Applying ecological and evolutionary approaches to support coastal marine conservation

DISSERTATION

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List of Figures and Tables

Acknowledgements

Chapter 1

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Sequence data are archived at the National Center for Biotechnology Information, accession number PRJNA955437. Data and code will be openly available at the Dryad Digital Repository. Previously published datasets used in all analyses are described in the code.

PDD, JLG, JAL, CDH and JBL designed the study. Fieldwork conducted by PDD, EAF, JLG, JAL, CDH and JBL. Sample processing by PDD, EAF, JAL, and JBL. JAJMW conducted bioinformatics. PDD, EAF, WEF and JBL prepared the original manuscript draft with support from CDH, JAL, JLG, and JAJMW provided valuable edits. EAF conducted statistical modeling. Figure preparation by PDD, EAF, and JBL. CDH, JLG, JAL and JBL acquired funding.

Chapter 2

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PUBLICATIONS

1. **Dawkins PD**, Fiorenza EA, Gaeckle J, Lanksbury J, van de Water JAJM, Feeney WE, Harvell CD, Lamb JB. (Manuscript in revision at *Nature Sustainability*). Global seagrass ecosystems as green urban infrastructure to mediate human pathogens in food from the sea.
2. **Dawkins PD,** Paz-Lacavex A, Fiorenza EA, Rush MA, Beas-Luna R, Lorda J, Malpica-Cruz L, Sandoval-Gil JM, McHugh TA, Han MK, Bracken MES, Lamb JB. (Manuscript and video protocol in revision at the *Journal of Visualized Experiments)*. Field collection and laboratory maintenance for restoration of canopy-forming giant kelp.
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Abstract

Climate change and expanding human populations are converging threats that impact marine ecosystems, leading to global declines in critical habitat and biodiversity. Targeted and innovative management solutions that can reconcile the objectives of supporting human livelihoods alongside those of environmental sustainability are urgently needed. My research highlights important management considerations for the conservation and restoration of two foundation marine ecosystems that dominate global temperate coastal regions, seagrass meadows and canopy-forming kelp forests.

Urban greening offers a strategic opportunity to reinforce the security and safety of food from the sea. Seagrass ecosystems can reduce human bacterial pathogens from terrestrial sources, however it remains unknown whether this health benefit is conferred to associated food fish. Marine bivalves are bioindicators of bacterial contamination and constitute over half of global seafood production. Following a three month deployment across 20 coastal urban locations in the greater Seattle Metropolitan Area, I found in **Chapter 1** that bivalves deployed and subsequently retrieved from locations with seagrass present had a 65% reduction in the abundance of bacterial species with known human pathogenicity when compared to locations without seagrass. My global models estimate that 1.1 billion people currently reside in over one-third (662 of 1,860) of urban agglomerations (populations over 300,000 people) and 19 megacities (populations over 10 million people) within 50 km of seagrass ecosystems, which I forecast to rise 15% by 2030. Given increasing reliance on food from the sea to meet supply and nutritional needs, these results highlight the global opportunity to support ambitious human health and biodiversity sustainability targets.

Canopy-forming kelps are foundation species that support biodiversity and provide ecosystem services valued at over USD$500 billion per year. Giant kelp forests are facing unprecedented loss due to climate-driven ecological stressors necessitating innovative restoration strategies. I produced a video for **Chapter 2** to illustrate a protocol and tools for restoring canopy-forming giant kelp forests. I include

field-based tissue collection and laboratory-based methods of sporulating, inoculating, rearing, maintaining, and monitoring substrates seeded with early life stages using the '*green gravel'* technique. The protocol simplifies and centralizes existing practices in this field of restoration to facilitate conservation objectives for underwater kelp forests by researchers, managers and stakeholders.

Climate change and anthropogenic disturbances are currently outpacing the adaptive capacity of natural kelp populations, challenging traditional conservation aims of restoration to historic states. Thus, conservation frameworks have expanded to include anticipatory management that proactively consider resilience and adaptive capacity. I conducted a >2-month long-term trial in **Chapter 3** to assess the effect of rearing temperatures (12°C, 16°C, 20°C, and 24°C) on early microstages of a canopy-forming giant kelp species from potentially locally adapted Santa Cruz and San Diego populations of origin in constant thermal conditions, followed by a simulated deployment for restoration at 16°C and a realistic marine heatwave of 22°C. Samples reared in cooler 12 and 16°C temperatures performed best in constant thermal conditions. However, these samples were significantly impacted by a simulated heatwave. Despite poor performance in constant thermal conditions, samples reared at 20°C were able to recover from reproductive dormancy given a simulated deployment for restoration at 16°C, and performed best after a 22°C simulated heatwave. There was no kelp survival in the 24°C rearing temperature, and thus this treatment was terminated. These findings suggest that rearing *M. pyrifera* at warmer temperatures may confer an advantage when deployed into warming oceans with increasing frequency and magnitude of marine heatwaves, and provides an avenue forward for marine managers looking to future-proof restoration efforts.

Critical marine habitats such as seagrass and kelp ecosystems continue to deteriorate at unprecedented rates, calling upon management solutions that pro-actively and simultaneously consider human populations and future environmental decisions.

Brief Introduction

Coastal Marine Ecosystems

Coastal marine habitats are diverse and productive zones, providing the foundation for ecosystems that are ecologically, culturally, and economically important. Seagrasses and kelps constitute two foundational temperate marine ecosystems, hosting considerable biodiversity and providing valuable services to adjacent wildlife and human communities. Found on every continental shelf except for Antarctica, seagrass meadows are composed of the only flowering plants in the marine environment. Seagrasses act as critical habitat for flora and fauna, including protected species such as dugongs, sea turtles, and sharks, and provide nursery habitat for over one-fifth of the world's largest fisheries[1]. Seagrasses also provide high-value nutrient cycling, water filtration, globally significant sequestration of carbon, and sediment retention[2,3]. Kelps are large brown algal seaweeds and are distributed in coastal regions of temperate and Arctic seas worldwide, requiring nutrient-rich, cool waters to grow. Canopy forming kelps are attached to the ocean benthos and create floating canopies on the ocean surface, which form multi-layered and complex underwater habitats. Kelps are among the most productive and extensive ecosystems at a global scale[4,5], with productivity outputs that rival those of tropical rainforests. However, both seagrass and kelp ecosystems face myriad stressors that are leading to global declines.

Threats and Declines

The coastal distribution of seagrass and kelp ecosystems means that they are vulnerable to a multitude of threats from both land and sea. Seagrasses are globally declining, with 29% of total known areal extent lost between 1879 and 1980 and rates of loss on the rise at 7% yr-1 since 1990[3.] Kelps are documented to have declined in 38% of the ecoregions that they inhabit[6,] associated with dramatic shifts in trophic dynamics and resulting in up to 95% canopy loss (e.g.,[7]). Threats to these ecosystems include habitat loss,

pollution and degraded water quality, eutrophication, disease, invasive species, and thermal stress[8]. The consequences of climatic change have been intensely documented in recent decades, with evidence that rising temperatures strongly affect survival, growth and reproduction of many marine species[10]. The warming of the ocean also drives greater stratification of the water column, reducing mixing in some parts of the ocean and consequently affecting nutrient availability and primary production. Given ocean conditions that are rapidly changing, strategic conservation interventions that integrate research into restoration are urgently needed.

Strategic Conservation Approaches

Climate change and expanding human populations are converging threats to the stability of marine ecosystems, leading to global declines in critical habitat and biodiversity. Sustainable Development Goal 14 of the United Nations aims to "conserve and sustainably use the oceans, seas and marine resources for sustainable development," which will require rebuilding and restoring these ecosystems that deliver the many co-benefits that society receives from a healthy ocean[11]. These co-benefits include the support of the growing seafood industry, improvement of water and air quality, in turn protecting human health[2], protection against coastal erosion[12], and mitigation of climate change impacts especially in degraded environments adjacent to human use[13]. Restoration of degraded ecosystems has been shown to be effective in re-building marine ecosystems, but can cost on average between USD $80,000 - $1,600,000 per hectare, with median total costs likely to be two to four times higher[14]. Restoration strategies and science-informed responses to global change ensure that necessary and costly restoration efforts will succeed in the face of changing ocean conditions and will maximize co-benefits for humans and nature.

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Chapter Overview

My research focuses on targeted, innovative management solutions that can reconcile the objectives of human needs alongside those of environmental sustainability. Throughout this thesis, I aim to (1) reinforce the value of marine ecosystems by quantifying an increasingly important natural service provided by seagrass meadows, (2) advance approaches for scaling canopy-forming kelp forest restoration to support coastal urban futures, and (3) assess the influence of assisted evolution techniques as restoration strategies to anticipate future environmental stressors.

Chapter 1

Urban seagrass ecosystems as green infrastructure to mediate human pathogens in food from the sea

With an estimated 1 billion people projected to reside within low elevation coastal zones by 2060, there is an urgent need for novel advances in affordable water treatment measures and infrastructure. In this chapter, I highlight the global potential for marine greening using seagrass ecosystems to reduce human health risks and support food from the sea for growing coastal urban communities. I conducted a large-scale survey and replicated field experiments across the greater Seattle Metropolitan Statistical Area to examine the influence of temperate seagrass filtration on pathogenic microbial communities at regional and local scales within food from the sea. My results indicate that seagrass ecosystems reduce putative human bacterial pathogens in shellfish bioindicators in urban regions. This study suggests seagrass meadows could provide an entirely novel ecosystem service by mitigating waterborne pathogens in seafood. I believe these findings will have broad and practical appeal to designers of coastal infrastructure, engineers, urban planners, medical and veterinary practitioners, economists, aquatic and

terrestrial scientists, and policy-makers addressing the impacts of rapid population growth on clean water and human health.

Chapter 2

Field collection and laboratory maintenance for restoration of canopy-forming giant kelp

Kelp forests along the California coast are facing unprecedented loss due to climate-driven ecological stressors necessitating innovative restoration strategies. Given global declines and documented 95% loss in California, effective application of these strategies will greatly benefit from the ability to harness citizen science efforts at the management scale. However, there remains a lack of resources for researchers, both technical and non-technical, that provide accessible guidelines for kelp restoration. Supported by California Sea Grant and the Ocean Protection Council, I have amended a newly developed and cost-effective restoration strategy for canopy-forming giant kelp in California, known as *'green gravel'*. In this chapter, I provide detailed written and video protocols for reproductive tissue collection, sporulation, inoculation, rearing, culturing, maintenance and monitoring. The overarching goal of these protocols is to simplify, centralize, and democratize existing practices in this field, to facilitate the up-scaling restoration of valuable underwater kelp forests. This project directly aligns with efforts to develop rapid and cost-effective restoration strategies for kelp forests worldwide. Outcomes will contribute practical protocols needed to manage and restore kelp forests in California and inform global-scale techniques as part of the *'green gravel'* restoration consortium. This project will directly engage with the local stakeholders by providing an accessible restoration tool that harnesses citizen science efforts at the management scale, and a multi-faceted educational outreach tool that simultaneously elucidates critical stages of kelp biology as well as current conservation methods.

To supplement written protocols for the culture and deployment of '*green gravel'* substrates with visual aids, I have developed a video protocol to guide management and restoration for the Southern

Californian canopy-forming giant kelp, *Macrocystis pyrifera*. Current kelp protocols outline technical methods for practitioners that are familiar with these processes and equipped with the necessary resources and facilities. My supplementary video protocols act as critical visual aids for those who are interested in kelp life history, culture, and restoration, without the need for prior knowledge or experience. Paired with comprehensive written protocols, these visual guides will help to facilitate broad implementation and uptake of this restoration method.

Chapter 3
Thermal acclimatization to future-proof giant kelp forest restoration

As critical marine habitats continue to deteriorate at unprecedented rates, it has been suggested that traditional techniques of reviving populations should be elevated to incorporate concepts of assisted evolution that consider ecological resilience and adaptive capacity to anticipate future environmental stressors. Failure to integrate such methods may result in failure of restoration efforts. Assisted evolution has an exciting possible application with kelp restoration through translocation of beneficial, locally adapted genotypes into threatened areas or acclimatization of sporophytes to forecasted environmental stressors.

For my final chapter, differentially prime early stage giant kelp individuals (*Macrocystis pyrifera*) in differential rearing conditions from two potentially locally adapted source populations and assess stress-tolerance later in the kelp life history. Studies suggest that under projected climate-change conditions, increased temperature is particularly detrimental for development of kelps beyond the germling stage. With artificial treatments during cultivation and rearing, I found that kelps reared in stressful thermal conditions performed better than those reared in optimal thermal conditions when exposed to heatwave conditions. These results suggest that rearing *M. pyrifera* at warmer temperatures may confer an stress memory-related advantage when deployed into warming oceans with increasing

frequency and magnitude of marine heatwaves, providing an avenue forward for marine managers looking to future-proof restoration efforts. Experimental manipulation of source populations and experimental hardening of restoration efforts will inform management strategies that will maximize survival and retention of transplanted kelps under projections of future climate stressors.

Chapter 1

Global seagrass ecosystems as urban green infrastructure to mediate human pathogens in food from the sea

Manuscript in revision at *Nature Sustainability*

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Main Text

Global food demand is accelerating at a rate that is projected to destabilize food security[1]. Food from the sea offers a promising option to meet global protein and nutritional needs[2,3], however urban production and harvest in adjacent coastal environments is challenged by food safety and public health concerns[4]. Urban greening – the strategic vegetation of urban landscapes – can deliver co-benefits to food security, public health, and the environment in terrestrial ecosystems[5]. Seagrasses form the largest coastal ecosystem and support global fisheries and aquaculture productivity[6], therefore their proximity to urban areas offers an opportunity to assess the utility of marine greening to enhance food systems. Evidence for

the filtration services provided by seagrass ecosystems to benefit public health outcomes is mounting[7], and a growing number of studies demonstrating the role seagrasses play in reducing waterborne bacterial species with human pathogenicity from terrestrial sources[8,9]. However, it remains unknown whether this ecosystem service is conferred to an associated marine species, let alone a globally important food source.

