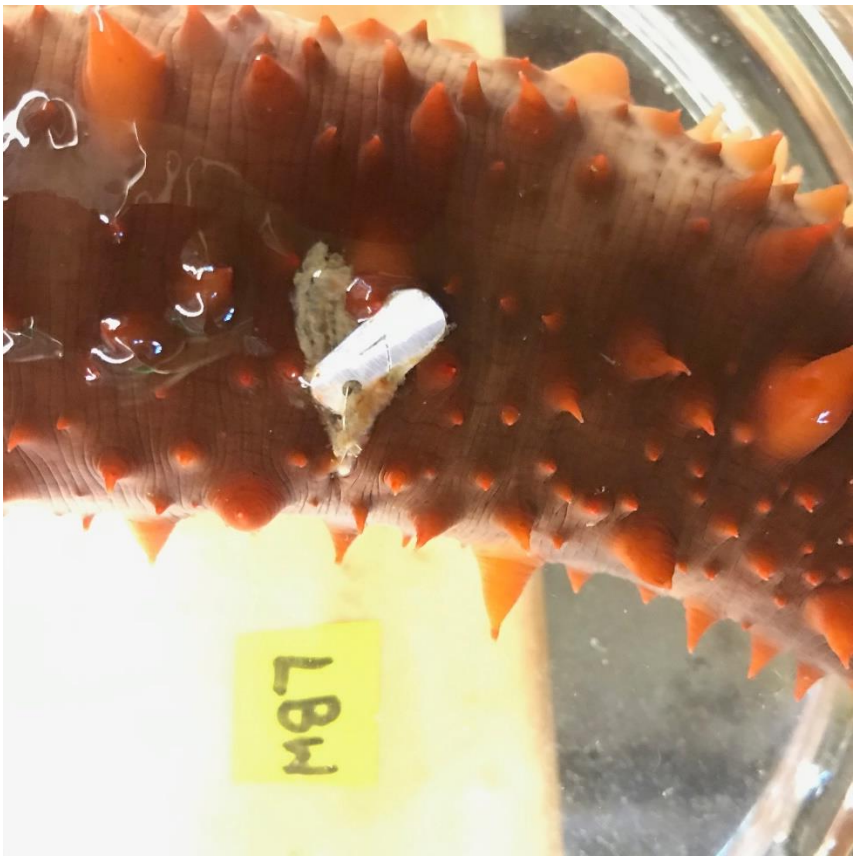

Figure 6. Grey film around the insertion site on a body wall tag.

Figure 7. Image at left shows a wound left after a body wall tag has been dropped. Image at right shows the progression of healing of the same wound.

Table 4. Best fit GLMMs for tagging experiment. The notation "1" signifies random intercepts for that term.

Best Models	Random Term	Conditional r^2
Days retained ~ size index * treatment	1|Table	0.50
Evisceration ~ treatment + days since tagged	1|Table	0.05

Respiration ~ treatment + days since tagged	1|Cucumber	0.30
Feeding ~ treatment + days since tagged	1|Cucumber	0.14

3.1.3 Stress Responses

Respiration

Sea cucumbers showed little change in respiration rate immediately after being tagged, except perhaps for those that were tagged through the body wall (Figure 8). Ten days after tagging, respiration rates were very low in the cloaca and PIT tag treatments. However, they were equally low in the untagged control individuals. Overall, respiration was variable by day across treatments (including the control group), with an absence of trend by treatment. A mixed model using a Poisson distribution with a log link identified the number of days after tagging as a significant predictor for cloacal openings per minute with a significant, though slight, decline in respiration rate over time (Table 5). This was probably driven by extremely low respiration rates of the control and PIT tagged sea cucumbers on the final day of sampling. The analysis showed no significant difference between any of the tagging treatments and the untagged controls (Table 5).

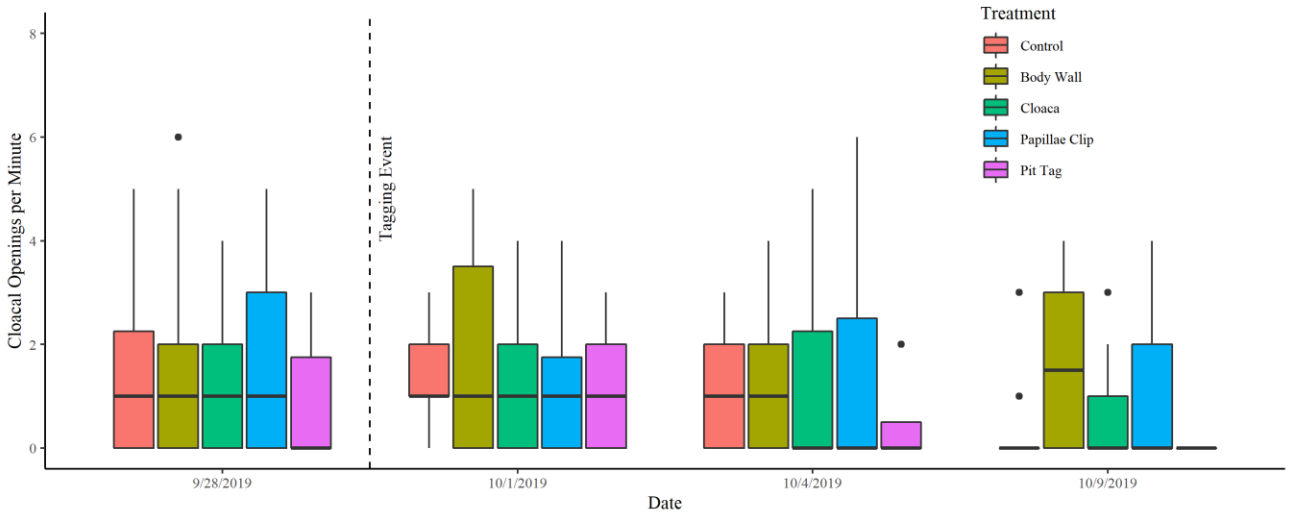

Figure 8. Respiration per minute for sea cucumber in tagging experiment. Size index was not a significant predictor of cloacal openings.

Table 5. Generalized Mixed Model results with log-link function for cloacal openings per minute. Random intercepts were included for each sea cucumber. Exponentiated coefficients are shown.

	Coefficient	z value	p
Intercept (Control Group on 9/28/2019)	2.31	8.20	< 0.01
Body Wall	1.23	1.71	0.09
Cloaca	0.99	-0.06	0.96
Papillae Clip	1.07	0.53	0.60
PIT Tag	0.78	-1.38	0.17

Number of Days	0.97	-2.73	0.01

Evisceration

Two sea cucumbers from different enclosures eviscerated prior to tagging on September 28, indicating that seasonal eviscerations may have already begun. There was an increase in eviscerations on 7 November 2021 (40 days after tagging), with the greatest increase in eviscerations from sea cucumber with a body wall tag, but there was no other obvious pattern nor significance in the GLMM (Figure 9, Table 6). Mixed model analysis with a Poisson distribution confirmed the absence of pattern. There was no obvious effect of time since marking and none of the tagging treatments appeared different from the untagged controls (Table 6).

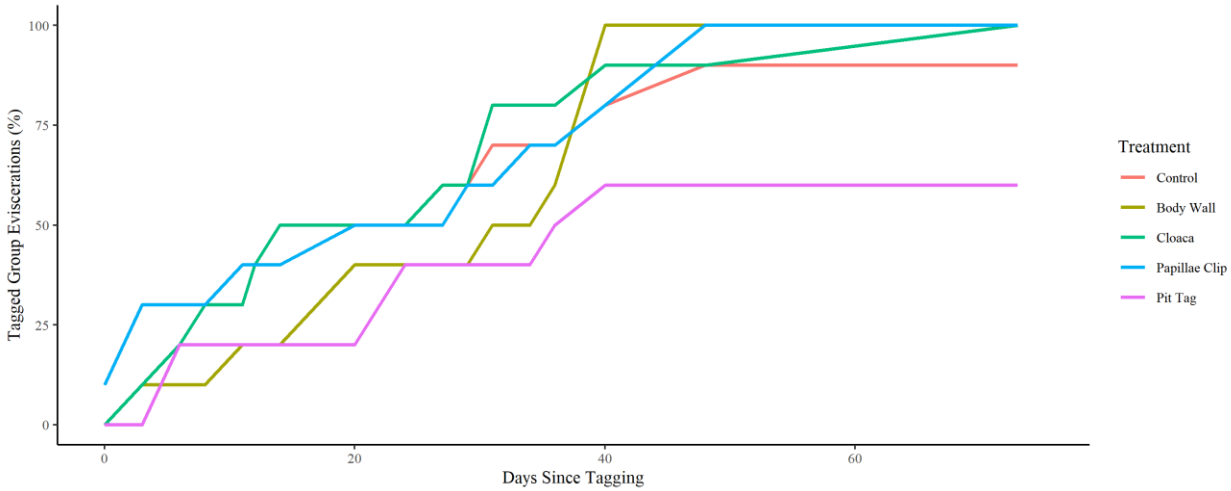

Figure 9. Cumulative percent of A. californicus *that eviscerated in each treatment in 75 days of observance. At the end of this period. 18 eviscerations (90%) had occurred among the control individuals, 20 (100%) in the body wall group, 20 (100%) in the cloaca group, 20 (100%) in the papillae group, and 6 (60%) in the PIT tag group.*

Table 6. Binomial Generalized Linear Mixed Model for sea cucumber evisceration. Random slopes for sea cucumber were included in the model. Exponentiated coefficients are displayed.

	Coefficient	z value	p
Intercept (Controls on 28th of September)	0.06	0.29	< 0.001
Treatment Body Wall	1.24	0.33	0.51
Treatment Cloaca	1.12	0.33	0.74
Treatment Papillae Clip	1.24	0.33	0.52
Treatment PIT Tag	0.61	0.48	0.31
Days Since Tagging	1.00	0.005	0.39

Feeding

Whether sea cucumbers were feeding or not appeared to be consistent across days except on day 11, nine days after tagging, when all treatment types except the papillae clip showed a drop in feeding, which was likely due to some environmental effect. Otherwise, there was no obvious difference in the number of sea cucumbers feeding in the tagging treatments. A binomial family GLMM (feeding vs. not feeding) failed to show significance in any factor, indicating that 1) feeding did not change significantly over time and 2) that none of the tagging treatments significantly changed feeding behavior relative to the control (

Table 7).

Table 7. GLMM results for feeding. The dependent variable was binomial (feeding or not feeding). Random intercepts for sea cucumber were included in the model. Exponentiated coefficients are included.

	Coefficient	z value	p
Intercept (Control Group, 9/27/2019)	1.03	0.09	0.93
Days Since 9/27/2019	0.98	-1.37	0.17
Body Wall	1.53	1.09	0.27
Cloaca	1.27	0.62	0.54
Papillae Clip	1.42	0.89	0.38
PIT Tag	1.53	0.99	0.33

3.1.4 Tag Retention

Tag retention was best for sea cucumbers with the body wall tag type. Kaplan-Meier survival curves showed average tag retention time at 87 and 72 days respectively for small and large sea cucumbers in that treatment (Figure 10). The second longest tag retention was observed for the cloaca tags and papilla clips (Figure 11), which exhibited a similar retention to one another. Papillae that were clipped slowly grew back into normal conical shapes. We did not observe two papillae replace any of the clipped ones as was reported in another study (C. Whitefield, University of Alaska Fairbanks, pers. comm., Dec. 2019). There was no significant difference in tag retention time between small and large sea cucumbers and no significant interaction between tag retention time and sea cucumber size (Table 8).