Marine bivalves contribute to more than half of global seafood production and are effective bioindicators for bacterial contamination because their tissues reflect the local environment[10]. Following a three-month deployment of mussels across 20 coastal urban locations (N = 11 locations with seagrass present, 9 locations with seagrass absent) spanning a latitude of 200 km, I assessed the bacterial communities associated with the mussel gill tissues using amplicon sequencing of the *16S rRNA* gene (1.7 million total bacterial sequences from 60 samples). First, I found that the composition of the overall bacterial community in mussel tissue was significantly different and distinct among the seagrass present and absent locations (perMANOVA: $F = 2.6$, $p = 0.001$, dispersion: $F = 2.8$, $p = 0.10$, **Figure 1.1A**). Next, I quantified 1,976 sequencing reads belonging to 30 species of bacteria with human pathogenicity and found that mussels from locations with seagrass present had a 65% lower relative abundance of bacterial pathogens when compared to locations where seagrass was absent (estimate = -1.06, standard error = 0.25, $z = 4.17$, $p < 0.001$, **Figure 1.1B**). Two-thirds of all potentially pathogenic bacterial species had a higher mean relative abundance at locations where seagrass was absent (21 out of 30 species) compared to locations where seagrass was present (**Figure 1.1C**). The mean relative abundance of three species of bacteria with known pathogenicity from the consumption of contaminated food (*Enterococcus faecalis, Listeria monocytogenes*, and *Acinetobacter radioresistens*)[10] was higher at locations where seagrass was absent, while one species (*Bacillus pumilus*) was higher at locations where seagrass was present. The mean relative abundance of two bacterial species with heightened pan-resistance to antibacterial agents (*Enterococcus faecalis* and *Pseudomonas aeruginosa*)[11] was higher only where seagrass was absent (**Figure 1.1C**).

The global co-occurrence of seagrass ecosystems, highly populated coastal urban regions, and bivalve harvest illustrates the relevance and an opportunity to mitigate public health risks associated with the contamination of food from bacterial species with known human pathogenicity (**Figure 1.2**). Our model estimates that over one-third (662 of 1,860) of urban agglomerations (populations over 300,000 people) and 19 megacities (populations over 10 million people) are currently within 50 km of a seagrass ecosystem. While I do not project the number of agglomerations to change by 2030, our estimates suggest the number of people living in urban agglomerations will increase by 15% (from 1.1 billion to 1.3 billion people) and include 24 megacities.

Financial returns gained by harnessing ecosystem services support innovative approaches to conservation of biodiversity, however determining the full valuation of these services remains a major hindrance to these efforts. Seagrass meadows are already recognized to provide high-value services, including acting as critical habitat for associated organisms, cycling nutrients, sequestering carbon, and providing protection to shorelines, which together are estimated to hold a value of up to US $770 billion per year[12]. We are only beginning to recognize the extent of the sanitation and public health services provided by seagrass ecosystems, with avoided cases of gastrointestinal illness estimated to reach 24 million each year (20% of global cases) when seagrass is present[7]. Here, I present the first evidence for seagrass ecosystems to reduce the abundance of bacterial species with known human pathogenicity in an associated marine species and globally important food product. Given that human infectious diseases in the marine environment from terrestrial sources are estimated to have an economic cost of US $12 billion per year[13], and recent predictions estimate heightened antimicrobial drug resistance could result in over 300 million deaths and a cost of $100 trillion to the global economy[11], seagrass ecosystems have significant potential to confer added public health benefits and economic savings.

Seagrass meadows rank among the most threatened ecosystems globally, with rates of loss estimated as high as 7% per year[14]. By showing that more than one-third of global urban agglomerations are adjacent to seagrass ecosystems, our study highlights the immediate opportunity to integrate existing

natural infrastructure into built solutions to address the urgent biodiversity and climate crises[12], while simultaneously improving human health. Marine and terrestrial environments are often managed as independent entities[15], but I suggest extending terrestrial urban greening networks to incorporate seagrass ecosystems could enhance large-scale strategic conservation and restoration consistent with numerous global sustainability targets.

Methods

I modeled the number of urban agglomerations adjacent to seagrass ecosystems by overlaying the range of global seagrass ecosystems obtained from the International Union for Conservation of Nature (IUCN) Red List of Threatened Species with current and projected urban agglomerations and population sizes obtained from the United Nations (UN) Department of Economic and Social Affairs, Population Division. Next, I applied a 50 km spatial buffer around seagrass ecosystems and filtered urban agglomerations that occurred within the buffered seagrass ecosystem range and calculated the number of urban agglomerations (populations over 300,000 people) and megacities (populations over 10 million people) from this filtered list. I used a classified gradient approach to identify 20 urban locations with either seagrass present (N = 11 locations) or absent (N = 9 locations) across the greater Seattle Metropolitan Area and deployed mussel bioindicators (*Mytilus trossulus*) at each location and collected them 90 days later. DNA was extracted from gill tissues of three replicate mussels at each location (N = 60 samples) and the bacterial community profiles were analyzed using *16S rRNA* gene amplicon sequencing and assessed for the presence of bacterial species with known pathogenicity to humans[10]. Please refer to the Supplementary Information for the full detailed methods.

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Figures

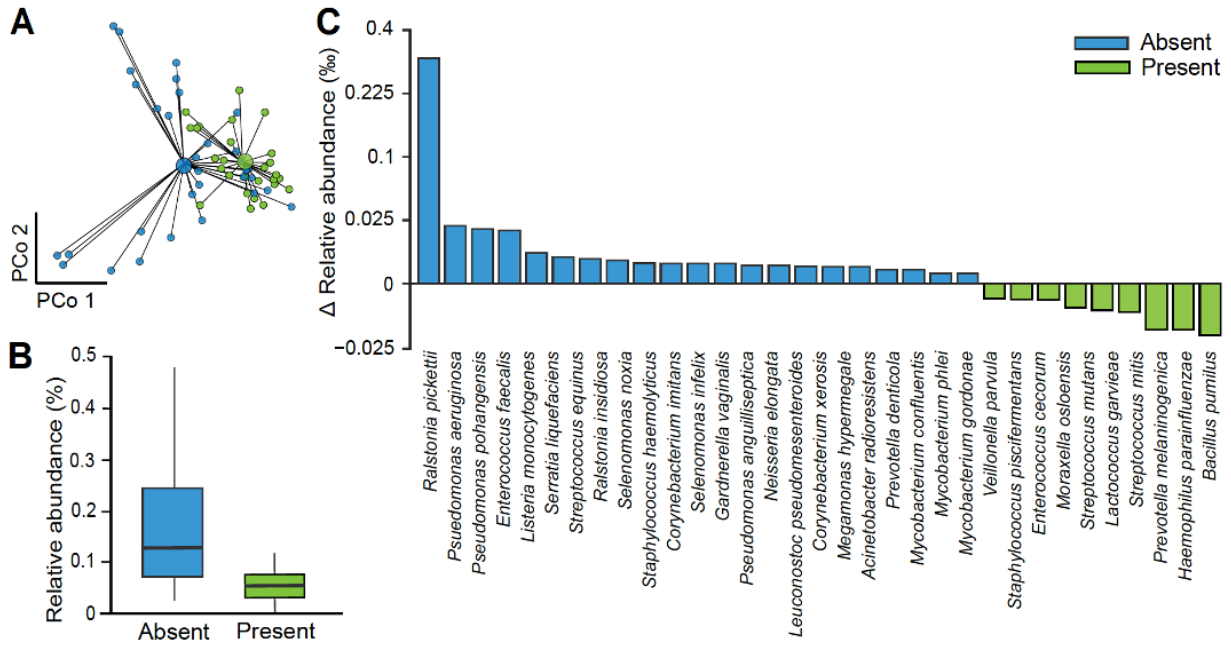

Figure 1.1. Urban seagrass ecosystems influence bacterial species with known human pathogenicity in seafood. (A) Bacterial community composition in mussels using a principal coordinate (PCo) analysis. Each point represents an individual sample at locations with seagrass present (N = 33 samples, 11 locations) and absent (N = 27 samples, 9 locations). (B) Total relative abundance of potential human bacterial pathogens in mussels at locations with seagrass present and absent. (C) Barplot represents the change in mean relative abundance of species-specific bacterial pathogens between seagrass present and absent locations. Species are ordered by increasing and diverging differences in mean relative abundance.

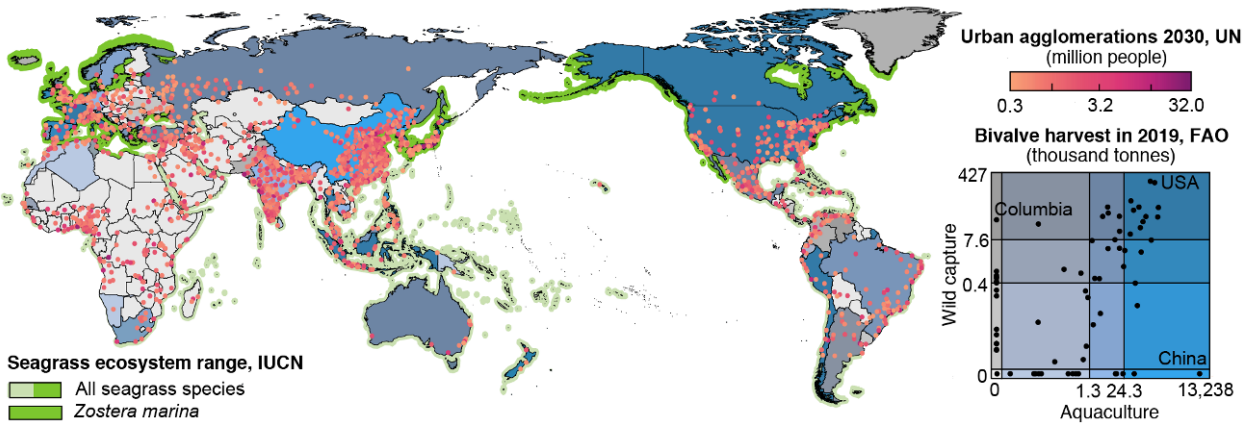

Figure 1.2. Global co-occurrence of seagrass ecosystems, wild capture and aquaculture bivalve harvest, and projected urban population agglomerations by 2030. World urbanization prospect data were obtained from the UN Department of Economic and Social Affairs, Population Division. Seagrass species distributions were obtained from the IUCN Red List of Threatened Species (v2019-3). Bivalve wild capture and aquaculture harvest dataset was synthesized from the Food and Agriculture Organization (FAO). Data available in **Supplementary Table 3**.

Supplementary Materials Text

Promising experimental evidence of chemical and biological pathogen regulation by seagrass has been shown in several field and laboratory studies, suggesting seagrass ecosystems offer a viable nature-based solution for urban filtration. For example, phytochemicals extracted from seagrass tissues kill or inhibit numerous bacterial pathogens that affect humans, fishes and invertebrates[1,2]. Distinct microbial biofilms dominated by cyanobacteria on seagrass blade surfaces and antimicrobial compounds from fungi found growing within the tissues of several seagrass species can inhibit multiple fish and human pathogens[3]. Tropical seagrass meadows mitigate waterborne pathogens of humans, fishes and invertebrates associated with inputs of untreated terrestrially-sourced wastewater in Indonesia, with seawater samples from seagrass present sites containing 50% less pathogens compared to paired seagrass absent sites[4]. Filtration is seen regardless of the predominant species of seagrass[5,6], demonstrating that regulation within seagrass ecosystems is conserved among several global temperate and tropical regions. Recently, the filtration service of seagrass was conservatively estimated to prevent 8 million cases of gastroenteritis globally each year[7].

Study location selection and defining urban status

Washington is the leading producer of farmed clams, oysters, and mussels in the United States, with an annual production of over 23 million pounds[8]. Seattle and several major coastal cities that border the Puget Sound had an estimated total population of 3.4 million people in 2020[9]. Seattle has experienced accelerating urban growth in the past decade, with a density of 2,800 people per square kilometer within the city center (2010 census estimate)[10]. This region has an estimated 23,000 hectares of seagrass coverage (*Zostera marina*)[11].

Urban agglomerations are defined as metropolitan areas with a population greater than or equal to 300,000 people[9]. I used a classified gradient approach to select urban locations[12]. I integrated information

on population density[13] and National Oceanic and Atmospheric Administration coastal change analysis program (NOAA C-CAP) land cover[14] by Washington State watershed administrative units (WAU)[15]. For each WAU, I found the mean population density using zonal statistics in QGIS. For land cover, I used the proportional composition of each land cover class provided by NOAA, with aggregated similar categories before calculation (forest, agriculture, and wetlands). I then assigned locations according to WAUs. Using the values for all locations, I then calculated a Mahalanobis distance matrix to account for differences in scale between population density (≥ 0) and proportional land cover composition ([0,1]) using function *vegdist()* from package *vegan*[12,16]. Using the Mahalanobis distance matrix, I then used a hierarchical clustering algorithm to delineate each location into urban and non-urban based on Ward's minimum variance method using function *hclust()* from package *stats*. I also conducted a principal coordinates analysis using function *cmdscale()* from package *stats* to visualize the clustering algorithm and determine which cluster defined urban and non-urban. To ensure that our use of WAUs, which vary in size from 21,136 to 179,879 acres, were representative of the conditions at each location, I also visually checked the location of each location using satellite imagery in Google Earth[17]. From our gradient clustering analysis, 20 locations were classified as urban. In consultation with the Washington Department of Fish and Wildlife (WDFW), I classified each urban location by presence or absence of seagrass[11]. Each location was characterized by high population densities and non-vegetative, impervious cover, representing areas that experience high volumes of surface water runoff as it moves across the urban landscape during and after rain events. On average, locations with seagrass present were located in WAUs with greater population densities (1,288 ± 197 and 1,055 ± 172) and proportions of high, medium and low density urban development compared to locations where seagrass is absent (**Supplementary Table 1.1**). I deployed commercially aquacultured Pacific blue mussels, *Mytilus trossulus*, as bioindicators in accordance with methods in[18]. I deployed five cages, each containing 80 adult mussels sourced from Penn Cove Shellfish on Whidbey Island, at each of the 20 urban locations for 90 days from December 2017 to February 2018.

At the end of the deployment period, a random selection of three mussels from each location (N = 60 samples) was allocated to this study, immediately placed on dry ice, and stored at -80ºC.

Seagrass ecosystems are threatened by numerous pressures, particularly poor water quality[19]. Following an assessment of seagrass ecosystems present within a 10 km^2 subset of the highly urbanized city center of Seattle we found that there were no significant differences in density of plant shoots among locations (mean = 244 shoots per m^2 ± 7.2 standard error, pairwise comparison estimate range [-0.13, 0.06], all p-values > 0.47 after false detection rate adjustment, **Supplementary Table 1.2**.

Human pathogen contamination

Gill tissues can be an entry point for pathogens, where disturbance of the gill microbiome has been shown to facilitate the establishment and growth of pathogens[20]. Mussels collected were thawed on ice immediately prior to processing (N = 60 samples). Shell width and length was measured using calipers at the widest points of each major and minor axis. Mussel tissue samples weighing approximately 500 mg were removed from the gill of each individual using sterile dissection blades and forceps and placed in 1.5 mL of DNA/RNA Shield (Zymo Research, Irvine, CA, USA) and stored at -80ºC.

Tissue samples were shipped to Zymo Research (Irvine, CA, USA) for DNA extraction, *16S rRNA* gene amplicon library preparation and amplicon sequencing. The ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA) was used for DNA extraction from 500 µl of sample. The *16S rRNA* gene amplicon libraries were prepared using the Quick-16S NGS Library Prep Kit (Zymo Research, Irvine, CA) and the Quick-16S™ Primer Set V3-V4, a proprietary version of the 341F/785R primer set that provides the best coverage of the *16S rRNA* gene amplicon while maintaining high sensitivity. ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA) was used as a positive control for each targeted library preparation and negative library preparation controls were included to assess the level of bioburden carried by the wet-lab process. The sequencing library was prepared using an innovative library

preparation process in which PCR reactions were performed in real-time qPCR machines to control cycles and therefore limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean & Concentrator (Zymo Research, Irvine, CA), then quantified on a TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit (Thermo Fisher Scientific, Waltham, WA). The final library was paired-end sequenced (2x300bp) on the Illumina MiSeq platform with v3 chemistry, and >10% PhiX spike-in.

Sequence data obtained from Zymo Research was processed using the UNOISE2 pipeline[21] as implemented in the USEARCH package (version 9.2; https://www.drive5.com/usearch)[22]. The raw forward (R1) and reverse (R2) sequence .fastq files of the 60 samples contained a total of 2,203,572 reads (ranging between 10,149 and 74,692 reads per sample). A total of 1,996,320 paired reads were merged using -fastq_mergepairs. Primer sequences were trimmed using -fastx_truncate and reads were quality filtered with the -fastq_filter script, generating a filtered .fasta file containing 1,831,551 reads with an average length of 420.8bp. A total of 434,793 unique sequences were identified using the –fastx_uniques script followed by denoising of the sequence dataset with the UNOISE2 algorithm, obtaining 82,583 denoised sequences or 'zero-radius Operational Taxonomic Unit' (zOTU). The -usearch_global script was then used to generate an OTU table at the 99% similarity level. The taxonomy was assigned to each OTU based on the All Species Living Tree Project (LTP) database (release LTP_09_2021) using the -sintax algorithm with the confidence cut-off set at 0.8. The OTU table was converted to the HDF5 biom format and the taxonomic assignment metadata was added. The final OTU table contained 1,816,464 reads belonging to 47,446 OTUs, with an average of 30,274 reads per sample (min 9,181; max 68,532). The OTU table, sample metadata, and representative sequences of each OTU will be available at the Dryad Digital Repository. Raw sequence data files were deposited in the National Center for Biotechnology Information Sequence Read Archive under BioProject accession number PRJNA955437.

Global urban agglomerations

Urban agglomerations are defined as metropolitan areas with a population greater than or equal to 300,000 people[9]. To determine the number of urban agglomerations that are adjacent to seagrass ecosystems, I overlaid the most recent estimated range of global seagrass ecosystems available from the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (v2019-3)[23] with the most current (2018) and projected (2030) urban agglomerations and populations sizes from the United Nations (UN) Department of Economic and Social Affairs, Population Division[9]. Using the overlaid datasets, I applied two different spatial buffers (10 km and 50 km) around the seagrass ecosystem layers to capture terrestrial areas where urban agglomerations could occur in QGIS[24]. I then filtered the urban agglomerations that occurred within the buffered seagrass ecosystem range for locations in the range of all seagrass ecosystems (multiple species) and specifically for the temperate eelgrass species *Zostera marina*. I then calculated the number of urban agglomerations in 2020 (greater than 300,000 people) and megacities (greater than 10 million people) from this filtered list[9] (**Supplementary Table 1.3**).

Statistical analyses

All models were constructed and fit in the R statistical environment v4.0.1[25]. A comprehensive list of pathogenic species of bacteria implicated in human illness were compiled from current online databases according to[26], and corresponding OTUs were subset from the OTU table. The composition of the bacterial human pathogen community was calculated as the proportion of the number of reads for each OTU normalized to the total numbers of reads for all OTUs identified as bacterial human pathogens.

To test for differences in the relative abundance of potential human bacterial pathogens, I employed generalized linear mixed effects models with beta binomial error distribution and logit link using the function *glmmTMB()* in package *glmmTMB*[27]. I modeled the joint pathogenic and non-pathogenic count of OTU reads and included seagrass status (present versus absent) and mussel shell area as fixed factors, while location was included as a random factor to remain consistent with the study design. I formed model

sets of all possible reduced models from the full model described above and compared models using Akaike information criterion (AIC) to determine the best fit model (**Supplementary Table 1.4**).

Metabarcoding sequence datasets are compositional, therefore I followed a compositional data (CoDa) framework according to[28,29]. As the compositional data framework does not allow for zeros, I used a Bayesian-multiplicative replacement and imputed a Dirichlet multinomial prior using function *cmultRepl()* in package zCompositions[30] for taxa that were detected in at least 5% of samples. I then performed a centered log-ratio transformation and calculated Euclidean distances in the transformed space to create an Aitchison distance matrix. I then ran a perMANOVA on the Aitchison distance matrix. I included location and seagrass status (present or absent) as factors and ran 999 permutations to assess differences in bacterial community compositions using function *adonis()* from package *vegan*[16]. I used a dispersion test to determine if the results of the perMANOVA were influenced by differences in dispersion among groups using the function *betadisper()* of package *vegan*. To visualize the results of the perMANOVA, I conducted a PCoA using the function *cmdscale()* of package *stats.* (**Supplementary Table 1.5**). Data and code will be available at the Dryad Digital Repository. Previously published datasets used in all analyses are described in the code.

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Supplementary Tables

Supplementary Table 1.1. Mean population density and proportional land cover categorization at sites with seagrass absent and present. Population density data was sourced from the Center for International Earth Science Information Network hosted by the National Aeronautics and Space Administration[13]. Land cover data was sourced from the National Oceanic and Atmospheric Administration coastal change analysis program[14].

	Population Density	High Density	Medium Density	Low Density	Open Space	Agriculture	Grassland	Forest	Shrub & Scrub	Wetland
Absent	1,055 ± 172	6.9 ± 0.7%	17.3 ± 1.9%	29.9 ± 2.7%	7.4 ± 1.1%	4.3 ± 1.8%	0.9 ± 0.2%	28.6 ± 4.2%	1.6 ± 0.3%	2.9 ± 0.9%
Present	1,288 ± 197	7.8 ± 0.7%	20.1 ± 1.9%	33.4 ± 2.9%	6.6 ± 1.2%	1.7 ± 0.8%	0.7 ± 0.2%	26.5 ± 5.0%	1.3 ± 0.4%	1.6 ± 0.2%

Supplementary Table 1.2. Density of seagrass at a subset of selected locations within a 10 km^2 region of the highly urbanized city center of Seattle. Mean density = 244 shoots per m^2 ± 7.2 standard error (SE), with no significant difference among sites.

Latitude	Longitude	Eelgrass density (shoots per m^2 ± SE)
Seagrass Present		
47.631	-122.400	247.6 ± 78.9
47.596	-122.385	232.4 ± 37.8
47.572	-122.418	252.0 ± 38.3
47.663	-122.432	252.4 ± 28.7
47.642	-122.420	237.8 ± 49.6
47.538	-122.398	265.3 ± 41.2
Seagrass Absent		
47.631	-122.397	-
47.595	-122.384	-
47.596	-122.418	-
47.663	-122.434	-
47.650	-122.426	-
47.537	-122.397	-

Supplementary Table 1.3. The number of coastal urban agglomerations within the range of seagrasses in the year 2030. Data on the range of seagrasses are from the International Union for the Conservation of Nature (v2019-3) and urban agglomerations are from the United Nations Department of Economic and Social Affairs (2018).

Spatial Buffer (km)	Seagrass Range	Number of urban Agglomerations	Projected Human Population in 2030	Urban Agglomerations (%)	Urban Agglomeration by population (%)	Megacities in 2030[b]
10	*Zostera marina*	141	266,144,320	7.58%	8.70%	4
50	*Zostera marina*	243	445,859,250	13.06%	14.58%	8
10	All species	424	862,034,750	22.80%	28.18%	15
50	All species	662	1,315,494,600	35.59%	43.01%	24

[a]Megacities are defined as urban agglomerations that have a population of greater than 10 million inhabitants.

Supplementary Table 1.4. Model selection table for assessing the influence of seagrass status on the relative abundance of putative human bacterial pathogens from highly urbanized locations.

	[1] Seagrass Status	[2] Null
Intercept	-6.47 ***	-6.94 ***
Intercept SE	0.15	0.16
Mussel Area	0.14	0.08
Mussel Area SE	0.09	0.09
Seagrass Status - Present	-1.06 **	-
Seagrass Status - Present SE	0.24	-
Location Variance	0.17	0.34
Log Likelihood	-245.1	-252.3
Degrees of Freedom	5	4
AIC	501.4	513.3

** P < 0.01, *** P < 0.001

Model Equations:

[1] $logit(Pathogens,\ Non-pathogens) \sim \beta_{1|Location} + \beta_2 Mussel\ Area + \beta_3 Seagrass\ Status$

[2] $logit(Pathogens,\ Non-pathogens) \sim \beta_{1|Location} + \beta_2 Mussel\ Area$

Supplementary Table 1.5. Output from a permutational multivariate analysis of variance (perMANOVA) investigating the influence of seagrass presence on bacterial community composition.

	Degrees of Freedom	Sum of Squares	R^2	pseudo-F	P > (pseudo-F)
Seagrass Status	1	15,828	0.036	2.365	0.001
Watershed Administrative Unit	7	83,778	0.19	1.789	0.001
Residual	51	341,267	0.77		
Total	59	440,375	1.0		

Chapter 2

Field collection and laboratory maintenance for restoration of canopy-forming giant kelp

In revision for the *Journal of Visualized Experiments*

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Introduction

Canopy-forming kelps are globally important foundation species, dominating coastal rocky reefs in temperate and Arctic seas[1]. These kelps form structurally complex and highly productive biogenic habitats known as kelp forests that support taxonomically diverse marine communities[2]. Kelp forests worldwide provide many ecosystem services to humans, including commercial fisheries production,

carbon and nutrient cycling, and recreational opportunities, with a total estimated value of US$500 billion per year[3].

Despite their substantial value, kelp forests face growing anthropogenic pressures in many regions[3]. Climate change presents one of the most significant threats to kelps, due to long-term ocean warming combined with the increasing frequency of temperature anomalies[3–7]. Increased ocean temperatures are associated with nutrient limitation[8], while exposure to heat stress above physiological thresholds can result in mortality[9]. In combination with variable regional local stressors[7], kelp populations are globally declining by approximately 2% per year[10] with significant losses and persistent shifts to alternate community states in certain regions[6, 11–14]. Natural recovery of kelp populations alone may not be sufficient to reverse the extent of current and projected losses[15–18], underscoring the importance of active restoration programs.

For regions where kelp spore limitation may be impeding kelp forest recovery, a relatively new kelp restoration approach called the *'green gravel'* technique was successfully trialed at the Flødevigen Research Station in southern Norway[19] and represents a promising restoration tool due to cost-effectiveness and scalability. The *'green gravel'* technique is currently undergoing trials by members of numerous international working groups (greengravel.org) across a number of different environments and several kelp species. This restoration framework is quite simple, where the workflow is as follows: (1) a spore solution is created from fertile tissue collected from reproductive adult kelps in the field and then seeded onto small substrates, such as gravel; (2) early stage kelps are reared in laboratory-controlled abiotic conditions on substrates; (3) substrates with visible sporophytes are deployed in the field on specific reefs as *'green gravel',* where sporophytes continue to grow.