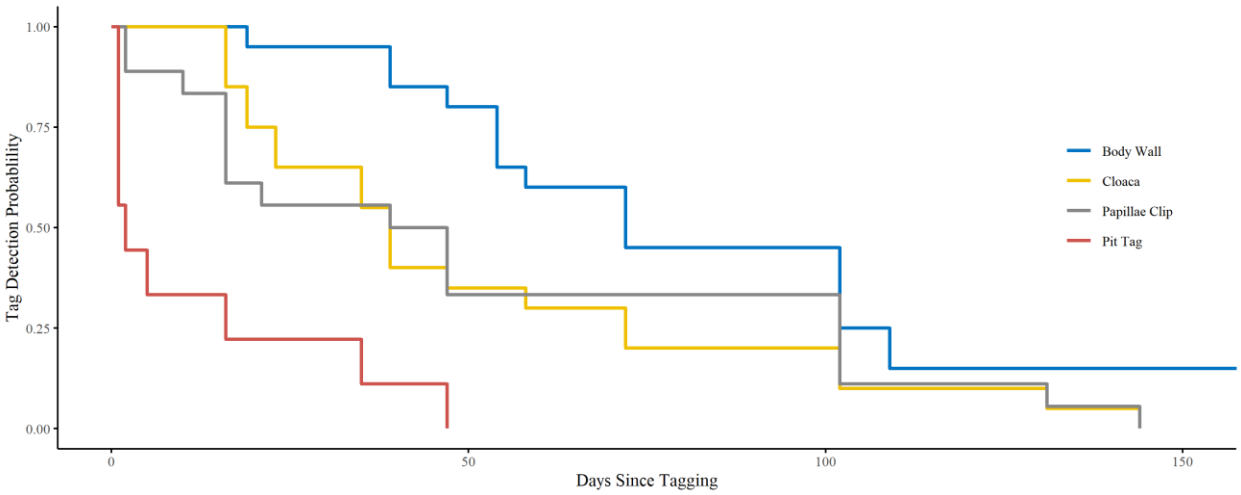

Figure 10. Tag retention curves for A. californicus *across all tagging treatments and sizes.*

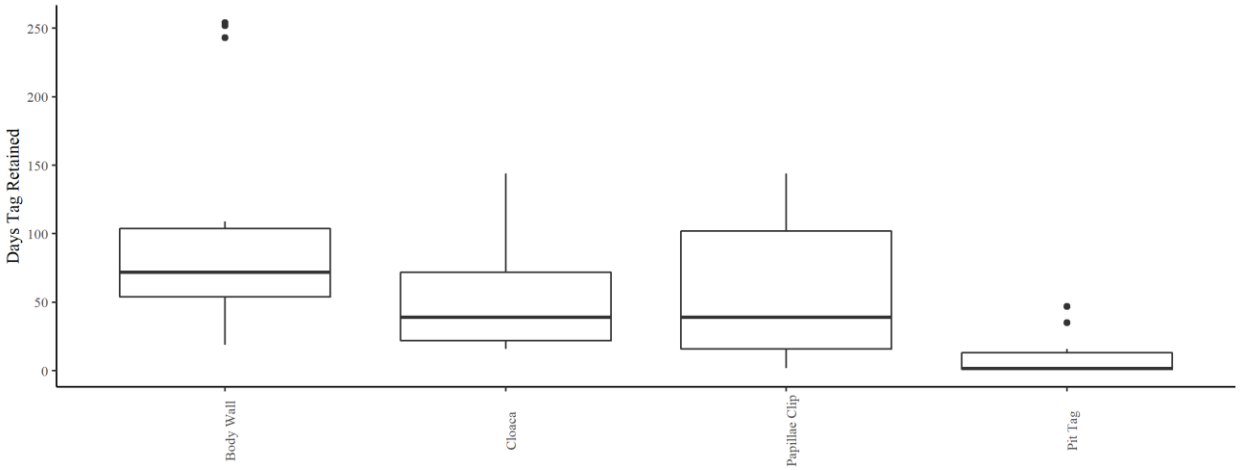

Figure 11. Median number of days tags were retained by treatment across all sea cucumber sizes.

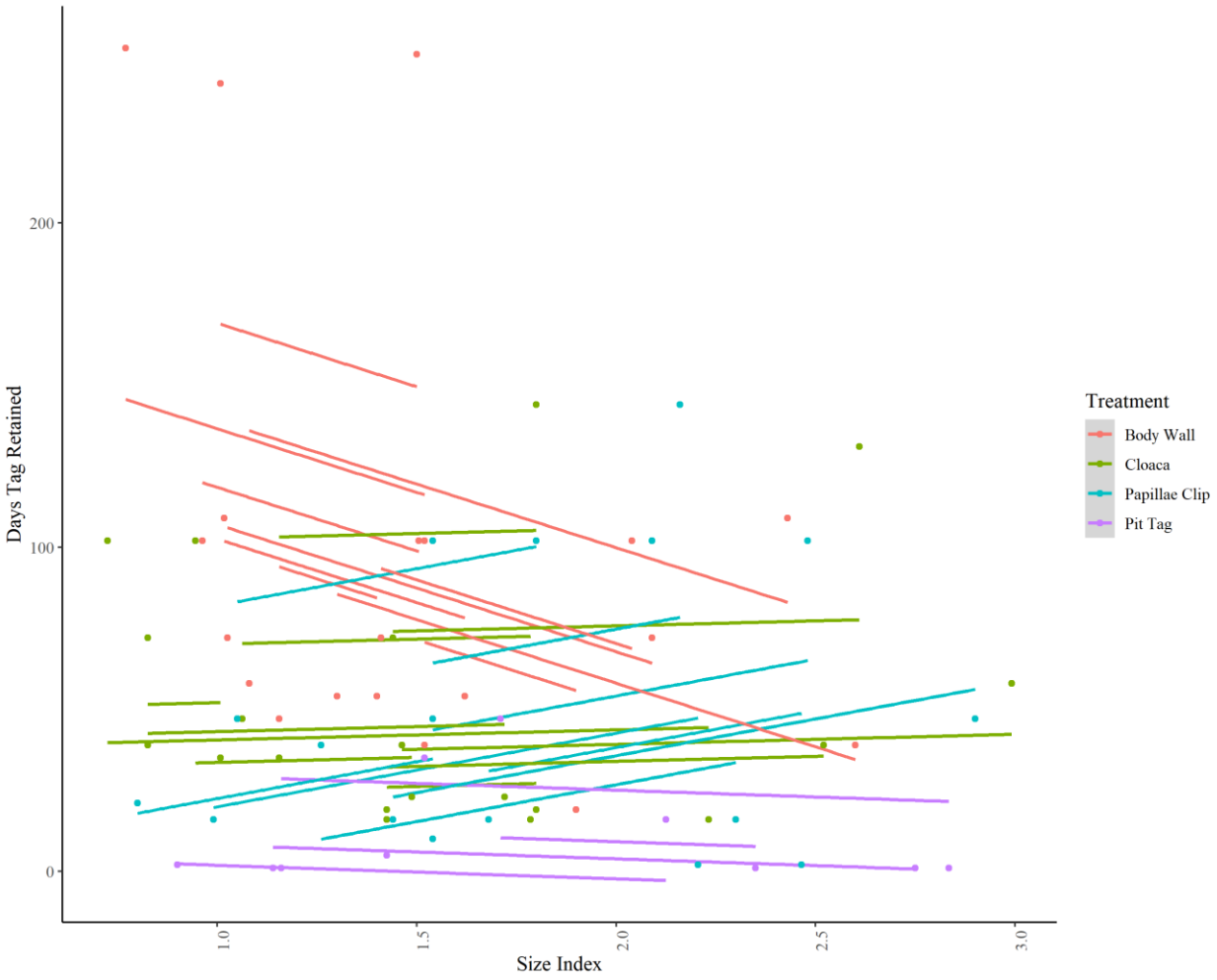

Figure 12. Number of days tags were retained plotted against sea cucumbers size index. GLMM model fits by treatment and random intercepts per sea table are shown. r^2=0.50

Table 8. GLMM for tag retention (number of days) as predicted by size index and tag treatment with random intercepts for sea table.

	Coefficient	DF	t value	p
Intercept (Body Wall Treatment)	156.36	50	4.84	<0.0001
Cloaca Treatment	-108.82	50	-2.72	<0.01
Papillae Treatment	-149.02	50	-3.20	0.00

PIT Tag Treatment	-124.35	50	-2.47	0.02
Size Index	-39.27	50	-1.98	0.05
Size Index : Cloaca Treatment	42.35	50	1.70	0.09
Size Index : Papillae Treatment	61.96	50	2.26	0.03
Size Index : PIT Tag Treatment	35.07	50	1.22	0.23

3.2 Sea Cucumber Laboratory Feeding

3.2.1 Water Parameters

From 25 June to 7 July, water temperature averaged 12.9°C in the laboratory feeding tanks, with a peak on 3 July when 70% of tanks measured over 14°C. There was up to 0.6 degrees of temperature variability among the tanks on each date and across time, probably as a result of differential flow due to build-up of sediment in the separate seawater supply lines. Flow rate averaged 148 ml/sec over the entire study period with higher rates in the later parts of the study. Dissolved oxygen decreased from a maximum average of 7.7 mg/l on 25 June to a minimum of 6.4 mg/l in early July. While there were fluctuations in temperature, flow, and dissolved oxygen, tank parameters were similar to one another.

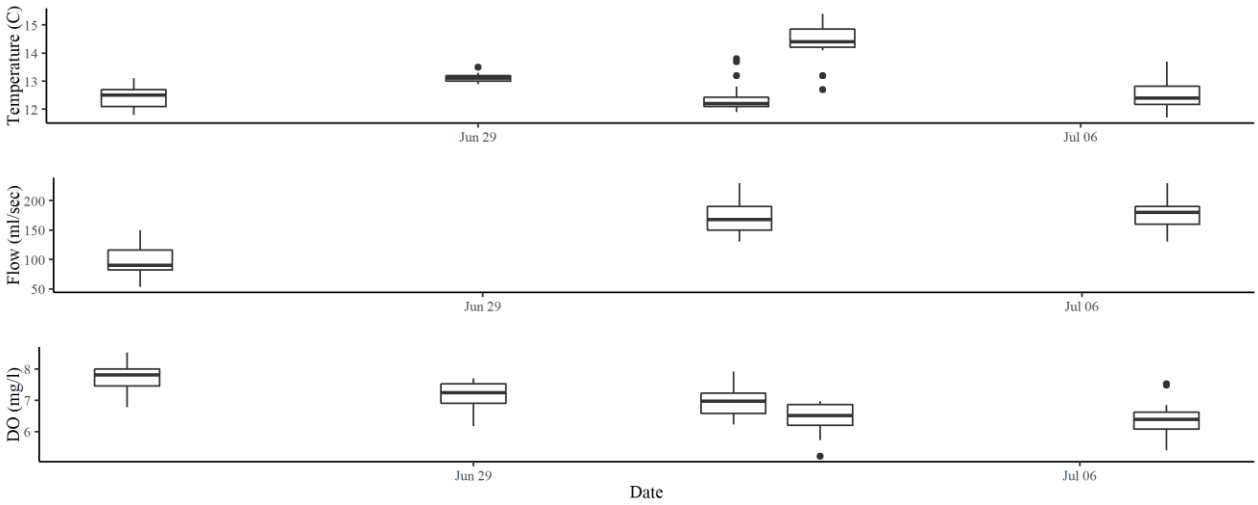

Figure 13. Temperature, flow rate, and dissolved oxygen values for all tanks over the course of the 11-day feeding experiment.

3.2.2 Sea Cucumber Sizes

Eighteen sea cucumber were used in feeding trials (mean = 200 g) with the smallest individual weighing only 9.8 g and the largest 509 g. The following quadratic model was created for their length-to-mass relationship (Figure 14).

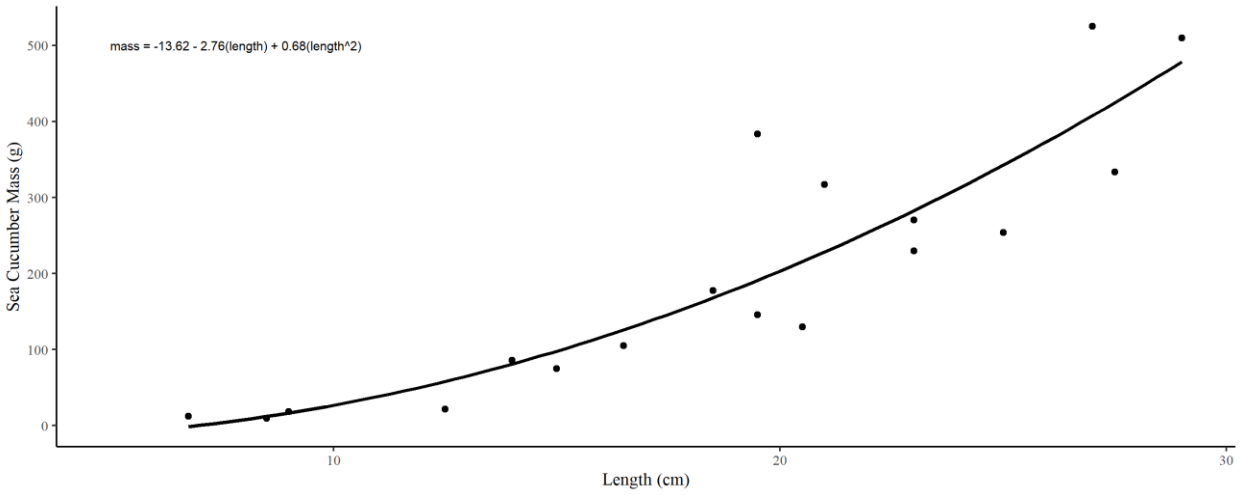

Figure 14. Polynomial length-to-mass relationship of the sea cucumbers used in the feeding study

Table 9. Best fit GLMMs for feeding experiment. The "1+Trial|Cucumber" indicates a random intercept for each cucumber and a random slope for each cucumber across the three trials.

Best Models	Random Term(s)	r^2
Diet Consumed (g) ~ diet * size index	1+Trial|Cucumber	0.95
% N ~ diet * form + diet * size index	1|Trial	0.74
% C ~ form *diet	1|Trial	0.71
Fecal Production Rate (g) ~ diet * size index	1|Cucumber	0.72

3.2.3 Food Consumption

Surprisingly, 54 feeding data points showed negative consumption values, and these instances all occurred for the dulse diet type. An explanation for these negative values is that sea cucumber fecal pellets had broken up and were indistinguishable from uneaten food. When the uneaten portion of food was siphoned and collected, dulse flakes from fecal pellets had potentially broken apart and were mistakenly included in the weight for uneaten dulse. If the sea cucumbers that this happened to had not fed much, the additional dry mass added to the uneaten portion would work out as more than what was fed to them in the first place. These points were removed from the consumption analysis and subsequent feeding analyses from this experiment.

Although Figure 16 suggests a negative relationship between sea cucumber size and consumption of the mussel biodeposits, the mixed model analysis (Table 10) indicates that the slope was not significantly different from 0. This suggests that, when fed mussel biodeposits, sea cucumber consumption was the same regardless of size. The slope for the mixed diet appears different from mussel biodeposits in Figure 16, but the non-significant mixed diet:size index interaction indicates that the slope of this relationship was no different from mussel biodeposits. A relationship between sea cucumber size and consumption of the dulse diet was significantly different from the mussel biodeposit and size relationship with a positive slope. Figure 16 shows that small sea cucumbers ate significantly less dulse than the other two diets, but that large sea cucumbers ate significantly more dulse than they did the mussel biodeposits diet.

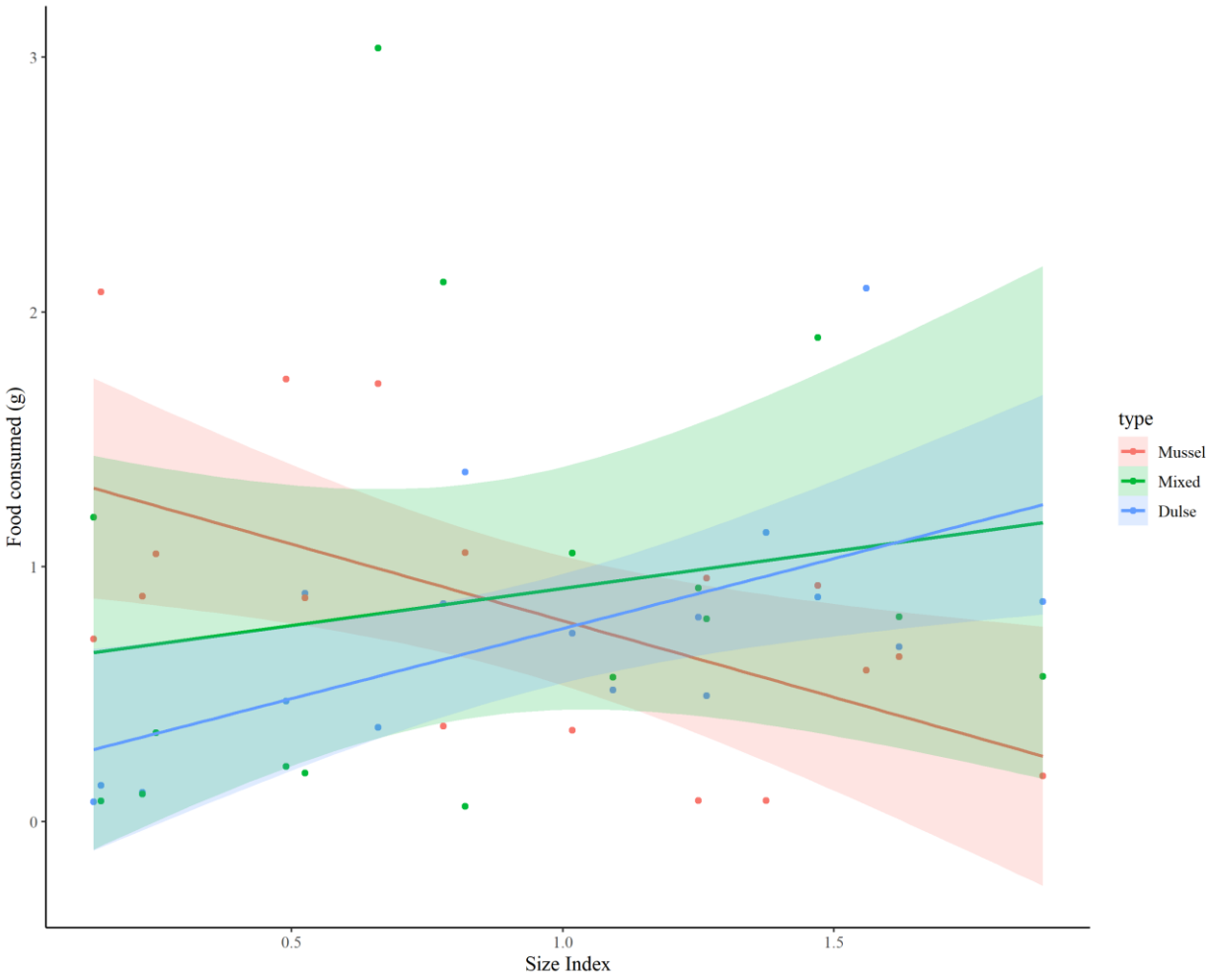

Figure 15. Food consumed in 24 hours by size index. Average regression are shown by diet and 95% confidence intervals are shown.

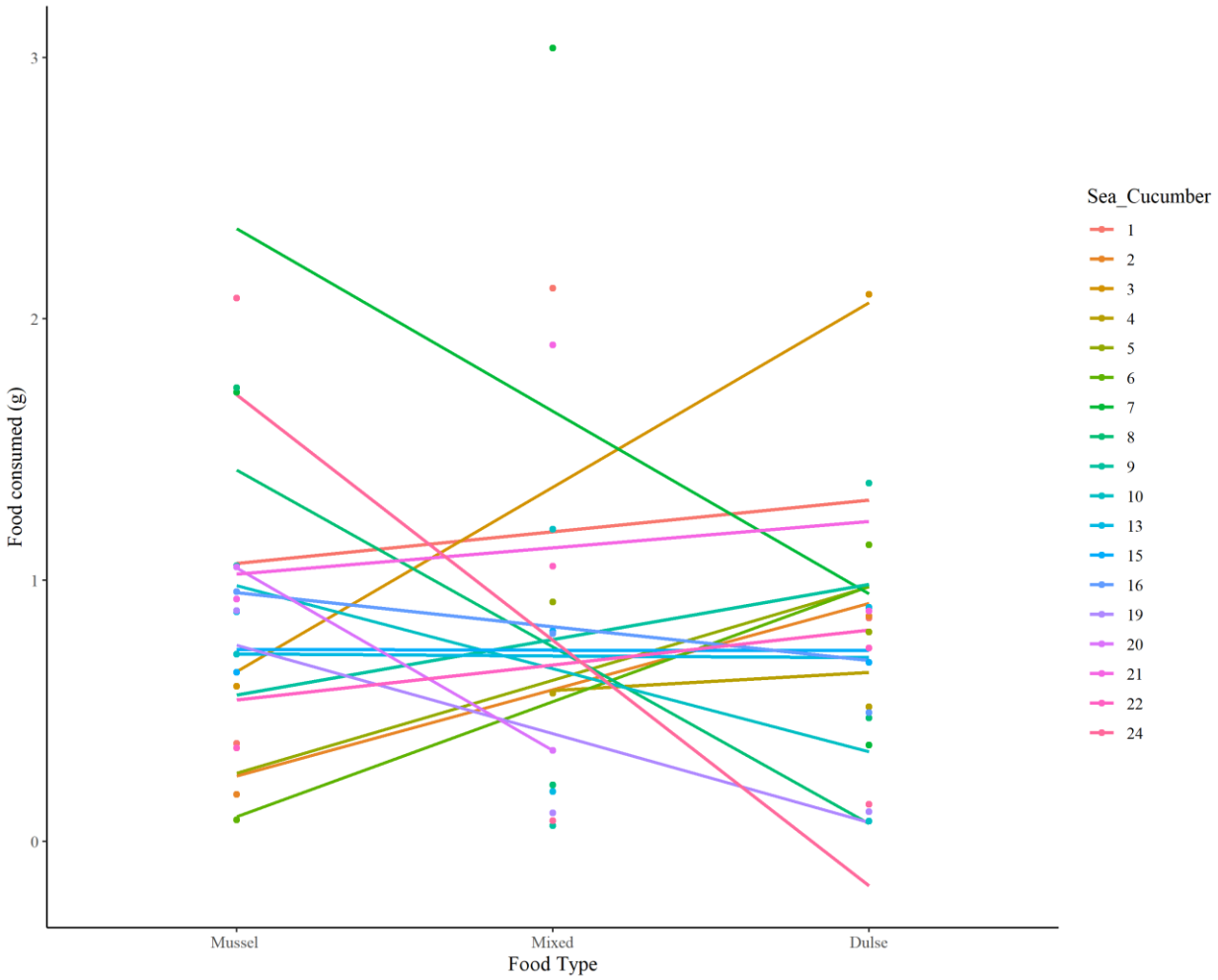

Figure 16. Mass (g) consumed in 24 hours by sea cucumbers as a function of diet. GLMM model lines (random intercepts for sea cucumber and slopes by trial) are shown for the three food treatments.

Table 10. GLMM model results for food consumed and the effects of diet type, sea cucumber size index, and the interaction of those two factors. Random intercepts per cucumber and slopes per trial were added to the model. SI=Size Index

	Coefficient	DF	t value	p

Intercept (Mussel Diet, SI 0)	1.01	28	4.41	< 0.001
Mixed Diet	-0.69	28	-2.34	< 0.05
Dulse Diet	-0.90	28	-3.39	< 0.01
Size Index	-0.17	16	-0.96	0.35
Mixed Diet:Size Index	0.33	28	1.36	0.18
Dulse Diet:Size Index	0.71	28	3.13	< 0.01

3.2.4 Percent Carbon and Nitrogen

The dulse diet material had a significantly higher percentage of carbon in it than did mussel biodeposits or mixed diet material to begin with (Figure 17, Table 11), and the dulse was the only material that dropped significantly in carbon content as a result of passage through the sea cucumber gut. Size of the sea cucumbers was not retained in the final statistical model, indicating that body size did not predict percent carbon assimilated.

These results were essentially the same for the nitrogen assimilation with only the dulse diet showing a drop in nitrogen as the material passed through the sea cucumber gut. However, size of the sea cucumbers did impact the assimilation (Figure 13, Table 12). The interaction of size index and the dulse food type showed a significantly negative slope compared to mussel biodeposits, which had a slope that was not significantly different from 0. Larger sea cucumbers

used the nitrogen in dulse more effectively than did small sea cucumbers, but size did not significantly affect efficiency of nitrogen use in the other two diets.

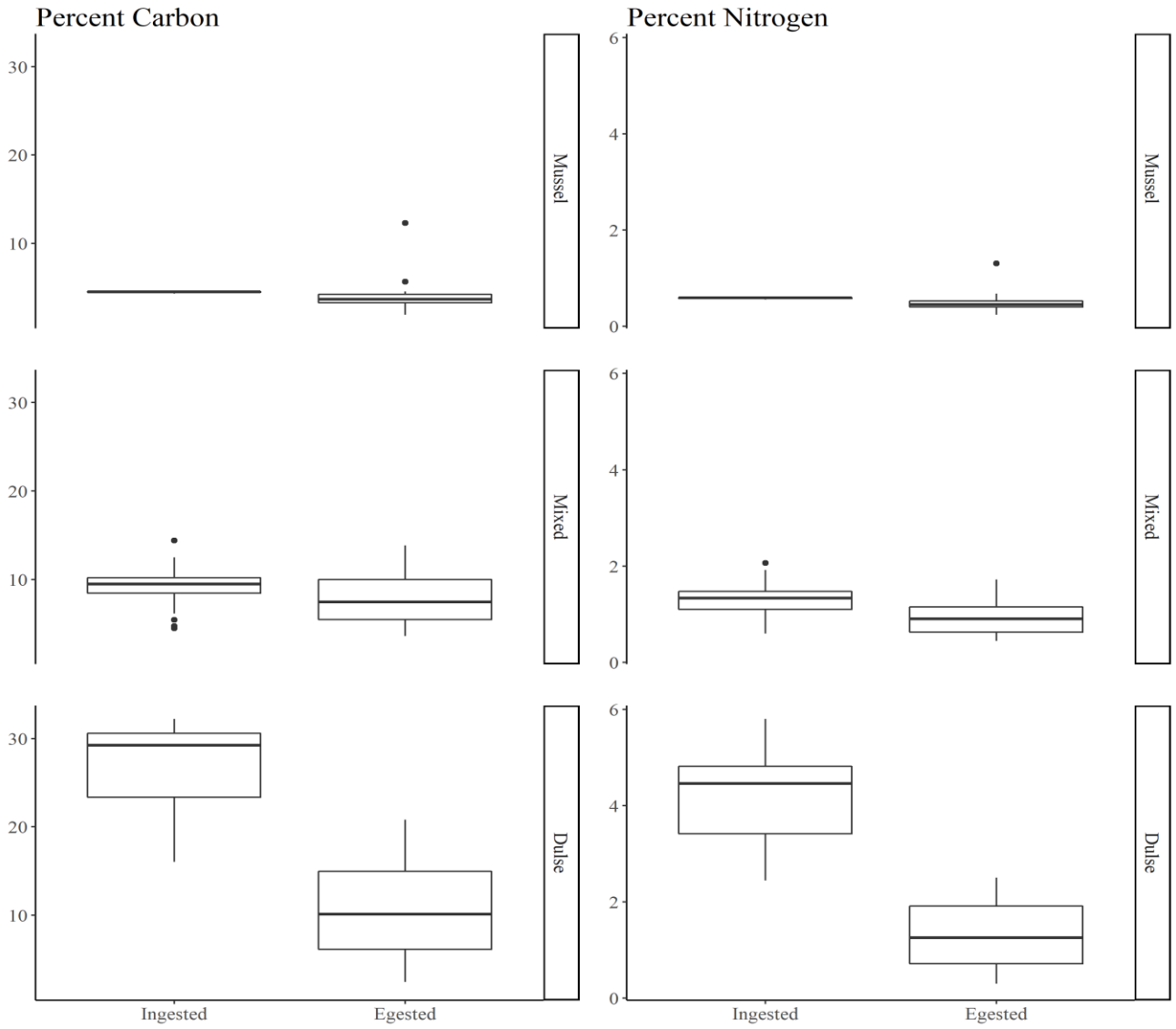

Figure 17. Percent carbon and nitrogen in uneaten food and in A. californicus *feces.*

Table 11. GLMM results for percent carbon as a function of diet type and whether the material had been digested by the sea cucumbers.