As this is a newly developed technique, the efficacy of this tool has only been exhibited using sugar kelp, *Saccharina latissima*, in Norwegian fjords. While *'green gravel'* is a promising restoration tool for canopy-forming giant kelp forests in California, modifications must be made to the published protocols and tested under a range of environmental conditions. For example, there are a number of

fundamental differences between *Saccharina* and canopy-forming kelps like *Macrocystis* and *Nereocystis* that necessitate adjustments of rearing and deployment (**Table 2.1**), particularly the ultimate size of mature individuals. Average *Saccharina* plants grow to be no larger than 3m tall, whereas *Macrocystis* plants grow up to 50m. This order-of-magnitude size difference requires the reassessment of current outlined methods and experimental testing of a suite of deployment strategies.

This protocol describes the field collection and regular laboratory maintenance using *'green gravel'* as a restoration technique for the canopy-forming giant kelp, *Macrocystis pyrifera.* Here I detail the required facilities, materials, and methods for tissue collection, sporulation, seeding, rearing conditions, regular maintenance, and monitoring of early stage kelp prior to deploying this restoration technique in the field.

Protocol

1. Preparations of Facilities and Materials

    1.1. Kelp culturing facilities must be able to control seawater temperature and provide full-spectrum light and filtered aeration. Incubator systems with a built-in outlet or an access port for wires and tubing can be adapted with lights and an air source (**Figure 2.1**). If an incubator system is not within the project's scope, budget, or intended scale, water baths tempered by either cool, natural seawater or a chiller can be used. Refer to the Table of Materials for specific details.

        1.1.1. Rearing temperatures should be site- and season-specific[20], and can range between 10-15 °C. Temperature of your growth media can be measured using thermometers or temperature guns.

        1.1.2. Aeration is important for haptera development and holdfast strength for *Macrocystis pyrifera* cultures, and can be added to cultures with the use of air pumps (see Section

5.2)[21]. Filters (0.2 μm pore size) are optional and recommended, as they help reduce air-borne bacterial contamination.

NOTE: Aeration pressure must be sufficient to circulate water in all culture containers while not disturbing the attachment of early stage kelp to seeded substrates.

1.1.3. Full-spectrum lights are set to a 12 h light : 12 h dark photoperiod, using the timing settings on the light source or by plugging the light source into a timer with a programmable cycle. Light intensities range between 0-180 μmol photons m^{-2} s^{-1}, if deploying with 1-2 cm sporophytes after approximately 6 - 8 wk, and are set based on the development of your kelp cultures (see Section 5.3). Light intensity can be measured with a PAR quantum meter and adjusted using a dimmable light source or by layering cellophane or mesh over the light source.

1.2. Materials and stations used for seawater filtration and enrichment (Section 2), sporulation and inoculation (Section 4), monitoring (Section 6), and maintenance (Section 7) should be sterilized and prepared ahead of time (see Table of Materials).

1.2.1. Clean surfaces using 70% isopropyl alcohol. Take out the trash often. Handle reproductive sori tissue and clean field equipment outside the *'green gravel'* nursery if possible.

1.2.2. Sterilization methods for equipment include:

a. Autoclave using appropriate settings (glassware or instruments).

b. Rinse using a lab-grade detergent such as 5% Alconox followed by a thorough rinse with distilled water.

c. Soak in a diluted bleach solution followed by a thorough rinse with distilled water. Make sure that materials do not smell like bleach or have a slippery feel.

Dilute bleach according to manufacturer directions. After sterilization, materials can be stored in a sealed container or wrapped with foil.

1.2.3. All gravel, even when marketed as pre-rinsed, should be thoroughly cleaned before seeding. Scrub and rinse gravel until the water runs clear to remove any dust or debris. Gravel should be soaked in a 10% diluted bleach solution for at least 24 hr, rinsed in freshwater, and finally rinsed in seawater.

NOTE: Gravel should have a textured or slightly pitted surface, since gametophytes are more likely to be retained on substrates with a high rugosity. Ideally, locally harvested substrate is used to reduce contamination of the restoration site. Alternatively, aquarium-grade gravel is recommended. Calcareous substrates such as limestone should be entirely avoided[22].

2. Preparation of filter-enriched seawater media

2.1. Large volumes of filtered seawater will be needed for your growth media. Calculate how much seawater you will need to replenish your culture containers each week (see Section 7) and schedule this filtration task accordingly. Large batches of filtered seawater can be prepared ahead of time and stored in dark containers for up to 6 months in 8-10 °C refrigerated conditions. If refrigeration is not available, store in a dark, cool area.

2.2. Using a clean flask, pour your unfiltered seawater into a vacuum filtration system (pore size of 0.55-1 μm), pull the unfiltered seawater into a sterile bottle. Turn your vacuum source off before all the water is pulled through to avoid damaging the filter, and pour the filtered water into a dedicated sterile container.

2.3. Alternatively, a flow-through filtration system can be used. Here, the seawater runs through a series of three pleated filters (10 μm, 5 μm, and 1 μm) arranged from largest to smallest pore size. This system is connected to a flow-through aquarium UV light, where the seawater

flows at a rate of less than 4 G min^{-1}.

NOTE: If access to natural seawater is limited, artificial seawater can be used. Using a refractometer or salinometer, mix Instant Ocean™ with distilled water (approximately 76 g Instant Ocean™ for 2 L of distilled water) for a salinity of 32-34 ppm. Alternatively, natural seawater that has already been filtered, sanitized, and pH balanced can be purchased from aquarium stores in bulk and does not require mixing, measuring, or adjusting. Media enrichment for these options is still necessary.

2.4. Enrichment of seawater with nutrients and vitamins are critical to *M. pyrifera* growth. Provasoli Enriched Seawater media (PES) is a widely used medium designed for algal cultures[23]. This media can be purchased from algal culture centers, but can be costly at scale. Preparations of PES and additional vitamins for *M. pyrifera* growth are described in[24]. Enrich every 973 mL of filtered seawater with 27 mL PES. Alternatively, industrial-level culturing media such as Fritz Aquatics PRO™ F/1 and F/2 can be purchased and mixed in a 1:1 ratio (30 mL F1 + 30 mL F2 for 227 L of seawater).

3. Field Collection

3.1. Determine the timing of sporophyll collections to mimic the natural reproductive cycle of local *M. pyrifera* populations. While certain regions produce mature sori in all seasons, others have a more constricted reproductive window. For example, in Southern California and Mexico, *M. pyrifera* sorus tissue can be collected all year. In Central and Northern California, sori mature predominantly in the Fall. Consult local experts (*i.e*. kelp researchers, managers, ecologists, citizen scientists, dive groups) to ensure appropriate timing for sporophyll collection.

3.2. By SCUBA, select 3-5 sporophyll blades from 10-15 fertile *M. pyrifera* individuals with visible sori, spaced 2-5 meters apart. Sporophyll blades are kept separately according to the

parent individual from this point forth. Select clean and intact sporophylls if possible, with little to no fouling or degradation.

NOTE: Sporophylls grow in a dense "skirt" at the base, above the holdfast of the adult kelp, and can be identified by their lack of gas-filled pneumatocysts[1]. Mature sorus tissue is often slightly raised and darker in color than surrounding tissue[1] (**Figure 2.2**).

NOTE: Obtain the necessary permits for kelp tissue collection that meet your region's criteria. Consulting with all applicable levels of government and obtaining the necessary permissions can be an incredibly time consuming part of the culturing process, and must be incorporated into project timelines.

3.3. Transport sporophyll blades in dark collection bags to avoid overexposure to sunlight, filled minimally with seawater from the site to keep blades wet, and stored in coolers at approximately 12 °C until arrival at the culturing space.

NOTE: Sporophylls can be shipped to or from other locations. Rinse sporophylls with filtered-sterilized seawater. Wrap blades collected from a single *M. pyrifera* individual in moist paper towels soaked in filtered-sterilized seawater, and again in aluminum foil to avoid light penetration and desiccation. This method of storage is known as "the burrito method." Place these packages (affectionately referred to as "kelp burritos") in a cooler with ice, with a protective barrier such as recycled bubble wrap or cardboard (**Figure 2.3**). Prepare the cooler for overnight shipment, and ensure that someone is available to receive the shipment and place the packages in refrigerated conditions.

4. Sporulation

4.1. Sporophylls should be processed in a temperature-controlled environment between 10-15 °C and away from any other cultures. Prepare and sterilize your instruments and stations ahead of time. Wear protective gloves when handling kelp tissue to reduce contamination.

4.2. Rinse sporophylls in sterile seawater and gently dry with paper towels, rubbing in one direction only, to remove any fouling on the blade.

4.3. After rinsing, sporophylls are optionally stored for 12-48 hr in 4 °C refrigerated conditions to desiccate, encouraging spore release from sorus tissue[25]. For storage, sporophylls are laid out separately on paper towels dampened slightly with sterile seawater, rolled up into a tidy wad, and wrapped in aluminum foil.

4.4. Select ripe sorus tissue, and cut into 25 cm^2 sections using sterile scissors (**Figure 2.4**). Aim to select 1-2 clean sori sections from 10-15 individual kelp parents to promote genetic diversity.

4.5. Gently rub in one direction only, to remove the mucus covering. Thoroughly rinse the section in seawater, scrub both sides with a sterile gauze, followed by a 30 seconds to 1 min freshwater bath, and another sterilized seawater rinse Refresh your freshwater bath and sterilize your materials in between the handling of different sori sections to reduce cross-contamination.

4.6. Submerge each sori section in enriched-sterilized seawater tempered to 10-15 °C within a sterile 50 mL Falcon tube. To trigger sporulation, place tubes at 4-12 °C in the dark for a maximum of 4 h. If no refrigerator is available, store in a low light, cool area.

NOTE: Alternatively, sori sections can sporulate in a single, sterile container.

4.7. Using a compound microscope and hemocytometer, observe the spore density of 3-4 samples every 30 min up to 4 h. Change pipette tips between samples. If densities are sufficient before 4 h, move on to the next step. If a sori section produces no spores after 4 h, discard the sample. Spores can settle within hours after release, but you may observe spores swimming in a circular motion.

4.8. Remove each of the sori sections from their Falcon tubes, combine the resulting spore solutions into a single, sterilized container, and quantify the final combined density (**Figure**

2.5).

5. Inoculation

5.1. Calculate the final volume of spore solution needed for inoculation, for a final concentration of approximately 500-1,000 spores mL^{-1} in your culture containers.

5.1.1. To calculate the concentration of your combined spore sample from your counts of the center grid of your hemocytometer, divide your count by 10^{-4} mL (representing the volume of solution viewed in the hemocytometer).

e.g. 50 spores / (10^{-4} mL) = 500,000 spores mL^{-1}

5.1.2. To determine the volume of spore solution to add to each container, determine the amount of filter-enriched seawater needed to submerge your substrates. In this example, we will use 300 mL of seawater.

5.1.3. To find the total number of spores in each container, multiply this seawater volume by your desired concentration. In this example, we use 1000 spores / mL.

e.g. 300 mL x 1000 spores mL^{-1} = 3 x 10^5 total spores

5.1.4. To determine the total volume of spore solution to be added, divide the total amount of spores by the concentration of spores per mL in your spore solution.

e.g. 3 x 10^5 total spores / 500,000 spores mL^{-1} = 0.6 mL spore solution

5.2. Inoculate the calculated volume of spore solution into your culture container using a sterile pipette tip, that contains (1) sterile glass slide(s) for monitoring kelp development, (2) aquarium-grade substrates submerged in (3) enriched filtered seawater media (**Figure 2.6**). Close the container and gently stir to distribute spores. Place the container into your incubator outfitted with lights and aeration lines for kelp culturing. For rearing conditions, see Section 6.

6. Rearing Conditions

6.1. Set your laboratory incubation system temperature between 10-15 °C based on the temperature at your deployment site.

6.2. After 1 day, light aeration with a filtered air source is provided.

6.3. Full spectrum LED lights for aquatic plants outfitted above each shelf are set to a 12 h light: 12 h dark cycle, with light intensities ranging between 0-180 µmol photon m^{-2} s^{-1}:

6.3.1. 5-10 µmol photon m^{-2} s^{-1} from 0-1 d, increased to 20 - 30 µmol photon m^{-2} s^{-1} through the end of 1 wk.

6.3.2. From this point on, increase the irradiance by 10-20 µmol photon m^{-2} s^{-1} every 3-4 d until reaching an irradiance of 180 µmol photon m^{-2} s^{-1} at the end of 6 wk.

6.3.3. Continue to rear cultures at 180 µmol photon m^{-2} s^{-1} through the end of 8 weeks, or when sporophytes have reached approximately 1-2 cm in length.

NOTE: See Supplementary File 1 for a calendar to track activities and expectations for Macrocystis cultures. It indicates the timing of adjustments to light and aeration, as well as weekly media changes.

7. Monitoring

7.1. Understanding the kelp life cycle is important to successfully rearing *'green gravel'* (see **Figure 2.7** for examples of developmental life history stages). Timelines for development may vary.

7.1.1. Once released from fertile tissue, spores will settle within hours. Settled spores are observed at 0-1 d.

7.1.2. Spores can germinate within a few hours, demonstrated by the formation of a germ tube. Germination is typically observed at 1-2 d.

7.1.3. The first gametophytic cell will develop as stores from the spore are transferred through the germ tube. Early gametophytes are typically observed at 1-4 d.