	Coefficient	DF	t value	p
Intercept (Raw Mussel Diet)	4.46	101	7.66	< 0.0001
Mixed	4.79	101	6.41	< 0.0001
Dulse	22.25	101	17.96	< 0.0001
Egested	-0.38	101	-0.72	0.48
Egested:Mixed	-0.82	100	-0.76	0.45
Egested:Dulse	-15.73	100	-8.89	< 0.0001

Table 12. GLMM results for percent nitrogen as a function of diet type, whether the material had been digested, and size of the sea cucumbers. The model included random intercepts by trial.

	Coefficient	DF	t value	p
Intercept (Raw Mussel Diet, SI 0)	0.57	98	7.29	< 0.0001
Mixed	0.92	98	5.96	< 0.0001
Dulse	4.04	98	13.60	< 0.0001

Egested	-0.09	98	-1.68	0.10
Size Index	0.02	98	0.33	0.74
Mixed:Egested	-0.24	98	-1.68	0.10
Dulse:Egested	-2.81	98	-10.69	< 0.0001
Mixed:Size Index	-0.22	98	-1.67	0.10
Dulse:Size Index	-0.48	98	-2.00	0.04

3.2.5 Fecal Production Rate

While fecal production rates declined with size index across all diets (Figure 19), the slope of the decrease was significantly greater for sea cucumbers feeding on mussel biodeposits than on either the mixed or dulse diets. However, this fit would be similar to the other diets if not for two very high measurements for sea cucumber with a low size index (Table 13, Figure 19). These high fecal production rates came from two sea cucumber that had exhibited the highest values

across all three diets (Figure 18). The difference in fecal production was greatest for the smallest sea cucumbers with rates converging as the size index increased.

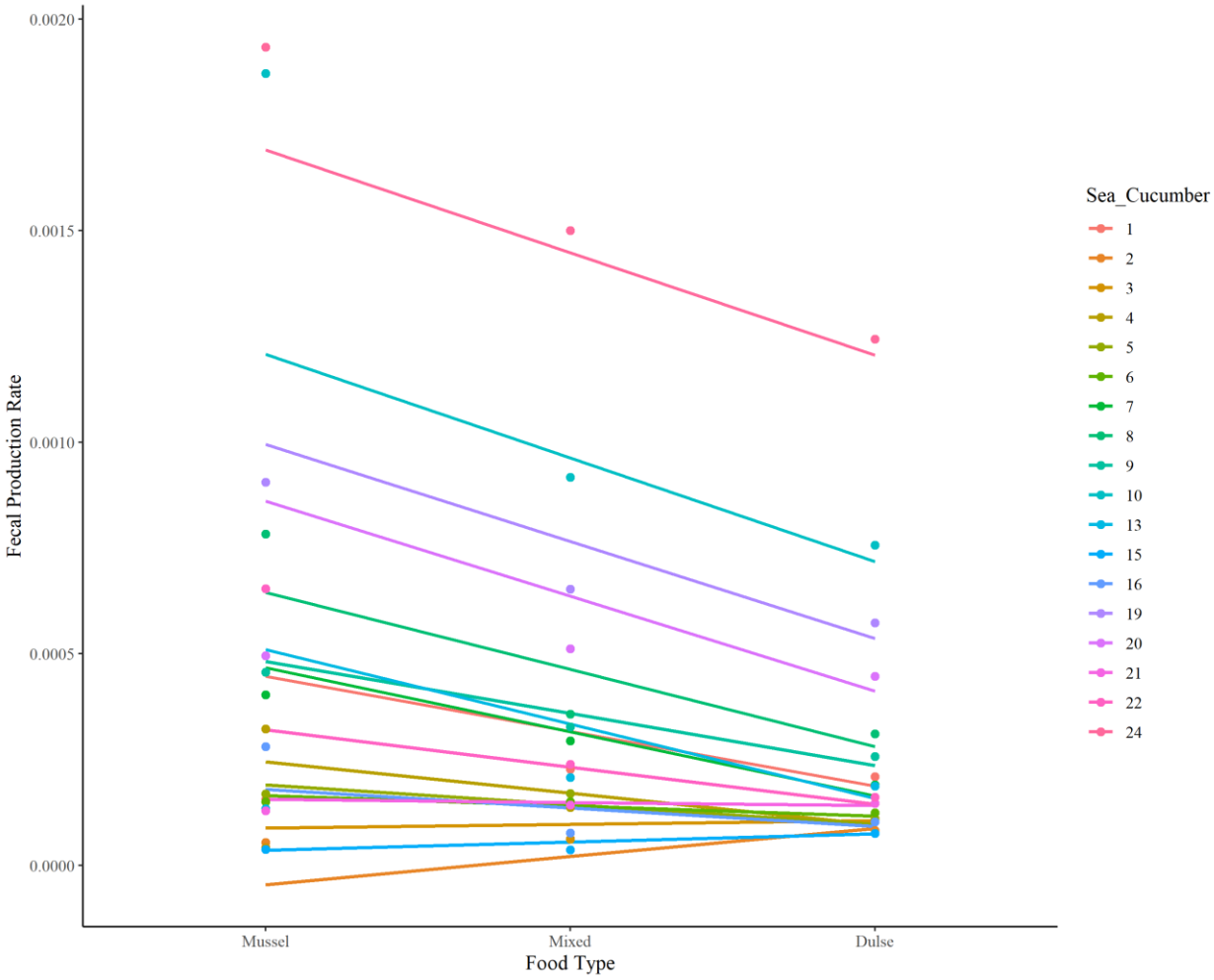

Figure 18. Fecal Production Rate as a function of diet. GLMM lines plotted with random intercepts per sea cucumber.

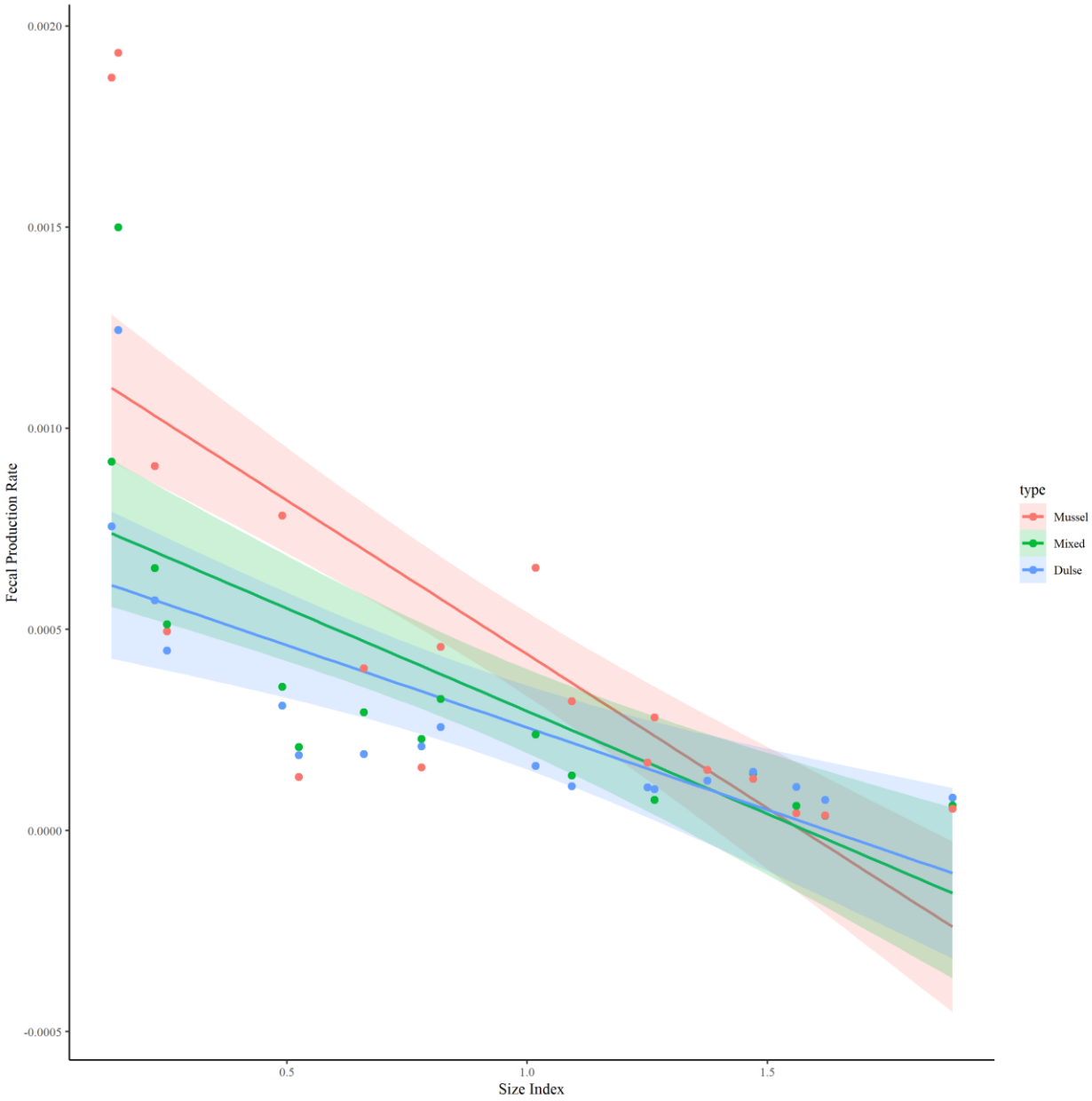

Figure 19. Fecal production rate plotted by sea cucumber size index. Average regression lines by diet type and 95% confidence intervals are shown for the three food treatments.

Table 13. GLMM for fecal production rate. Random slopes per trial and random intercepts for sea cucumber were included in the model.

	Coefficient	DF	t value	p
Intercept (Mussel Diet, SI 0)	0.0012	32	7.88	< 0.0001
Mixed Diet	-0.0004	32	-3.28	< 0.01
Dulse Diet	-0.0005	32	-4.55	< 0.01
Size Index	-0.0008	16	-5.34	< 0.001
Mixed Diet:Size Index	0.0003	32	2.25	.03
Dulse Diet:Size Index	0.0004	32	3.21	< 0.01

3.3 Totten Inlet Sea Cucumber Feeding and Growth Study

3.3.1 Temperature, Dissolved Oxygen, and Flow

Over the study period in Totten inlet, temperature steadily decreased over time at both the control and raft sites from 18°C to a minimum of 11.6 °C on November 3, as is seasonally expected. There was no apparent difference between the sites. Dissolved oxygen was normoxic during the study period, but relative to the control site, mean dissolved oxygen was approximately 1.5 mg/l (16%) lower at the raft site for the first month of the study (Figure 20). After September 15, the oxygen levels converged and were very similar except for a short period in late October.

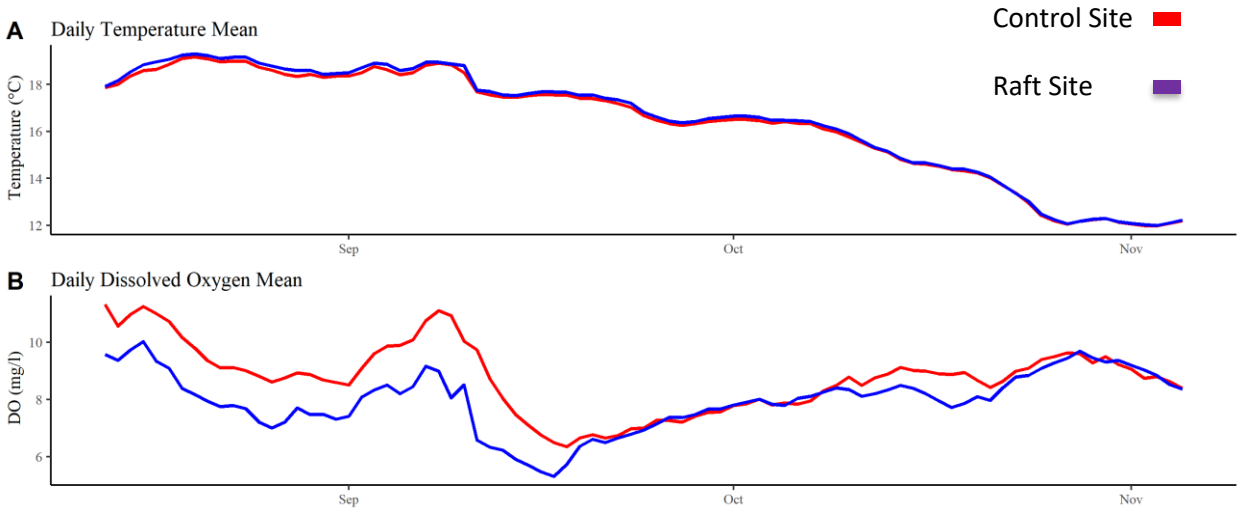

Figure 20. Temperature and dissolved oxygen daily means measured at the center of both the control and the raft site in Totten Inlet.

Dissolution blocks, used to measure relative water flow over a two-week period from 27 August to 10 September, showed greater variability but lower median mass loss at the mussel raft site. This suggests that water movement was higher and spatially more consistent at the no-mussel control site than beneath the mussel rafts.

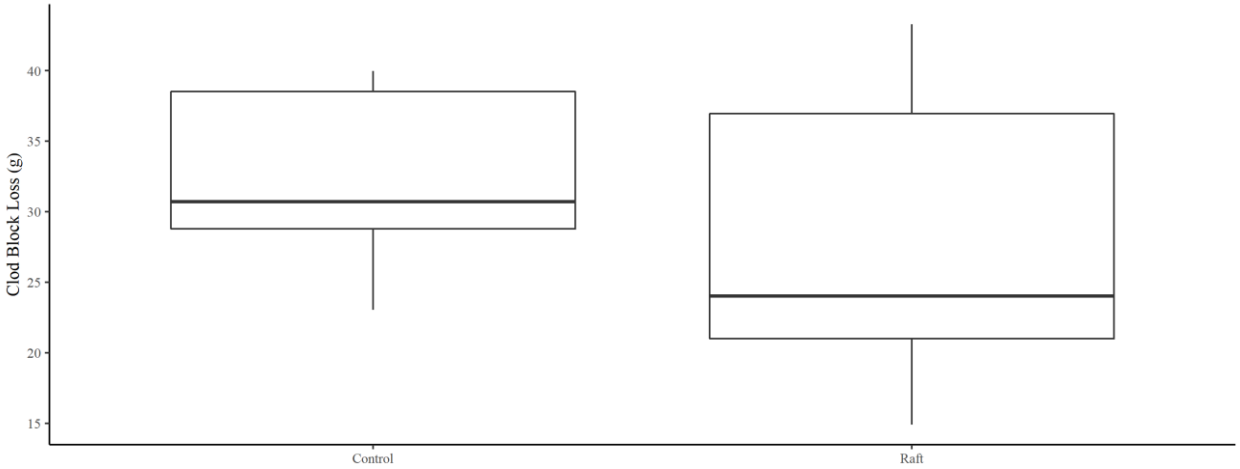

Figure 21. Dissolution block mass lost over two weeks at control and raft sites in Totten Inlet (n = 23).

Table 14. Best fit GLMMs for in-situ density experiment

Best Models	Random Term(s)	r^2
Sea Cucumber Mass (g) ~ site * enclosure density + days since outplant	1+Size Index|Cucumber	0.79
Dry Mass Enclosure Sediments (g) ~ site * days since outplant	1|Enclosure	0.53
% C ~ enclosure density * site *days since outplant	1|Enclosure	0.92

% N ~ enclosure density * days since outplant + site * days since outplant	1|Enclosure	0.91

3.3.2 Sea Cucumber Growth

Sea cucumbers placed into the cages under the sites on 27 July did not differ in initial wet weight across site or across density treatments based on a 2-way ANOVA with fixed predictors of site and density (Figure 22, Table 15). The mean wet weight of all sea cucumbers used for outplant was 221.3 g ± 1.1 (SE). Over the period of the study, three sea cucumbers (all at the raft site) died. The dead individuals were identified from previous measurements and within the specific enclosure and replaced as soon as they were discovered to maintain the assigned density in that enclosure. Growth data from the dead and replacement individuals were removed from all statistical analyses.

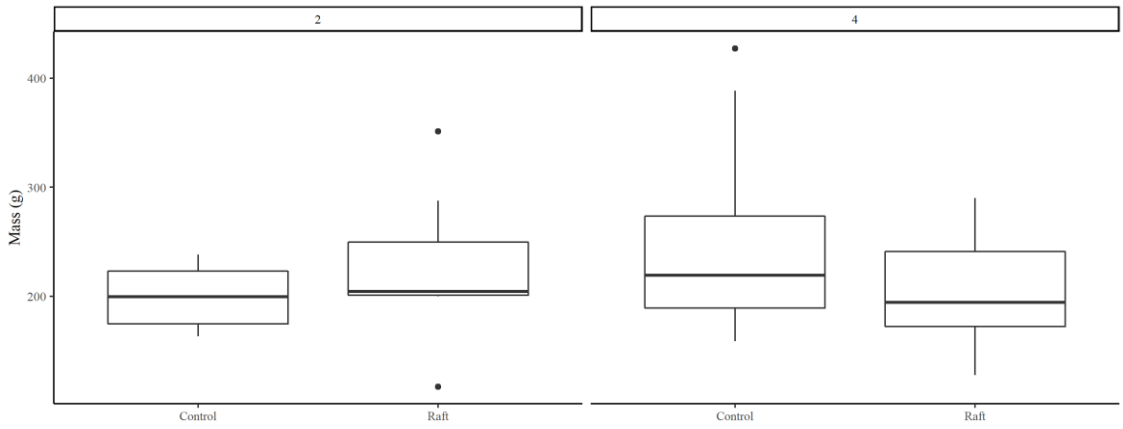

Figure 22. Sea cucumber wet weight measured on day of outplant 27 July 2020. The numbers over the figures indicate the number of individuals in that cage treatment.

Table 15. ANOVA results for sea cucumber wet weight measured on the day of outplant 27 July 2020.

	DF	SS	MS	f value	p value
Density	1	2748	2748	0.68	0.41
Site	1	3716	3716	0.93	0.34
Density : Site	1	10,595	10,595	2.64	0.11
Residuals	41	164,615	4015		

Statistical analysis of sea cucumber mass over the entire duration of the field study showed a significant interaction between site of the deployment and density of the sea cucumbers in the cages. That pattern resulted from greater growth of the sea cucumbers in the high-density cages at the control site and the opposite pattern at the raft site where sea cucumbers appeared smaller when stocked at the higher density (Figure 23). Number of days in the treatments was not included in the final statistical model (Table 16), indicating the absence of a consistent linear pattern in growth over the monitoring period. However, a graph of the average running mean over time (Figure 24) confirmed the reversal of the density effect at the two sites while also suggesting seasonality to the growth patterns, with differences between the control and raft sites.

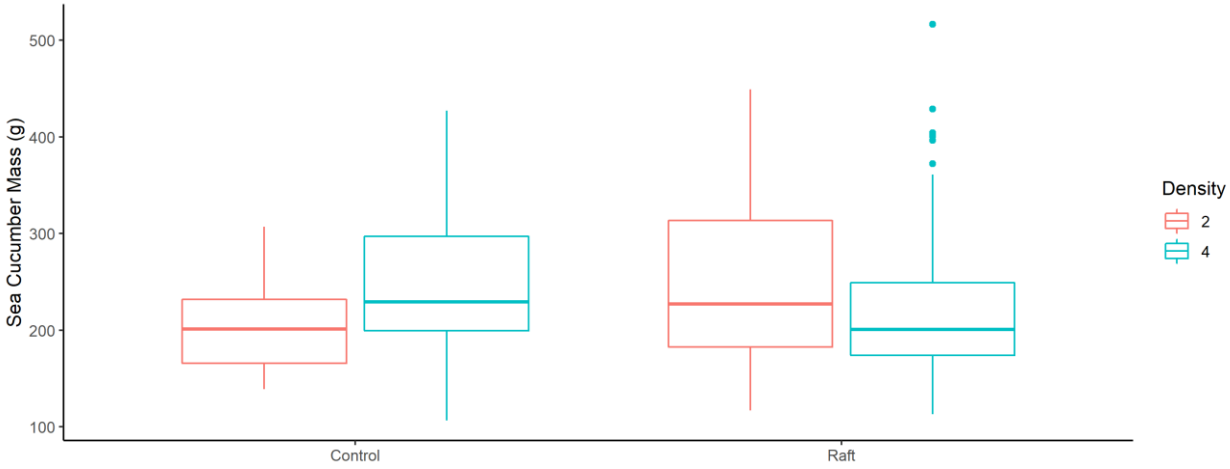

Figure 23. Sea cucumber masses by site and density measured over outplant, n=350.

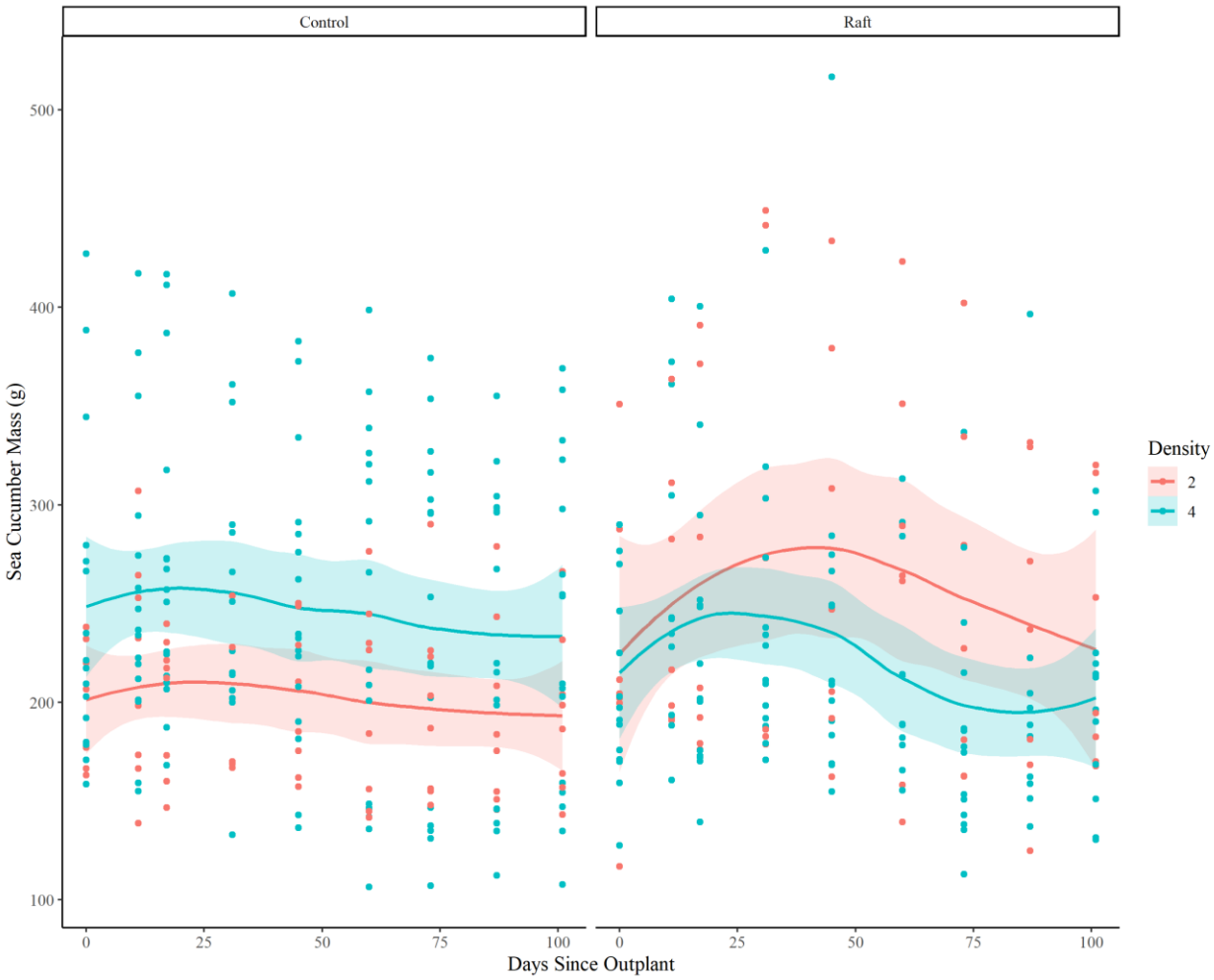

Figure 24. Sea cucumber masses by site and density over time. Mean loess regression lines and 95% confidence intervals are shown for the two density treatments. A smoothed line was used here to illustrate a non-linear relationship for sea cucumbers mass, likely due to seasonal aestivation after peak mass.