7.1.4. Gametogenesis, the process by which cells undergo division and differentiation to form male and female gametes, is typically observed within the first two weeks. Gametophyte sex can be distinguished based on size and shape. Female cells are 5-7 times larger than male cells. Male gametophytes grow thin, filamentous branches, whereas females are more round or ovoid in shape. Female gametophytes produce oogonia, which contain eggs or ova. Eggs are typically observed within 2-3 wk.

7.1.5. Sperm released from the male gametophytes swim to the female gametophytes, where they fertilize the eggs, resulting in the formation of diploid zygotes. Sperm travels for a few millimeters of water. Having the right density will ensure successful reproduction by proximity[26,27]. The fertilized eggs develop into embryonic sporophytes, which remain attached to the female gametophytes. However, the ovum may detach and settle elsewhere, forming a new attachment. Sporophytes are typically observed within 2-4 wk.

7.1.6. The zygote undergoes rapid cell division, resulting in the growth of 1-2 cm blades within approximately 6-8 wk.

7.2. Glass slides within containers are monitored daily - every other day for the first two weeks to assess germination success and gametophyte development. To monitor, handle the glass slide with sterilized tweezers, and place in a clean Petri dish containing enough sterilized seawater to submerge the slide. Use an inverted microscope at 100-400x magnification to observe early-stage kelps. Do not return glass slides to cultures after being removed to avoid cross-contamination.

7.3. After 2 wk, slides are monitored once or twice weekly for healthy growth and contamination until sporophytes reach 1-2 cm in size. Healthy growth is characterized by golden-brown

coloration, as opposed to green or transparent. Common contaminants include diatoms, red algae, fungi, and ciliates. Glass slides should be handled with sterilized tweezers and monitored within a clean Petri dish.

7.4. There are a number of quantitative metrics that can be observed on glass slides with an inverted microscope, including survivorship, germination rate, vegetative development, reproductive maturity and fecundity, and sex ratio[28].

7.5. Contamination by bacteria, fungi, ciliates, and diatoms should be assessed with a microscope. Isolated contamination should be removed. A treatment with germanium dioxide (GeO_2) can be used to control early signs of diatom contamination (see Section 8.3).

8. Maintenance

8.1. Filter-enriched seawater media is drained from culture containers and re-filled weekly to replenish necessary nutrients and minerals for *M. pyrifera* growth. Enriched media may be prepared ahead of time and stored in a refrigerator.

8.1.1. Remove media from the refrigerator ahead of time (up to 1-2 h before media change, depending on volume and storage temperature) to let it temper to the appropriate temperature. Keep track of liquid temperatures with a sterilized thermometer or handheld infrared thermometer.

8.1.2. Media should be siphoned out of your culturing containers to avoid the disturbance of seeded substrates. Let the media drain until the container is nearly empty. Tilt growth containers slightly when refilling, so that media runs down the side of the culturing container to minimally disturb substrates during the media changing process. Work in manageable batch sizes to ensure that your cultures and your new enriched media do not exceed 15°C. This process should take less than a few minutes as juvenile sporophytes are prone to desiccation if removed from the water for extended periods.

8.1.3. Randomly shuffle container or tub positions during weekly media changes to account for differences in light irradiance.

8.2. Light conditions are adjusted according to observed developmental stages (see Section 5.2).

8.3. A treatment of germanium dioxide (GeO_2) can be used to control diatom contamination. This chemical reduces the amount of bioavailable silica in the water, which is an important structural component of diatom cell walls. Add 0.3-0.5 mL of 250 mg/mL GeO_2 to each 1 L of seawater added to seeded substrates to reduce widespread diatom contamination.

NOTE: Diatom contamination is one of the most common challenges to producing successful kelp cultures. When provided with adequate nutrients and light, diatom colonies can grow rapidly and out-compete early life stages for substrate and smother existing growth. Diatoms can be introduced at any stage of the culturing process, from the fresh tissue handling process pre-sporulation, to weekly seawater media changes. Precautionary measures of rigorous tissue cleaning, proper sterilization of surfaces and materials, as well as the use of GeO_2 will reduce initial levels of diatom contamination on your seeded substrates. GeO_2 is a relatively expensive chemical, so using this preventative method in large quantities of water is not advised.

NOTE: GeO_2 may inhibit algal gamete production. It is recommended to use GeO_2 in the short window after germination and before peaks of egg and sperm production (1-7 d) or after egg fertilization and sporophyte observations (>21 d), followed by a media change 48 h after to remove the chemical. These timelines may vary given culture conditions, so monitoring life stage development with microscopy is the best way to assess the timing of GeO_2 application. If diatom contamination persists in your culture containers and overgrowth onto early stage kelps is observed, consider re-seeding your substrates.

9. Giant Kelp Vegetative Culturing Seed Bank

9.1. To reduce lab culturing dependency on reproductive tissue collection from the natural reef, axenic, high-quality gametophyte stocks can be vegetatively propagated indefinitely and be readily available for reproduction, growth, and deployment. For vegetative conditions, gametophytes are stored in 1 L round, flat-bottomed flasks filled with filtered-enriched seawater (pore size 0.5 µm) at 4-12°C in red light at an intensity of 5-20 µmol photon m^{-2} s^{-1} in a 12 light: 12 dark cycle. In these conditions, gametophytes produce biomass asexually without gamete production.

9.2. To bulk-up gametophyte biomass, gametophytes can be fragmented every two weeks using a sterilized food processor or coffee grinder. Pulse your gametophyte solution for 1-2 s approximately 15 times. Filter-enriched seawater should be changed every week, with constant aeration.

9.3. To induce gametophyte reproduction, fragment gametophytes as explained above. Increase full spectrum LED light gradually from 5-20 to 45-60 µmol photon m^{-2} s^{-1} (+10 µmol photon m^{-2} s^{-1} daily for photo-acclimation). Finally, increase light by +10-20 µmol photon m^{-2} s^{-1} every 3-4 d until reaching an irradiance of 180 µmol photon m^{-2} s^{-1}.

10. Deployment

10.1. After 6-8 wk of laboratory culturing, the juvenile sporophytes are around 1-2 cm in length and are ready to be deployed. Filter-enriched seawater media in culture containers should be 24 h before deployment.

10.2. Transport '*green gravel*' in trays placed in insulated coolers that are then covered with towels soaked in seawater to keep the kelp hydrated. Trays holding *'green gravel'* should not be in direct contact with ice or ice packs, and cardboard can be used as a barrier. Depending on space availability, substrates can also be transported in their culture containers or tubs to reduce handling, but seawater media should still be mostly drained and covered with paper towels

dampened with seawater. The *'green gravel'* can be transported for up to 6 h in a dark cooler to the restoration site. Make sure that the *'green gravel'* is tightly packed together to avoid rolling of the material and sporophyte detachment from the substrate during transportation.

10.3. The *'green gravel'* should remain chilled and shaded while transported by boat to the site. Deployment should be timed to avoid the most direct sunlight. Utilize a shaded structure on the boat to avoid direct sun during the deployment process. At this point, *'green gravel'* can be carefully scattered from the surface or brought via SCUBA onto the reef below.

Discussion

Anthropogenic climate change is a growing threat to the health of the world's oceans[32–36], resulting in major disturbances and biodiversity loss[37–40]. To accelerate the restoration of degraded ecosystems, the United Nations has declared 2021 through 2030 the "UN Decade on Ecosystem Restoration," coinciding with the "UN Decade of Ocean Science for Sustainable Development" which aims to reverse deterioration in ocean health[41]. In line with this global call to action, the Kelp Forest Alliance (www.kelpforestalliance.com) has launched the Kelp Forest Challenge to restore 1 million hectares and protect 3 million hectares of kelp forest by the year 2040. Marine restoration is undervalued[42], and kelp ecosystems receive considerably less attention compared to coral reefs, mangrove forests, and seagrass meadows[43]. Restoration of degraded ecosystems has been shown to be effective in re-building marine ecosystems but can cost on average between USD $80,000 - $1,600,000 per hectare, with median total costs likely to be two to four times higher[44]. Current and projected losses call upon developing scalable, feasible, and cost-effective kelp restoration methodologies as urgent conservation interventions.

Current kelp restoration can use a combination of methodologies in efforts to re-establish coastal rocky reefs[3, 45]. Depending on geographic context and specific impediments to kelp recovery, there are a number of restoration methodologies to address site-specific concerns[11]. Strategies to recover or protect

kelp forests depend on the stressor at specific locations in addition to local governance structures. For example, where herbivore pressure or interspecific competition are driving decline or suppressing recovery (e.g. by sea urchins[13] or turf algae[9,39]), restoration may focus on the removal of these biotic stressors[47]. However, these methods require substantial resources and continuous maintenance[11]. To catalyze kelp species recovery, some efforts have a direct seeding approach, weighing mesh bags filled with fertile kelp blades to the benthos that release zoospores into the environment[48]. Still, this method is time intensive and requires technical underwater installation and removal. Other cases focus on transplanting large quantities of whole adult donor plants, which may compromise closely associated and vulnerable donor populations and are often limited to small scales due to reliance on continual transplantation[49]. The *'green gravel'* technique overcomes important limitations that currently constrain kelp restoration[19]. This innovative approach provides a promising restoration tool, urging for extensive trials across diverse locations and environments to unlock its full potential[22]. The *'green gravel'* technique applies simple deployment from the surface requiring no underwater installment or technical knowledge, and scalability at relatively low costs USD $70,000 per hectare comparable to other restoration techniques including methods of transplantation and recruitment enhancement[50].

Climate change and anthropogenic disturbances are currently outpacing the adaptive capacity of natural populations, challenging traditional conservation aims of restoration to historic states[51–56]. Conservation frameworks have expanded to include anticipatory management that proactively consider resilience and adaptive capacity[57]. Failure to integrate such methods may result in the failure of restoration efforts under projections of future climate stressors. Anticipatory management practices are already being implemented for tree species to address climate change in forest ecosystems[58] and has been suggested for other large-scale restoration efforts to maximize evolutionary potential of outplants[59,60]. While these strategies are inherently easier to manipulate in terrestrial systems, there are a number of studies beginning to explore their potential and feasibility in marine systems[55, 61–63]. For instance, coral reefs are important foundational ecosystems that are threatened by a myriad of anthropogenic stresses that

have led to unprecedented losses[64,65]. In response to the deterioration of coral reefs globally, intervention strategies, such as active restoration and assisted adaptation techniques have been increasingly advocated to protect remaining reefs and their associated functions[55, 66, 67]. One such technique involves translocation of individuals between conspecific populations within the current distribution range of the species to increase tolerances to heat stress[68]. Another promising feature of *'green gravel'* is its customizable framework to explore assisted adaptation techniques such as translocation of resilient genotypes to vulnerable areas, non-genetic manipulation such as hybridization, or acclimatization of individuals to environmental stress[55] with outcomes aimed towards obtaining more resistant strains for restoration programs[69, 70].

Harnessing local support to enhance restoration endeavors is crucial to sustain kelp ecosystem conservation success. Engaging local stakeholders can increase local buy-in for restoration needs[6,38] and promote coastal stewardship that could subsequently result in increased funding and longevity of kelp ecosystem protection. As with all other kelp restoration methodologies, structured decision-making frameworks that integrate diverse ecological, socio-economic, and conservation objectives will help to achieve optimal outcomes for kelp ecosystems and the communities they support[11].

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Figures and Tables

Table 2.1. Differences in species-specific life history characteristics of sugar kelp (*Saccharina latissima*) versus canopy-forming species, including giant kelp (*Macrocystis pyrifera*) and bull kelp (*Nereocystis luetkeana*).

Life history characteristic	*Saccharina latissima*	*Macrocystis pyrifera*	*Nereocystis luetkeana*
Maximum Growth Rate	5 cm day^{-1}	60 cm day^{-1}	6 cm day^{-1}
Thermal Limits	< 20 ºC	< 20 ºC	< 17 ºC
Depth Range	0 - 30 m	2 - 30 m	4 - 22 m
Lifespan	2 - 5 years	6 - 8 years	1 year
Reproductive Season	Annual (primarily winter)	Perennial	Annual (primarily autumn)
Maximum Size	< 3 m	< 50 m	< 45 m
Holdfast Size	< 10 cm	< 1 m	< 40 cm