Table 16. GLMM for sea cucumber mass over entire outplant period random intercepts per sea cucumber and random slopes for size index were included in the final model.

	Coefficient	DF	t value	p
Intercept (Control Site, Density 2)	206.21	348	12.67	< 0.0001
Raft Site	33.88	41	1.40	0.17
Density 4	29.94	41	1.49	0.15
Days Since Outplant	-0.23	348	-4.72	<.0001
Raft Site:Density 4	-57.71	41	-1.95	0.06

3.3.3 Sediment Accumulation

Over the course of the study, sediments accumulated in the cages beneath the raft site at a much greater rate than at the control site, where sediment accumulation actually decreased over time (Figure 25, Table 17). Density was not a significant fixed factor in the models we tested, indicating that there was no consistent pattern of sediment accumulation in cages holding 2 or 4 sea cucumbers.

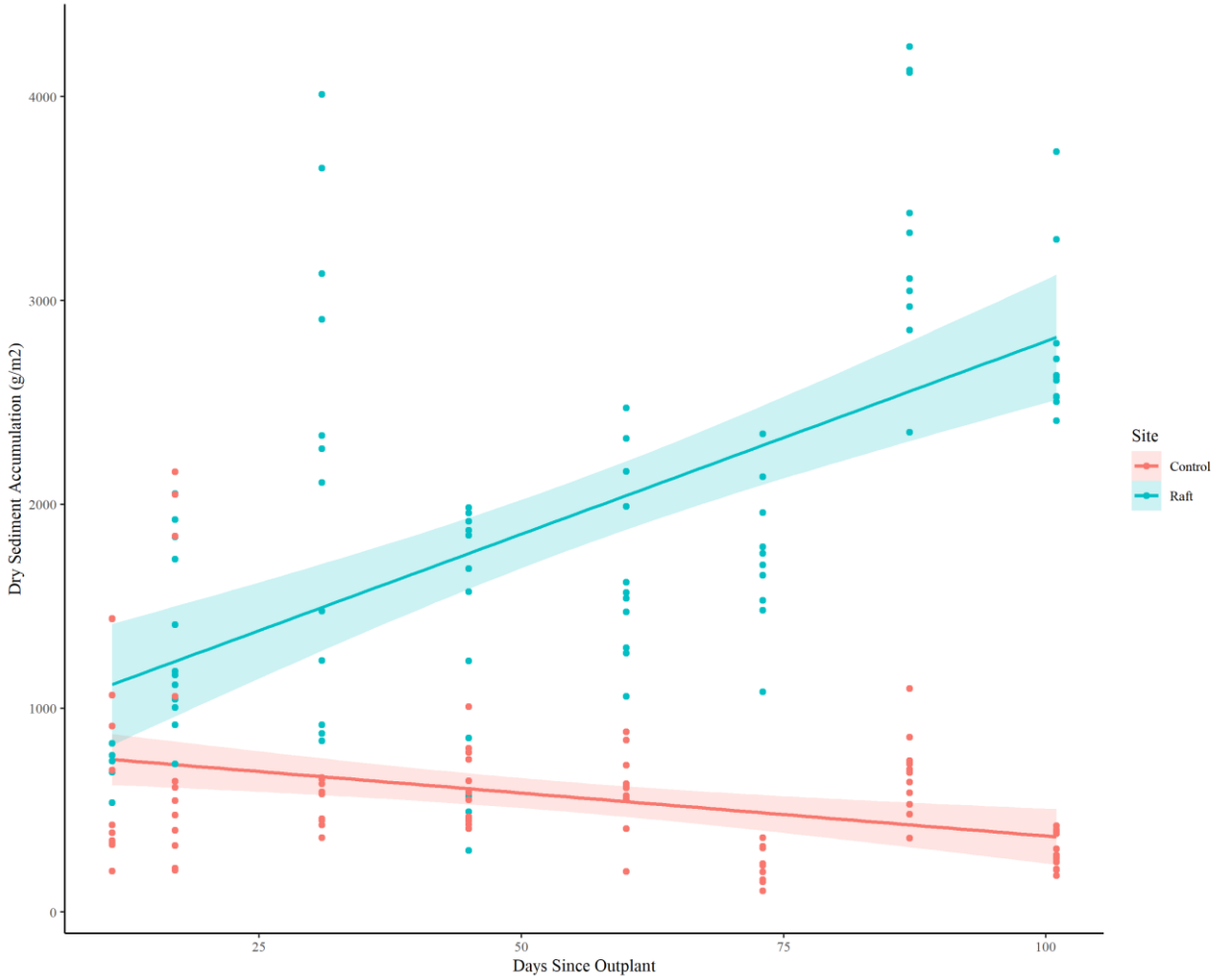

Figure 25. Dry mass of sediment accumulated per 2-week interval in the cages at the raft and control sites, n=176. Average regression lines and 95% confidence intervals are shown for the two density treatments.

Table 17. GLMM for sediment accumulation within sea cucumber cages with random slopes for enclosure.

	Coefficient	DF	t value	p

Intercept (Control Site Day 0)	770.92	149	8.94	< 0.0001
Raft Site	112.32	21	0.59	0.56
Days Since Outplant	-4.17	149	-3.47	< 0.001
Density 2	-30.52	149	-0.37	0.71
Density 4	95.22	21	1.17	0.26
Raft Site:Days Since Outplant	23.21	149	7.54	< 0.0001

3.3.4 Carbon and Nitrogen within Cage Biodeposits

The patterns in % carbon and % nitrogen in the cage sediments closely mirrored one another (Figure 26). At the control site, both showed minimal change over time and little obvious difference between empty cages and those with two or four sea cucumbers. The patterns in % carbon and % nitrogen at the raft site were again remarkably similar to each other but were dramatically different from the control site (Figure 26, Tables 18 and 19). In the two weeks immediately following outplant of the sea cucumbers, the levels of both carbon and nitrogen in the cage sediments was nearly twice as high under the mussel rafts as it was beneath the control site (Figure 26). The levels of both elements declined over time under the rafts until, by the end of the experiment in late October, they had reached levels similar to those found in the control cages, possibly linked to the fact that there was not as much organic matter in the water by November for sea cucumbers to assimilate. In the early stages of the experiment, there were greater levels of both carbon and nitrogen in the empty cages than in the cages with 2 or 4 sea

cucumbers at the raft site, but those differences decreased over time until all cages shared similar levels at the end of the experiment (Figure 26).

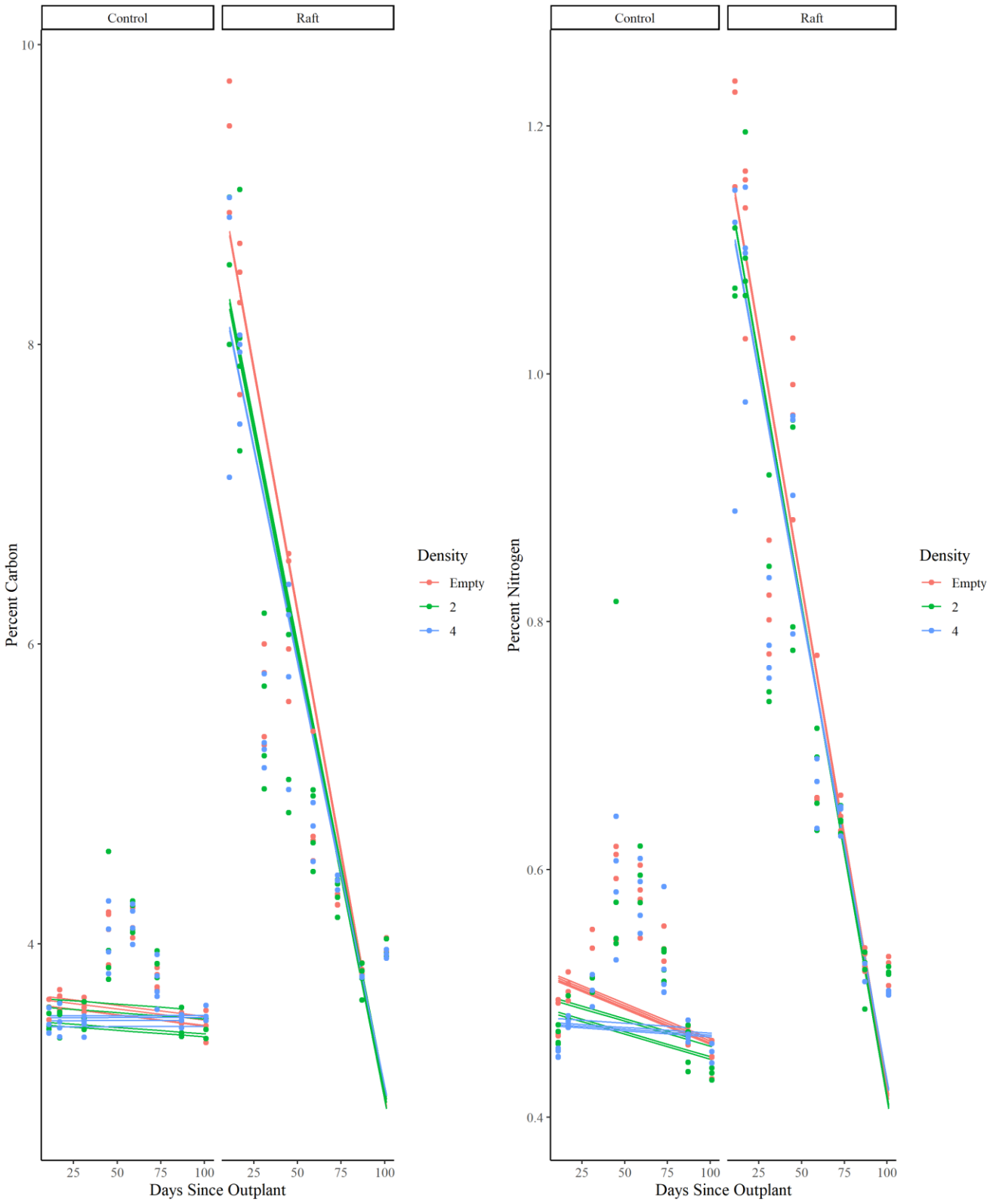
Control
Raft
Percent Carbon
Days Since Outplant
Density
Empty
2
4
Percent Nitrogen

Figure 26. Percent carbon and nitrogen measured from cage sediments at the control and raft sites, n=185. GLMM model lines with random intercepts by enclosure are shown for the three density treatments.

Table 18. GLMM for percent carbon measured from enclosure sediments at the three density treatments for control and raft sites. Random intercepts for enclosure were included in the model.

	Coefficient	DF	t value	p
Intercept (Day 0 at Control Site in Empty Cages)	3.60	151	44.90	< 0.0001
Density 2	-0.11	18	-1.01	0.33
Density 4	-0.14	18	-1.20	0.25
Raft Site	5.92	18	27.31	< 0.0001
Days Since Outplant	0.002	151	2.05	0.04
Density 2:Raft Site	-0.44	18	-1.42	0.17
Density 4:Raft Site	-0.62	18	-2.00	0.06
Density 2:Days Since Outplant	0.003	151	2.03	0.04
Density 4:Days Since Outplant	0.001	151	0.95	0.34
Raft Site:Days Since Outplant	-0.07	151	-24.14	< 0.0001
Density 2:Raft Site:Days Since Outplant	0.01	151	1.17	0.24
Density 4:Raft Site:Days Since Outplant	0.01	151	2.39	0.02

Table 19. GLMM for percent nitrogen measured from enclosure sediments at the three density treatments for control and raft sites. Random intercepts for enclosure were included in the model.

	Coefficient	DF	t value	p
Intercept (Day 0, Control Site, Empty Cage)	0.52	153	44.51	< 0.0001
Density 2	-0.03	20	-1.60	0.13
Density 4	-0.04	20	-2.70	< 0.05
Days Since Outplant	0.0006	153	-4.11	< 0.001
Raft Site	0.71	20	44.28	< 0.0001
Density 2:Days Since Outplant	0.0002	153	0.92	0.36
Density 4:Days Since Outplant	0.0005	153	2.50	< 0.05
Days Since Outplant:Raft Site	-0.01	153	-37.31	< 0.0001

Discussion

4.1 Tag Retention

As many other studies have found when attempting to tag sea cucumbers, none of the four tag types tested in this experiment provided a long-term marking solution. Rather than encysting or permanently incorporating tag materials into their skin, sea cucumbers we tagged either rapidly healed so that physical marks were indistinguishable or quickly pushed out any foreign objects and rapidly healed from the tagging wounds. Body wall and cloacal tags simply pulled through

the skin, leaving an open wound that healed within a few days. The mechanism by which PIT tags were dropped is unknown, but it seems likely that the tags were pushed out the same hole through which they had been inserted. The healing processes we observed are linked to the remarkable ability of echinoderms to regenerate organs or dropped body parts as part of a defensive function. Studies of other sea cucumber species have shown that such tissue repair is facilitated by specialized cells called morulae that quickly invade the area around an injured portion of epidermis and provide much of the ground structure matrix for tissue repair of deep injuries (Byrne 1986, San Miguel-Ruiz and Garcia-Arreras 2007).