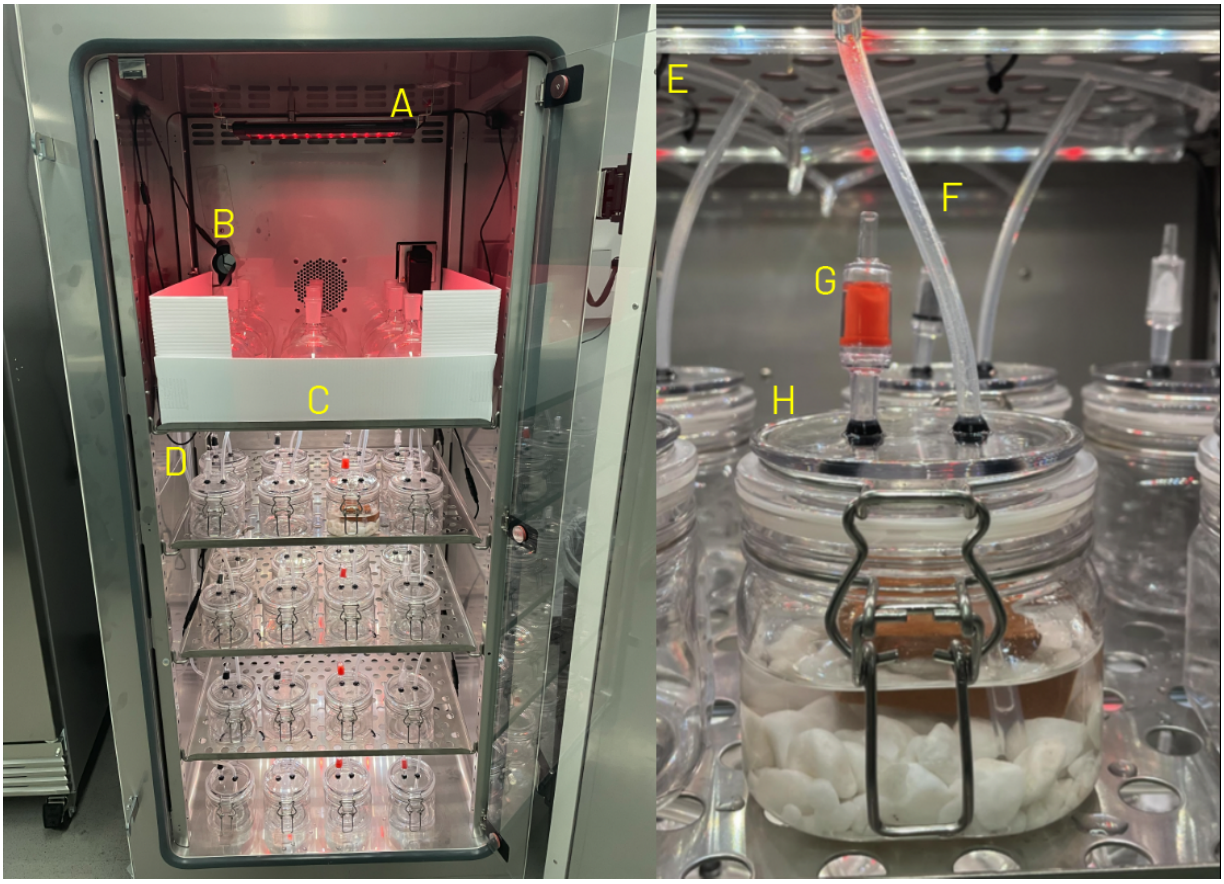

Figure 2.1. Diagram of kelp culture incubator system. (A) Red light source for vegetatively bulking gametophyte cultures. (B) Access port for electrical wires and tubing, leading to an external outlet. (C) Structure to block full-spectrum light out of the red light section. (D) A *'green gravel'* culturing section. (E) full-spectrum light sources. (F) Tubing lines connected to an external filtered air source. (G) Check valves to reduce airborne contamination. (H) Individual culture containers that minimize contamination.

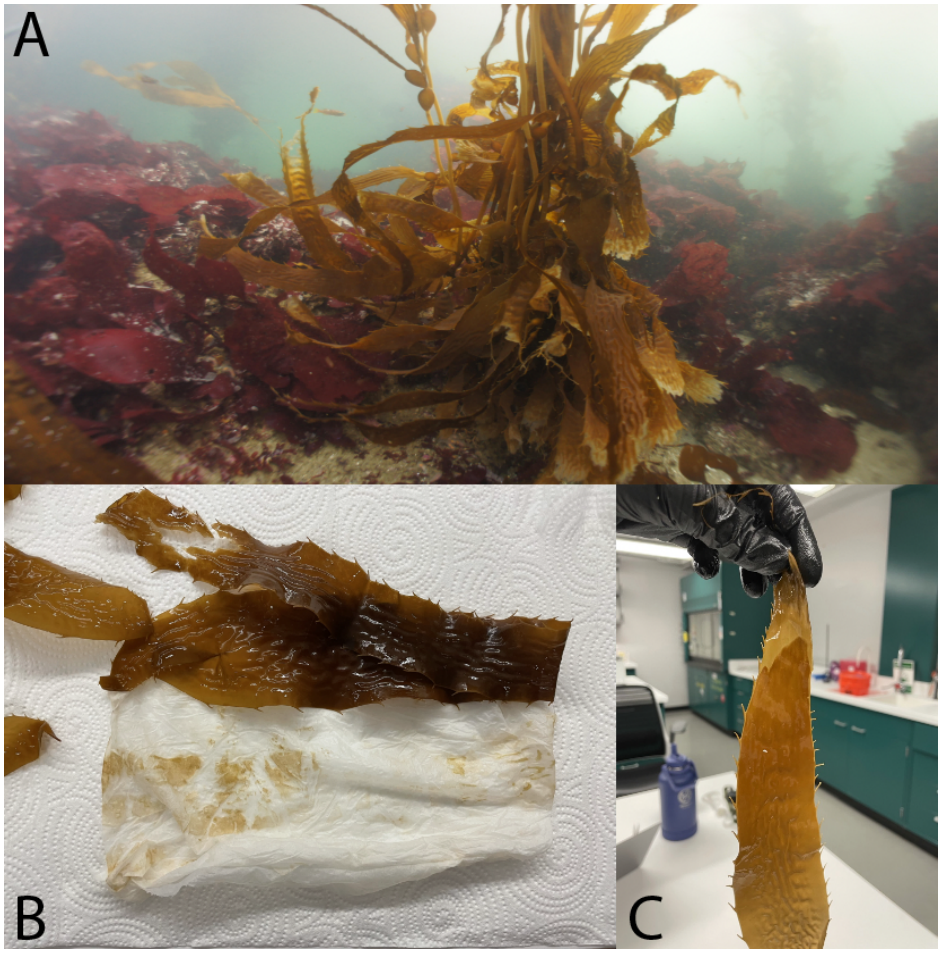

Figure 2.2. (A) Sporophyll "skirt" found at the base of an adult kelp. Photo by Steve Clabuesch. (B) Sporophylls selected for sporulation. Evidence of partial sporulation on damp paper towels used to keep tissue hydrated during transportation. (C) Visible sori found on reproductive sporophyll.

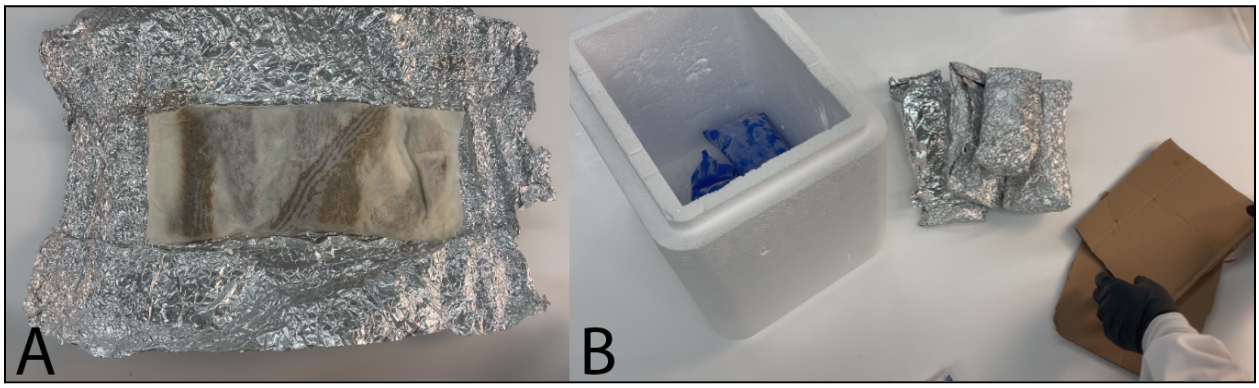

Figure 2.3. (A) Sporophylls wrapped in moist paper towels, to be wrapped again in aluminum foil. (B) Preparation of a sporophyll shipment, using cardboard as a barrier.

Figure 2.4. Sori selection and sporulation. (A) Sporophylls post-desiccation. (B) Spray bottle with sterile seawater (C) Freshwater and sterile seawater rinse trays. (D) Chemicals used to sterilize instruments. (E) Sterile scissors. (F) Sori sections are placed in 50 mL Falcon tubes to sporulate. Caps are numbered to indicate kelp parent.

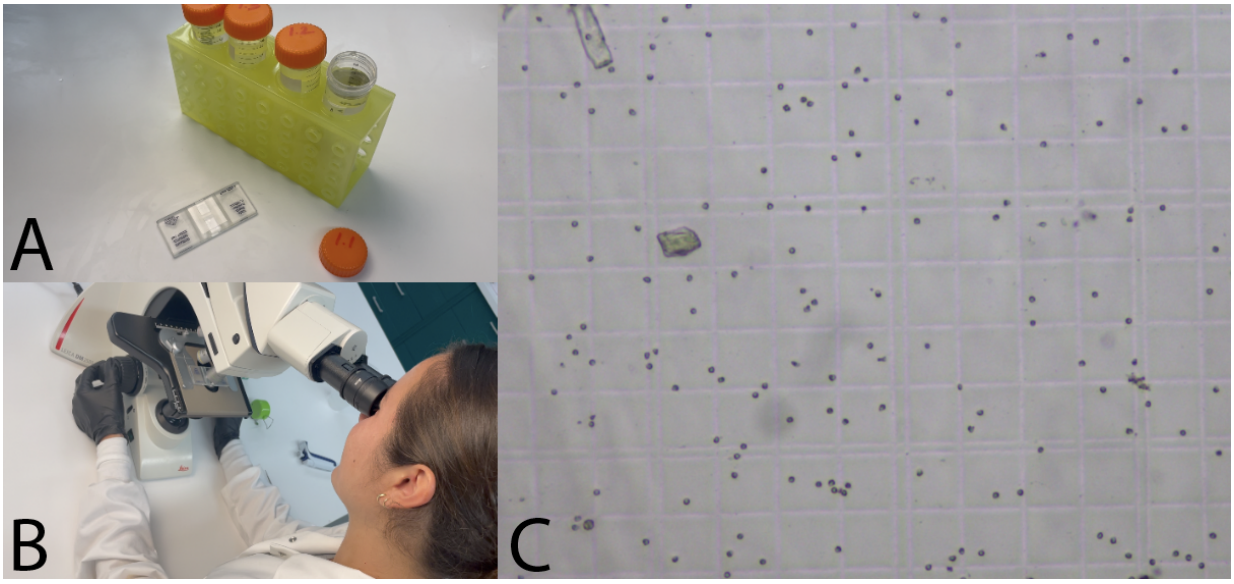

Figure 2.5. (A) A hemocytometer can be used to observe spore densities. (B) *'green gravel'* grower views hemocytometer with a compound microscope at 100x - 400x magnification. (C) Example of hemocytometer grid with spores.

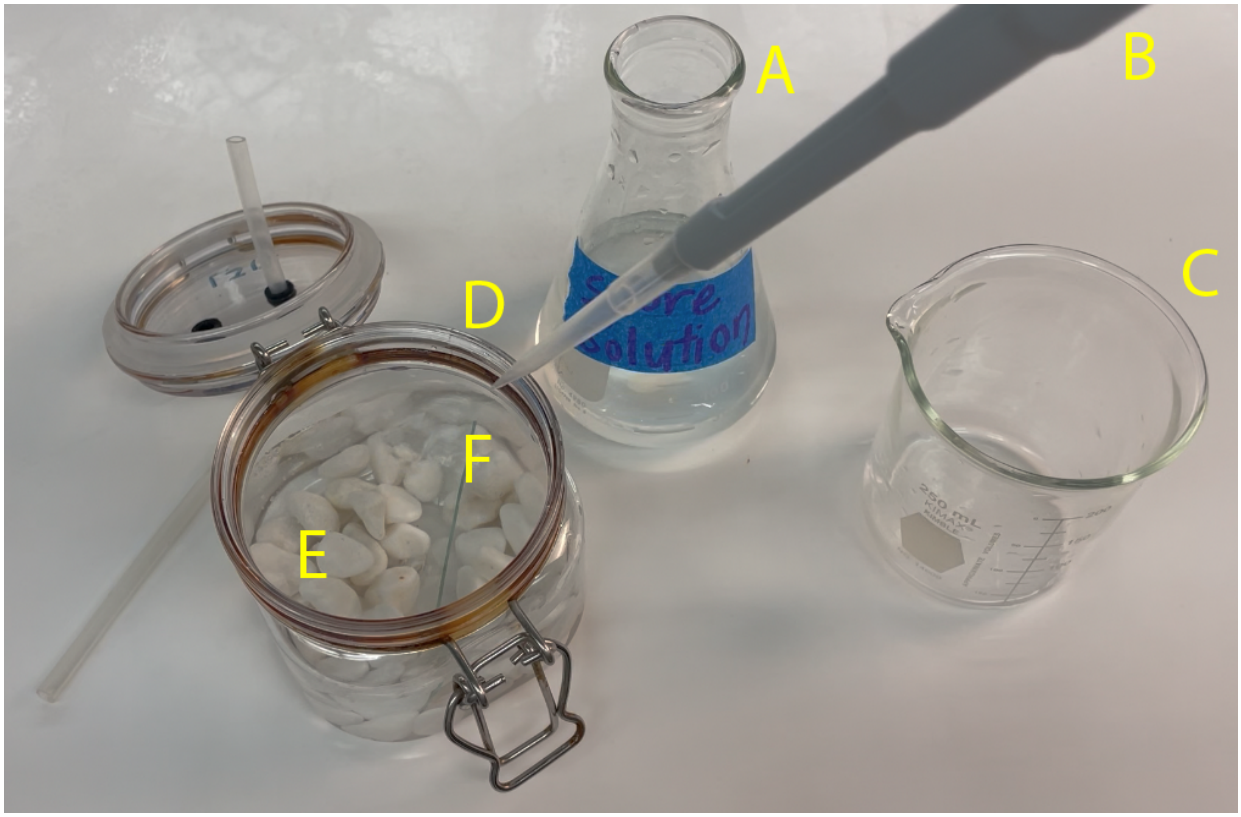

Figure 2.6. Seeding of *'green gravel'* substrates. (A) Spore solution. (B) Inoculating culture container with spore solution. (C) Beaker used to measure tempered enriched seawater. (D) Culture container used to hold seawater. (E) gravel. (F) Glass slide to monitor kelp development.