Although all tags in our study were eventually dropped by the *A. californicus*, the sea cucumbers tolerated different body tags to different degrees. PIT tags had the shortest retention time of all four tagging methods, with an average of only 12 days. The short retention time for the PIT tags was in part due to the delicate nature of *A. californicus* feeding tentacles. Because the sea cucumbers would readily withdraw their tentacles when they were touched, we found it extremely difficult to place the tags inside their tentacle stalks without significantly disturbing the animals. It is possible that we created additional tentacle damage in our attempts to insert the PIT tags. Ten individuals were tagged successfully, but it is likely that those tags did not migrate down the feeding tentacle to lodge inside the plates of the pharyngeal bulb as we intended. This was simply a very difficult way to tag the individuals.

Although we had difficulty placing PIT tags effectively, and those we did place did not persist for very long, precisely placed tentacle PIT tags have been successful in some other species of sea cucumbers. The more substantial feeding tentacles of the dendrochirote *Cucumaria frondosa* provide a good pathway into the aquapharangeal bulb, and Gianassi et al. (2015) had 70% retention of tentacle-placed PIT tags after 300 days in large individuals. To increase placement

accuracy in future tagging efforts with *A. californicus*, sea cucumbers could be anaesthetized beforehand with a < 1% solution of propylene phenoxetol and sea water (Lincoln and Sheals 1979). This could allow enough time to precisely insert a PIT tag. On the other hand, anesthetization could also complicate PIT tag placement as it renders the tentacles flaccid. Using smaller PIT tags could also provide a more successful outcome. The 8-mm long tags we used were the smallest commercially sold PIT tag available. These may have been too large to easily move down the feeding tentacle and into the aquapharangeal bulb. Though PIT tags were not successful in our experiment, PIT tags have been highly effective in other organisms (especially fish), and we hold out hope that continued studies will lead to methods that will permit their use in *A. californicus*.

Cloacal tags were the second least successful method of marking in our study. Tags that had been fastened through the wall of the cloaca were rejected within a few weeks. We attempted to ensure these tags encircled the cloacal muscles that connect the cloaca to the posterior body wall and believe we accomplished that goal. The low retention of the tags there may have been related to the high activity of the cloacal muscles related to the respiratory water pumping that takes place there.

Though perhaps a little more predictable in the duration of the marking, papilla clips were not much better than cloacal tags. Papillae that had been completely removed regrew into pointed features that, within the first few weeks of tagging, were indistinguishable from papillae that had never been altered. Papilla clipping shows little potential for long-term marking of *A. californicus*.

The body wall tags showed the longest retention of the four tag types tested, with one individual shedding the tag only after 36 weeks had passed. It is possible that cucumbers that retained the body wall tags longer did so because the loop of the stitched tag completely encircled one of the five longitudinal muscle bands running lengthwise inside the body wall of *A. californicus*. When inserting the body wall tags, we attempted to encircle the longitudinal muscles by observing difference in skin tension and assuming that the muscle lay beneath the area where the epidermis was taut and fringed by softer-looking skin. It was easier to completely encircle the muscle band in smaller individuals, which could explain why smaller sea cucumbers tended to retain the tags longer. We attempted to keep suture loops as small as possible so that they would not interfere with any internal organs, however there is a chance that they were too small to completely surround a muscle in larger individuals. Increasing the diameter of the loop may provide better encircling of a target muscle. Gianasi et al. (2015) used a portable x-ray machine to confirm placement of PIT tags in the cucumbers they tested in their study. In future work, a similar approach might help better identify the location for the placement of body wall tags in *A. californicus*.

A handful of researchers have attempted to tag sea cucumbers with plastic markers that are quick to deploy and easy to identify. Most of these are similarly short lived, but in the most successful, Ciecel et al. (2009) found that a plastic single t-bar tag (Floy FD-94) inserted through the body wall into anterior dorsal muscle showed 70% retention after 16 weeks, although dermal sores around the insertion site were common. Our test of a stainless-steel product resulted in retention times shorter than those described by Ciecel et al. (2009). Unlike the plastic tags reported there, we saw minimal tissue necrosis around the stainless-steel sutures in this study. However, there was no obvious increase in retention time based on the sutures' biologically inert properties.

While our study showed the best results with a stainless-steel body wall tag that could be retained by *A. californicus* up to 9 months, most body wall tags were lost much sooner. In fact, the average retention time was only 98 ± 15 days. This was almost double the retention time of cloacal tags or detection of clipped papillae but is still inadequate for the kind of long-term tagging necessary to monitor *A. californicus* populations in the field or in an aquaculture situation.

Many questions about the basic biology of *A. californicus* will remain unanswered until a long-term tagging option is available. Recent studies focused on the growth of hatchery individuals in Manchester, Washington have found that growth can be highly variable and dependent on the diet the sea cucumbers are consuming, making comparisons to the growth rates of wild sea cucumbers less straightforward (Andy Suhrbier, Pacific Shellfish Institute, pers. comm. December 2019). An ideal tag should provide a long enough retention period to track movement, understand age classes, and serve to differentiate between IMTA cultured product and wild individuals. This will be necessary to achieve the goal of sustainably managing a wild sea cucumber fishery in Washington. While none of the tag types we trialed live up to these expectations, the body wall tag did persist for several months and can be added to Floy Tags as a cheap and easy way to uniquely identify individuals for short-term studies. While PIT tags have shown the best success in other sea cucumber tagging studies, they did not work well for us. In addition, they are not readily visible like the body wall tag we tested. No diving rated PIT tag reader yet exists, so identifying sea cucumbers with PIT tags requires bringing them to the surface and scanning them there. Clearly, an external identifier like the body wall tag trialed in this study may still have some application for shorter-term studies.

4.2 Feeding Trials

In laboratory feeding trials, feeding sea cucumbers removed a small fraction of the total carbon and nitrogen contained in mussel biodeposits. This may have been due to the overall low organic content in the mussel fecal matter to begin with; there was simply not a great deal of material to assimilate. While the amount of total carbon and nitrogen in the *A. californicus* fecal matter was statistically indistinguishable from what was present in the mussel biodeposits they fed on, sea cucumbers have been shown to assimilate a good portion of the organic carbon and nitrogen from the waste products of floating aquaculture in other studies (Palzat et al. 2008, Hannah et al. 2013). For example, in trials where *A. californicus* were placed below sablefish aquaculture, the sea cucumbers removed 60.3% of the total organic carbon and 62.3% of the total nitrogen from the sablefish wastes (Hannah et al. 2013). Palzat et al. (2008) found the assimilation efficiency of carbon by *A. californicus* feeding on Pacific oyster (*Crassostrea gigas*) biodeposits to be 40 to 50%. It appears that for any measurable change in total carbon, the organic content of the food type must be relatively high to begin with. The nutritional quality of mussel feces in the Puget Sound is known to change throughout the year with organic content being highest during springtime phytoplankton blooms. The low organic content of the mussel fecal matter we collected in the late summer may be a consequence of collection timing (Callier et al. 2006).

When the *A. californicus* were fed a diet of dulse alone, 59% of the total carbon and 61% of the total nitrogen were assimilated by the sea cucumbers indicating that, in fact, they can digest this material and receive significant nutrition. In an IMTA situation, where the falling material was dulse or something similar, *A. californicus* could be well suited both to reduce nutrient loading from the deposited matter and have a high potential for growth as a secondary aquaculture product. An important consideration is that the specific culture methods of one farm may be

different from another, and the local oceanography influencing each farm will have effects on the available organic matter below them. More specifically, the vertical flux of organic matter may be lower for a farmed vegetation than it is for farmed shellfish, or nonexistent if the vegetation is completely harvested before it begins to degrade.

Other studies of sea cucumber feeding have shown that sea cucumbers will consume more of a low-quality diet than they will a high-quality diet (Zamora 2011). This is linked to the need to ingest larger amounts of low-quality food to gain the same net energy (Zamora 2011, Yuan et al. 2006). We did not find that pattern in our study. Sea cucumbers ate the same quantity of food regardless of type. This discrepancy may have resulted from a carryover effect related to the fact that the same sea cucumbers were offered all three diets with only 48-hour gaps between the treatments. It is possible that there was simply insufficient time for *A. californicus* to modify their feeding or digestive behavior in such a brief treatment period. Perhaps also, sea cucumbers that had been fed dulse had fulfilled nutrition requirements for a period of time that was greater than 48 hours, affecting their feeding behavior when offered a lower quality diet. Clearly, if we repeated the study, we would want to allow for more time between feeding trials or use enough sea cucumbers in the experiment so they were eating only one diet.

If we consider our nutrition results, we predict that *A. californicus* would need to consume less of a higher nutrition macroalga like dulse in an aquaculture situation than they would biodeposits falling from a mussel raft. It also seems likely that higher stocking densities of sea cucumbers could be sustained under a dulse farm given the greater nutritional value and that individual sea cucumbers might grow faster or reproduce more under these conditions.

Interestingly, when dulse was added to the mussel biodeposits, effectively increasing the organic content, there was still no significant drop in total carbon and nitrogen in the sea cucumber's fecal matter. It is possible that the mixed diet type was not different from mussel biodeposits treatment because, when offered both mussel biodeposits and dulse, sea cucumbers may have consumed a higher proportion of biodeposits, selecting for the granular type particles of biodeposits over flakes of dulse.

In our study, large and small sea cucumbers ate similar amounts of mussel feces, but large sea cucumbers ingested more dulse than small cucumbers did. This result may be linked to mechanical feeding advantages that larger sea cucumbers have. Dulse particulate flakes ranged in size from approximately 1 to 4 mm in maximum dimension, much larger than fine-grain mussel biodeposits. Both Yingst (1982) and Slater (2011) found that deposit-feeding sea cucumbers show no grain size selectivity when feeding on sediment. Instead, it has been suggested that some sea cucumbers may be able to identify and select for particles that are higher in organics with specialized chemoreceptors on their feeding tentacles (Zamora and Jeffs 2012). The dimensions of the decomposing dulse flakes we fed to sea cucumbers were much larger and irregular than sediment particles. It is possible that instead of selecting for smaller particles, small sea cucumber could not physically move particles of a certain size through their mouth and consumed less dulse due to this mechanical inability.

Our findings show that *A. californicus* operates similarly to many other sea cucumbers in assimilating organic material present in aquaculture waste products. The assimilation that *A. californicus* exhibits for dulse is similar in magnitude to that reported beneath other types of floating aquaculture (Palzat et al. 2008, Hannah et al. 2013). We found that *A. californicus* has the ability to significantly reduce the carbon and nitrogen in waste beneath a farm with a product

like dulse and that large sea cucumbers may easily consume large detrital flakes, whereas small individuals cannot. This has implications for the stocking strategy one would implement to best suit an IMTA operation that includes both sea cucumbers and macroalgae.

4.3 Sea Cucumbers Under Rafts

Over the course of summer 2020, *A. californicus* outplanted in Totten Inlet beneath established rafts of mussels saw heavier sedimentation levels than did *A. californicus* in a nearby control site without mussels. This is similar to other studies that found increased sedimentation within a 12m footprint of mussel raft cultures (Callier et al. 2006). Clearly, mussels in Totten Inlet were concentrating material in the seawater and transporting it to the seafloor as feces and pseudofeces. Sedimented material at the raft site in July was high in nutrients, but the organic content declined through the study period to a low in November. Callier et al. (2006) observed a similar pattern of increasing sedimentation from June to September, coupled with a declining organic quality of the deposited material. Our results suggest that mussels at a commercial raft site increased their filtration rates to compensate as the quality of suspended particulate matter dropped. In Totten Inlet, similar conditions produced increasing sedimentation rates of declining quality biodeposits from the mussels over time. The change in quality of the mussel biodeposits appeared to impact the sea cucumber's growth. The peak and subsequent decline we observed in the sea cucumber's mass under the raft site appears related to an initial deposition of abundant high quality organic matter, followed by a subsequent decline in organic content, a consequent drop in sea cucumber biomass, and the start of a natural aestivation process. Multiple studies have shown that sea cucumbers undergo seasonal aestivation independent of food availability (Palzat et al. 2008, Hannah et al. 2013). While the trigger for aestivation is still relatively

unstudied for *A. califonicus*, it has been linked to high temperatures in the Japanese sea cucumber *A. japonicus* (Ji et al. 2008).

We believe the increase in growth the sea cucumbers exhibited under mussels leading up to aestivation was caused by abundant food during that time. Primary productivity is driven by nutrient availability, and levels are elevated in the Salish Sea from the beginning of April until November. Mussels consume the most phytoplankton during the spring and early summer blooms (Hatcher et al. 1995, Khangaonkar et al. 2012). The total organic content in mussel biodeposits changes over time with the availability of this phytoplankton - becoming less rich after peaks in July in the Northern Hemisphere (Callier et al. 2006). We found that maximum increases in wet-weight body mass occurred in September for sea cucumbers under the raft site in Totten Inlet and are best explained by the tail end of this heightened primary productivity culminating in a late summer phytoplankton bloom. The same growth rates were not measured for sea cucumbers at the control site, where there were no mussels present to concentrate and transfer organic particulate matter to the benthos in the form of feces and pseudofeces.

Unfortunately, chlorophyll measurements to confirm a late fall bloom at the study site were not available as the Covid-19 pandemic paused a Washington Department of Ecology (DOE) monitoring program. Aerial images collected by DOE's Eyes Over Puget Sound program do, however, confirm the presence of algae blooms in South Sound in late September (Appendix 3). Carbon-to-nitrogen ratios experienced a low point between August 22nd and September 16th, indicating an influx of nitrogen to nutrient-starved phytoplankton (Appendix 1). The C:N ratios from sediments at the raft site dip lower than at the control site when mussels were feeding on this abundance of phytoplankton. Soon afterward we saw dissolved oxygen plummet at both the control and the raft sites, which is indicative of bacterial respiration following this bloom. During

this dip, the raft site experienced lower dissolved oxygen levels than the control site, most likely due to enhanced bacterial activity and accumulation of organic rich biodeposits concentrated below the mussel raft culture.

Hannah et al. (2016) found that under a sablefish aquaculture operation in Canada, large *A. californicus* (100 – 565 g) grown at a density of 12 m^{-2} started to grow in January and had a maximum increase in body mass of 25% during March. In the month of August, they measured a further 5% increase in wet weight (Hannah et al. 2013). Comparatively, sea cucumbers in our experiment saw increases of 24% and 14% for density 2 and 4 treatments respectively in the month of August and September. It is likely that sea cucumbers in Totten Inlet would have exhibited higher growth rates had they been deployed earlier in the year when primary productivity was highest. A combination of permitting delays and pandemic restrictions dictated that sea cucumbers were placed in Totten Inlet at the end of July, well after the best conditions for elevated organic matter in mussel feces and maximum growth of the sea cucumbers. If we assume growth starting in January after seasonal aestivation with the same growth rates we measured in late summer, total increase in body mass for the low-density *A. californicus* treatment (2 cucumbers) could have been even higher under mussel aquaculture than what was reported with sablefish. It is also possible that sea cucumbers could be removed from underneath rafts, placed in temperature-controlled tanks, and supplemented with a high quality diet to overwinter poor conditions with low available organic matter. In this scenario, the sea cucumbers may not go through the process of aestivation, and more growth could be achieved. To our knowledge, a study to confirm that this could be achieved with *A. californicus* has not yet been carried out.

In addition to placing *A. californicus* earlier in the year, better growth may be produced by establishing a longer interval between measurements. Recent studies have suggested that sea cucumbers grow better if subjected to less handling like they experienced during our monitoring events (A. Suhrbier, Pacific Shellfish Institute.). While this may be true, we found that fouling organisms completely covered cages from mid-August until October, making it critical to clear cage sides and tops biweekly to ensure water flow and an unobstructed supply of mussel biodeposits to the sea cucumbers. One option that may decrease cage fouling without having to manually pull the cages to the surface would be to deploy grazing organisms such as sea urchins within or on the outsides of the cages. Sea urchins have been utilized in IMTA to effectively reduce fouling on the sides of netting surrounding floating mussel aquaculture (Lodeiros and Garcistera 2004, Sterling et al. 2016). Sea urchin gonad is of high value and can provide an additional product for farmers. However, studies on sea urchins cultured in IMTA have reported gonads that are of inconsistent quality (color, taste, firmness) compared to wild populations that are primarily consuming kelp (Sterling et al. 2016). These studies have suggested that the gonad quality of urchins grown in IMTA could be increased by providing a finishing diet of macroalga before market (Sterling et al. 2016).

Interestingly, the quantity of sediments measured from cages of different density was not statistically different at the sites. One would expect that because more sea cucumbers were within higher density enclosures, there would also be a higher quantity of sediments from sea cucumber defecation. A likely explanation for this is that any differences were overshadowed at the raft site by high quantities of mussel bio deposits and conversely that the sea cucumber were simply not feeding at the control site. *A. californicus* feeding reduced the carbon and nitrogen levels in biodeposits, but only during part of the year. When the organic matter in the deposits

was high enough, the sea cucumbers assimilated nutrients as others have found (Palzat et al. 2008, Hannah et al. 2013). This assimilation effect was independent of the cage stocking densities we tested, and disappeared around day 65 when the organic content of cage sediments fell below 15%. It is apparent that the sea cucumbers in the cage were re-ingesting deposited material (perhaps multiple times) and effectively reducing the carbon and nitrogen from within those sediments.

Further progress towards understanding *A. californicus* growth, genetics, reproduction, and disease will ensure that commercial implementation of sea cucumber IMTA in the Salish Sea will be done responsibly. Our study demonstrates that *A. californicus* will grow better at lower density and can effectively reduce nutrient deposition below mussel aquaculture. While we saw these effects for sea cucumbers within cages, the benefits would be even greater if sea ranching were utilized. Sea ranching *A. californicus* would reduce costs associated with maintenance of cages and allow for sea cucumbers to access a higher quantity of rich organic matter deposited on the seafloor. This access would allow for higher stocking densities while at the same time enhancing the growth of *A. californicus*. The challenge of this is that we must first establish a long-term marking technique so that hatchery produced sea cucumbers are not mistaken for wild individuals. As our understanding of the biology of *A. californicus* broadens, and multi-trophic aquaculture with echinoderms becomes more prevalent, resource managers will benefit from understanding growth potential, nutrient assimilation, and best practices for stocking density underneath different types of aquaculture.

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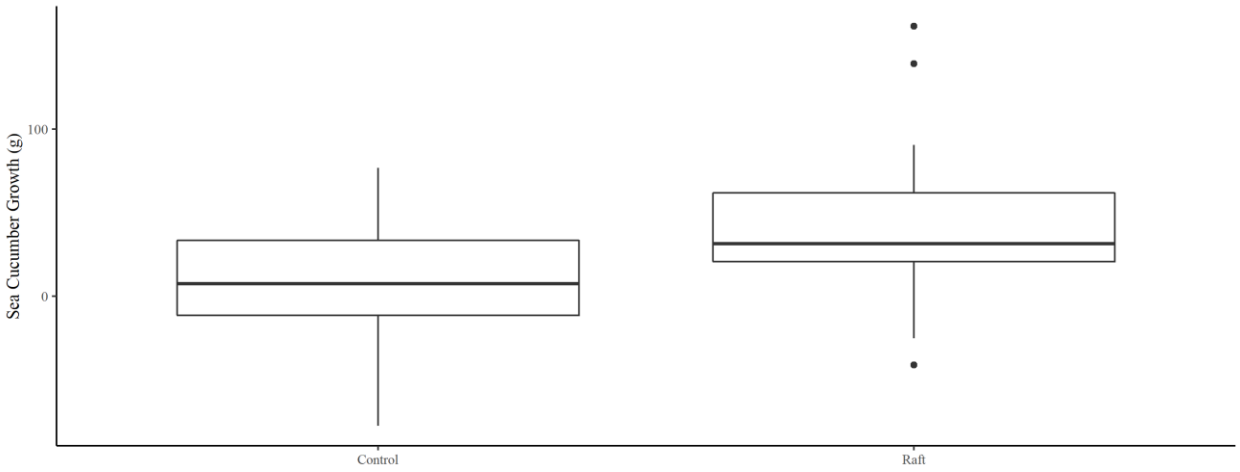
Appendix

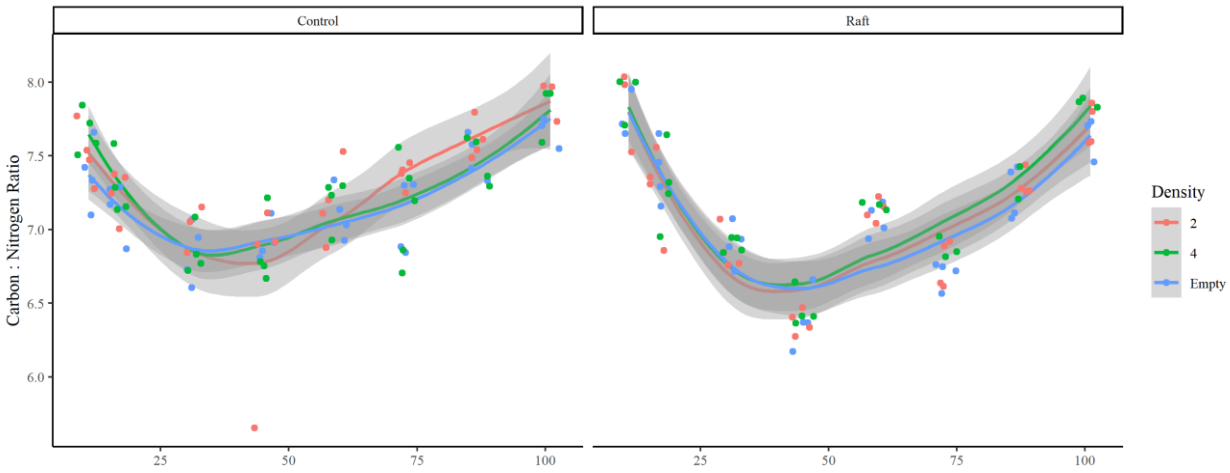


Appendix 1. Sea cucumber growth (mass day 31 - mass day 1) in first 31 days of outplant, n=44.

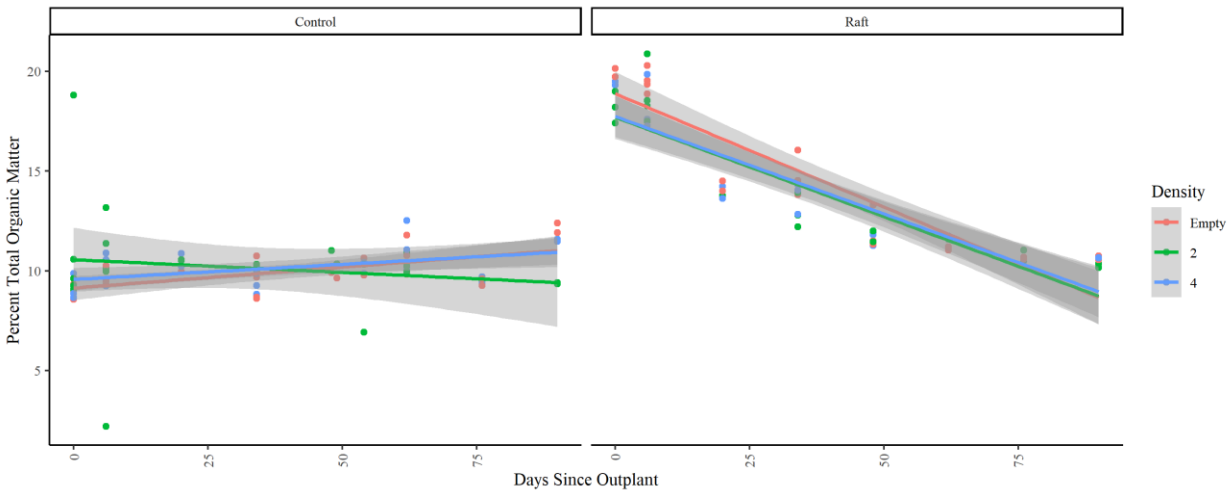
Appendix 2. GLMM for mass change from day 0 to day 31 in the field. random intercepts for sea cucumber were included in the model.

	value	df	t value	p value
Intercept (Control Site)	7.63	43	.85	0.39
Raft Site	33.11	43	2.52	0.02

Appendix 3. Aerial image taken by Washington Department of Ecology's Eyes Over Puget Sound Program. The photo indicates a Fall red-brown phytoplankton bloom.



Appendix 4. Carbon-to-Nitrogen ratios within cage sediments at the control and raft sites. 95% confidence intervals are shown.



Appendix 5. Total organic matter of sea cucumber measured within cages of different density sea cucumbers. 95% confidence intervals are shown.

Appendix 6. GLMM model for percent total organic matter in enclosure biodeposits.

	Coefficient	DF	t value	p
Intercept (Empty Enclosure, Control Site,	10.11	109	36.29	<.0001
Density 2	0.92	18	2.32	0.03
Density 4	0.54	18	1.34	0.20
Raft Site	5.83	18	14.41	<.0001
Days Since Outplant	-0.01	109	-1.69	0.09
Density 2:Raft Site	-1.98	18	-3.44	<0.01
Density 4:Raft Site	-1.12	18	-1.93	0.07
Density 2:Days Since Outplant	-0.01	109	-1.95	0.05
Density 4:Days Since Outplant	-0.01	109	-0.89	0.38
Raft Site:Days Since Outplant	-0.06	109	-9.67	<0.0001
Density 2:Raft Site:Days Since Outplant	0.03	109	3.08	<0.01
Density 4:Raft Site:Days Since Outplant	0.01	109	1.33	0.19