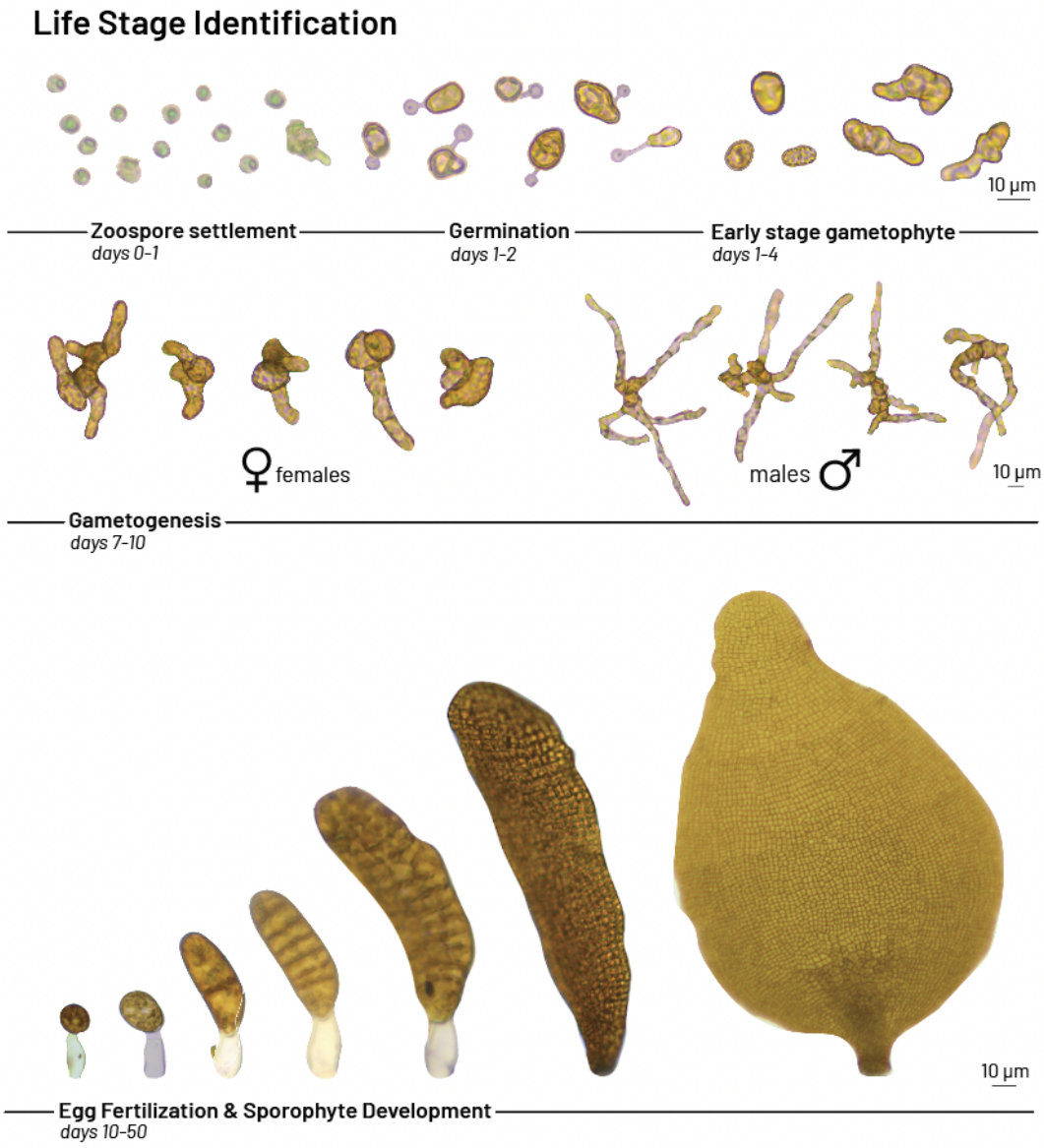

Figure 2.7. Developmental life history stages of giant kelp from laboratory growth trials.

Chapter 3

Thermal acclimatization to future-proof giant kelp forest restoration

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Introduction

Kelp forests are foundational ecosystems dominated by large brown seaweeds (Order Laminariales) and are distributed along 25% of temperate and polar coastlines worldwide[1]. Kelps provide services valued at USD $500 billion per year[2] through the provisioning of complex biogenic habitat for valuable fish and seafood species and removal of nitrogen from contaminated seawater[3]. Despite their ecological importance and substantial value to human populations, kelps are experiencing declines in many regions across the world[4–6], raising concerns for the future of these foundation ecosystems and underscoring the importance of their strategic management[7].

Thermal stress has emerged as a particularly important driver of kelp decline on a global scale, with an accelerating loss of these ecosystems at many warm range margins[8–10] and in response to extreme marine heatwave conditions[11–14]. For instance, in the eastern Pacific ocean anomalously high temperatures between 2014 - 2016 resulted in catastrophic declines of >90% in certain regions13 and prolonged shifts in the community structure of California kelp forests15. Persistent and gradual ocean warming combined with acute marine heatwaves (MHWs) of increased frequency, duration, and severity[16,17] threaten the persistence of kelp forest ecosystems in coming decades. As climate change outpaces the adaptive capacity of natural kelp populations, there is an urgent need for conservation to employ anticipatory,

future-proofing management strategies[18].

Conservation frameworks have necessarily expanded to include assisted evolution methods that proactively consider ecological resilience and adaptive capacity. These methods utilize adaptation and acclimatization processes that may alter performance and impact an organism's susceptibility to warming[19–21]. While genetic rescue focuses on simply enhancing the genetic diversity of restored populations, assisted gene flow introduces adapted or tolerant individuals into threatened populations in order to increase resilience to an identified stressor. Genetic manipulation and assisted expansion are more advanced strategies that utilize genetic techniques to meet specific restoration goals[7]. Acclimating individuals to stressful conditions is known to lead to increased tolerance to stress later in life — also known as stress memory, hardening or priming—has been shown to yield enhanced stress tolerance beyond the sexual generation as well[22]. The common goal of these future-proofing strategies is to facilitate naturally occurring evolutionary processes to boost resilience of restored kelp populations against environmental change[23].

The application of future-proofing methods are already being implemented for tree species to address climate change in forest ecosystems[24] and has been suggested for other large-scale restoration efforts to maximize evolutionary potential of outplants[25,26]. While these strategies are inherently easier to manipulate in terrestrial systems, marine ecologists are beginning to explore their potential and feasibility as well[27–30]. In response to widespread deterioration of coral reefs, intervention strategies such as active restoration and assisted evolution techniques have been increasingly advocated to protect remaining reefs and associated functions[29,31,32]. One such technique involves translocation of locally adapted individuals between conspecific populations within the current distribution range of the species to increase tolerances to heat stress[33]. Another method involves acclimatization of bacterial associates[34] or the algal symbionts[27] to temperature extremes to yield heat-tolerant hosts. Coral reef conservation has offered a solid framework for the use of assisted evolution in other threatened marine systems, including kelp forests. Understanding the scope of adaptation and acclimatization processes is critical to future-proofing kelp

restoration, as it may affect individuals used as breeders or transplants.

Kelp forests along the southeastern Pacific coast are dominated by giant kelp (*Macrocystis pyrifera*), creating canopy-forming marine habitats in central California extending southward along the coast. Ocean warming unequivocally threatens this region as populations are distributed towards the upper limit of this species' thermal distribution, and thus restoration should proactively consider processes of adaptive capacity throughout its complex multi-stage life cycle. Here, I explore the impact of different thermal rearing conditions on two potentially locally adapted populations of *M. pyrifera* kelp forests following realistic thermal simulations of a restoration to a warmer region and a marine heatwave. In this >2-month long-term trial, I quantify density of different kelp life stages through time and hypothesize that: (1) kelp from distinct populations of origin are locally adapted and will exhibit higher densities of early microstages when reared in temperatures that reflect local thermal environments, and (2) kelp reared in stressful thermal conditions will have higher reproductive output compared to kelp reared in optimal thermal conditions in response to simulated restoration and marine heatwave conditions.

Methods

Site Selection

Connectivity of giant kelp populations are typically limited to several kilometers due to relatively short durations of spore dispersal and high spore densities required for successful post-settlement fertilization[35,36]. However, oceanic connectivity may explain levels of genetic connectivity observed in kelp populations between Santa Cruz and San Diego[37,38]. In a study investigating *Macrocystis pyrifera* population genetic structure, distinct California geographic clusters (with regions spanning from Santa Cruz to San Diego, CA) were identified with genetic continuity throughout[38] (**Figure 3.1**). The warmer, southerly region (referred to as K4 and encompassing San Diego kelp populations) is particularly

susceptible to temperature anomalies and sites in this region experienced the greatest kelp loss during the 2014-2016 El Niño event (Northern range = San Clemente, California, Southern range = Ensenada, Mexico[11,38] (**Figure 3.1**).

Experimental Design

Beginning on 6 March 2022, I conducted a >2-month long-term trial to assess the effect of 12°C, 16°C, 20°C, and 24°C rearing temperatures on early microstages of *Macrocystis pyrifera* originating from Santa Cruz (36.602°N, 121.885°W) and San Diego (32.850°N, -117.276°W) populations in constant thermal conditions [days 0 - 40], followed by a simulated deployment for restoration at 16°C (average SST of warmest most southerly population ; **Figure 3.1**) [days 40 - 52], and a realistic marine heatwave of 22°C[39] [days 52 - 68]. In preparation for these experiments in our newly constructed aquarium system, I developed and constructed a multi-level racks system to fit 50mL Falcon tube replicates, each containing a glass slide with standardized grid cells (fixed fields) used to monitor and quantify early life history stages using high resolution images from our Leica DMi1 inverted microscope and Leica C1 FLEXACAM.

Effect of rearing temperature on early stages of canopy-forming giant kelp

For the first 40 days of this experimental trial (6 March 2022 - 16 April 2022), I assessed the effect of rearing temperature on two distinct donor kelp populations (**Figure 3.2** and **Figure 3.3**). I reared Santa Cruz and San Diego kelps in 4 distinct thermal conditions (12°C, 16°C, 20°C, and 24°C; see rationale **Table 3.1**), with nutritional and light conditions held constant for each treatment (N = 144 cultures). I imaged fixed fields of glass slide replicates at 3 timepoints (4 thermal conditions x 3 replicate tanks x 2 populations x 6 culture replicates x 5 fixed fields = 720 images per time point x 3 timepoints = 2,160 photos). There was no kelp survival in the 24°C thermal condition, and thus this treatment was terminated

and excluded from further trials.

Effect of rearing temperature following a simulated field restoration deployment

In days 41-52 of growth (17 April 2022 - 28 April 2022), temperature of 'treatment' samples was brought to 16°C (average SST in the most southern population, and the average of remaining thermal conditions) at a rate of 1°C per day over 4 days (**Figure 3.3**). 'Treatment' samples remained at 16°C for 8 days, (N = 72 cultures). 'Control' samples remained at their respective initial rearing temperatures (N = 36 cultures). I imaged fixed fields of glass slide replicates on day 52 (3 thermal conditions x 3 replicate tanks x 2 populations x 6 slide replicates x 5 fixed fields = 540 images per time point = 540 photos).

Effect of rearing temperature following a simulated marine heatwave

In days 53-68 of growth (29 April 2022 - 18 May 2022), 'treatment' samples were brought to 22°C, which is a realistic MHW condition that occurred in the southern limit of *M. pyrifera* ranges in the northern hemisphere (www.marineheatwaves.org) at a rate of 1°C per day (**Figure 3.3**). Duration of MHWs vary considerably across the global ocean. The eastern tropical Pacific, a region located just south of the San Diego population of origin, has an average MHW duration of up to 60 days, while other tropical regions are typically characterized by events of 5–10 days[39]. 'Treatment' samples remained at 22°C for 10 days, (N = 72 cultures in 'treatments'). 'Control' samples remained at their respective initial rearing temperatures (N = 36 cultures in 'controls'). I imaged fixed fields of glass slide replicates on day 68 (3 thermal conditions x 3 replicate tanks x 2 populations x 6 slide replicates x 5 fixed fields = 540 images per time point x 2 timepoints = 540 photos).

Life history trait estimations and data analyses

All Laminarian kelps, including *Macrocystis pyrifera*, demonstrate a complex heteromorphic

haploid-diploid life cycle. Fertile tissue releases kelp spores that settle and germinate, demonstrated by the formation of a germ tube. The first haploid gametophytic cell will develop as stores from the spore are transferred through the germ tube. Female gametophytes produce eggs that, once fertilized, produce diploid sporophytes from which adult giant kelps grow. Life history categories were defined based on developmental characteristics and reproductive structures: (1) microscopic germinated spores, identified by a germination tube, (2) microscopic gametophytes of >2 cells [male or female], (3) microscopic next-generation [combined egg and sporophyte counts], and (4) visible/macroscopic sporophytes >1 mm in length.

Observations for life stage categories were made on days 2, 24, 32 and observations for life stage category (3) were made on day 52 and day 68. Life history stages were counted on five evenly spaced fixed visual fields per slide. A small dot was etched using the chemical media (Armour Etch Glass Etching Cream), to mark the top-right point of each of the five microscopy fields and ensure fixed fields through time. All microscopy photos were taken with the Leica Inverted Microscope DMi1 and FLEXACAM C1 Camera, and analyzed with ImageJ software[40].

Statistical Analyses

To determine if there were differences in the different life stages of *M. pyrifera* (germinated spores, gametophytes, and next-generation [combined egg and sporophyte counts]) across rearing temperature and population of origin, I used a generalized linear mixed effects model. For models concerning density of the life stages, I used a Poisson distribution and log link with counts of the life stage as the response variable. For models concerning the sex ratio of gametophytes, I used a Binomial distribution and logit link with the joint counts of the females and males as the response variable. To test the effect of rearing temperature on early stages of canopy-forming giant kelp, I included site, temperature, and the interaction of site and temperature as the fixed effects for the full model using function *glmmTMB()* from package

glmmTMB[41]. To test the effect of rearing temperature following a simulated field restoration deployment and a marine heatwave, my full model included site, temperature, and a treatment effect and the interaction of all three fixed factors. I also included tank ID and sample ID as random effects to account for study design across all Aims. An offset of the logarithm of area sampled was also included to account for sampling effort resulting from different microscope lenses (4x and 5x) for data from days 24 - 52. Across all aims, I also tested a model that treated temperature as a factor to allow for non-linearity in response to the different temperature treatments. I then compared the models treating temperature continuously and as a factor using Akaike Information Criterion (AIC). The best model was chosen as the model with the lowest AIC, unless the models were within two AIC units of each other, then the continuous temperature model was kept. The best supported model treated temperature as a continuous variable for germinated spore counts at day 2, and gametophyte counts at day 24. The best supported model treated temperature as a factor for next generation life stages and count of sporophytes >1mm in length at day 32, next generation stages at day 52, and next generation stages at day 68. Counts from the 24°C treatment were excluded from all models since this treatment failed to produce any viable kelp gametophytes.

Results

Effect of rearing temperature on early stages of canopy-forming giant kelp

Germinated spore counts declined with increasing temperature (Estimate = -0.045, standard error (SE) = 0.017, Z = -2.66, P = 0.008) similarly for both populations of origin (Estimate = -0.027, SE = 0.024, Z = -1.11, P = 0.27; **Figure 3.4A**). Furthermore, there was no effect of population of origin on observed counts (Estimate = 0.40, SE = 0.38, Z = 1.06, P = 0.29). I also found that no spores germinated at 24 °C.

Gametophyte counts at day 24 were similar across all temperatures for samples originating from San Diego (Estimate = -0.11 SE = 0.11, Z = -0.74, P = 0.46) but were negatively correlated with

temperature for samples originating from Santa Cruz (Estimate = -0.16, SE = 0.022, Z = -7.33, P < 0.001; (**Figure 3.4B**). Furthermore, samples originating from Santa Cruz had greater estimated gametophytes at lower temperatures (Estimate = 2.03, SE = 0.36, Z = 5.63, P < 0.001).

The count of next generation life stages at day 32 were similar for 12°C and 16°C (Estimate = -0.056, SE = 0.27, Z = -0.21, P = 0.84) and lower at 20°C (Estimate = -2.53, SE = 0.35, Z = -7.16, P < 0.001; **Figure 3.4C**). Patterns across temperatures were similar for both populations of origin at 12°C (Estimate = -0.13, SE = 0.27, Z = -0.50, P = 0.62), 16°C (Estimate = -0.39, SE = 0.40, Z = -0.97, P = 0.33) and 20°C (Estimate = -19.44, SE = 3692.54, Z = -0.005, P = 0.996).

The count of sporophytes >1mm in length at day 32 were lower at 16°C (Estimate = -0.48, SE = 0.17, Z = -2.76, P = 0.0058) and 20°C (Estimate = -5.24, SE = 0.35, Z = -15.14, P < 0.0001) than at 12°C (**Figure 3.4D**). Samples originating from Santa Cruz had lower counts at 12°C and 20°C compared to San Diego (Estimate = -0.83, SE = 0.17, Z = -4.73, P <0.0001), though less so at 16°C (Estimate = 0.58, SE = 0.25, Z = 2.34, P = 0.019).

Effect of rearing temperature following a simulated field restoration deployment

The simulated deployment had no effect on counts of next generation stages for 'treatment' samples reared at 12°C (Estimate = 0.20, SE = 0.25, Z = 0.81, P = 0.42) or reared at 16°C (Estimate = -0.55, SE = 0.37, Z = -1.53, P = 0.13) compared to their respective ‘control’ samples. For 'treatment' samples initially reared at 20°C, counts of next generation life stages were increased compared to ‘control’ samples (Estimate = 1.66, SE = 0.40, Z = 4.13, p<0.0001). The pattern remained consistent for both populations of origin from 12°C (Estimate = -0.31, SE = 0.36, Z = -0.87, P = 0.39) and 16°C (Estimate = 0.49, SE = 0.51, Z = 0.97, P = 0.33), though 'treatment' samples from Santa Cruz and 20°C saw a larger increase compared to San Diego (Estimate = 2.45, SE = 0.74, Z = 3.33, P = 0.00086) (**Figure 3.5A**). Next generation stages were similar for 'control’ samples at 12°C and 16°C (Estimate = -0.27, SE = 0.29, Z = -0.93, P = 0.35) and lower at 20°C (Estimate = -2.73, SE = 0.34, Z = -7.96, P < 0.001). Patterns across

temperatures were similar for 'control' samples for both populations of origin at 12°C (Estimate = -0.32, SE = 0.29, Z = -1.11, P = 0.27) and 16°C (Estimate = -0.19, SE = 0.42, Z = -0.46, P = 0.65) though 'control' samples from Santa Cruz at 20°C were lower (Estimate = -1.70, SE = 0.67, Z = -2.52, P = 0.012) (**Figure 3.5A**).

Effect of rearing temperature following a simulated marine heatwave

'Treatment' samples that experienced a simulated marine heatwave of 22°C had lower counts of next generation stages from 12°C and 16°C (Estimate = -2.06, SE = 0.26, Z = -7.94, P < 0.001), though counts from 20°C were increased compared to 'control' samples (Estimate = 4.21, SE = 0.38, Z = 11.00, p<0.0001). Counts for Santa Cruz exhibited less of a decline with the simulated heatwave at 12°C and 16°C (Estimate = 1.28, SE = 0.36, Z = 3.56, P = 0.00037), though samples from Santa Cruz and 20°C exhibited less of an increase compared to San Diego (Estimate = -2.42, SE = 0.55, Z = -4.37, p<0.0001) (**Figure 3.5B**). Next generation stages were similar for 'control' samples at 12°C, 16°C (Estimate = -0.43, SE = 0.28, Z = -1.50, P = 0.13) and lower for 20°C (Estimate = -2.34, SE = 0.31, Z = -7.42, p<0.0001). Patterns across temperatures were similar for 'control' samples for both populations of origin at 12°C (Estimate = -0.11, SE = 0.28, Z = -0.38, P = 0.70), 16°C (Estimate = -0.30, SE = 0.41, Z = -0.74, P = 0.46) and 20°C (Estimate = -0.03, SE = 0.46, Z = -0.070, P = 0.94) (**Figure 3.5B**).

Discussion

In this >2-month long-term experimental trial, I first assessed the effect of constant rearing temperature treatments (12°C, 16°C, 20°C and 24°C) on *Macrocystis pyrifera* microstages of two potentially locally adapted source populations that experience distinct thermal environments in the wild. Given significantly warmer local environments of San Diego populations closer to the equatorward trailing range edge compared to cooler, more northerly kelp populations of Santa Cruz, I expected that kelps from distinct

populations of origin will perform best when reared in temperatures that reflect local thermal environments. As kelps continue to decline worldwide, so do sources of spores to support establishment of microscopic propagules that mature into the succeeding generations of macroscopic wild kelp populations[42]. In a study of Atlantic kelp (*Laminaria longicruris*) approximately 9 x 10^6 recruited to the microscopic gametophyte stage from an estimated total of 9 x 10^9 spores[43]. Therefore, spore survival and subsequent germination represents an important population bottleneck that may be impeding natural kelp forest recovery. In constant thermal rearing conditions, spore germination declined with increasing temperature similarly for Santa Cruz and San Diego kelps with no effect of population of origin, and no spores survived the 24°C treatment. This result is supported by other cases in the literature, where spore germination was negatively correlated with increasing temperature[44,45] and the thermal threshold for spore and germling development was determined to be between 21.7°C and 23.8°C[46]. There are relatively fewer studies that have focused on this early life stage of *M. pyrifera*, with one demonstrating that temperature can also be positively correlated with the release of spores by fertile adult plants[46]. Evidence of spore germination sensitivity to higher temperatures in my study highlights the vulnerability of this preliminary stage of *M. pyrifera* development to ocean warming.

Gametophytes reared in constant thermal conditions originating from the cooler Santa Cruz population exhibited declines with increasing temperature, whereas gametophytes originating from the warmer San Diego population were similar across all temperature treatments (12°C, 16°C and 20°C), providing initial evidence of local adaptation. Kelps demonstrate a complex heteromorphic haploid-diploid life cycle[47], where haploid microscopic gametophyte stages are considered to be more stress tolerant than diploid macroscopic sporophyte stages[48]. Kelp gametophytes may serve as a crucial component to the persistence of kelp populations, acting as a 'bank of microscopic forms' able to withstand conditions considered unfavorable for derived reproductive and sporophytic life stages. In this vegetative form, gametophyte banks act as reservoirs of genetic diversity and a source of reproductive material that allows for the regeneration and survival of kelp populations under variable environmental

conditions[42]. This ecological role is especially important as global declines in kelp populations persist, and there are diminishing sources of new spores. Thermal tolerance of this microstage may be particularly important at the southern limit of the range in the Northern Hemisphere impacted by severe warming and El Niño conditions[49,50], including at the San Diego, California site used in this study.

Next generation life stages and macroscopic sporophytes (>1mm) exhibited similar patterns of local adaptation, whereby kelps originating from San Diego performed better than kelps from Santa Cruz at 16°C and 20°C treatments in all instances. *M. pyrifera* is a relatively short-lived kelp species, producing eggs and derived sporophytes continuously rather than risk delaying reproduction[51]. Thus, densities of next generation life stages of eggs and sporophytes act as a useful metric for assessing performance of *M. pyrifera* through time in response to varying thermal conditions. Observed densities of next generation life stages trended higher for San Diego kelps at 16°C and 20°C, while macroscopic sporophyte (>1mm) densities were significantly higher for San Diego kelps at 16°C and 20°C. In another thermal trial that assessed the scope of local adaptation of *M. pyrifera* from contrasting thermal environments and different global regions, kelps that were crossed with low-latitude populations experienced reduced mortality of sporophytes in response to a heatwave[52]. Results of this trial further support that emerging *M. pyrifera* restoration approaches, such as those that use spore or gametophyte solutions to seed rocky reef sites, should proactively consider the provenance of source population, especially in restoration scenarios of environments at the upper thermal limit of the species distribution.

Kelp aquaculture standards outline thermal rearing conditions generally ranging between 5 - 15°C, a thermal window considered physiologically optimal for cellular processes such as protein stability and enzyme activity[53]. Similarly, culturing protocols developed for restoration grow-out efforts recommend rearing kelps within this range, and not to exceed temperatures of approximately 10 - 15°C throughout the culture period (e.g. [54–56]). However, for restoration to be effective given the scale and rate of ongoing climate change worldwide, rearing conditions during the nursery phase must take into account imminent thermal conditions of ocean warming that exceed optimal thermal windows for kelps in

controlled culture conditions[16]. Climate change and anthropogenic disturbances are currently outpacing the adaptive capacity of natural populations, challenging traditional conservation aims of restoration to historic states[29,57–61] and underscoring an urgent management priority to develop and employ anticipatory, future-proofing strategies[7,18].

Following a simulation of a restoration deployment at 16°C for 8 days, kelps that exhibited relatively poor performance in constant thermal rearing conditions at 20°C were able to recover from reproductive dormancy, producing similar densities of next generation life stages to kelps reared at 16°C treatments and similar or slightly lower densities to kelps reared at 12°C treatments. Kelp gametophytes serve a vital ecological function similar to seed banks in terrestrial plants[62,63], whereby dormant seeds in the soil can germinate and contribute to the population's regeneration when favorable conditions arise. In stressful abiotic conditions, kelp gametophytes can persist in a vegetative state and do not undergo gametogenesis (i.e. produce gametes) until environmental conditions are favorable[47]. Studies have explored the impacts of long-term, cold storage of gametophytes in vegetative states on viability post-storage[64,65]. In a study using kelp gametophytes held in cold storage for 5 years, viability was observed to be as high as 89%, with viability differences between genetic populations[64]. Careful development of cold storage protocols for kelp gametophytes has facilitated the preservation of genetic diversity for targeted breeding initiatives and the development of seed stock for subsequent cultivation and specific crossbreeding. This study demonstrates that *M. pyrifera* can be cultured for at least 40 days in unfavorably warm conditions at 20°C and recover reproductively, but further research is needed to determine how long gametophytes can survive under such hot temperatures and what, if any, impacts there are on future viability from prior exposure.

Following a simulation of a realistic marine heatwave (MHW) of 22°C for 10 days, kelps that were reared at 20°C exhibited significant increases of next generation life stages compared to respective controls, whereas those that were reared at 12°C and 16°C temperature treatments experienced significant declines. This result supports my hypothesis that kelps reared in stressful thermal conditions will have

higher reproductive output compared to kelps reared in optimal thermal conditions in response to simulated restoration and marine heatwave conditions. The most extreme declines were observed at 12°C, where San Diego kelps produced 5-fold fewer and Santa Cruz kelps produced 1.25-fold fewer next generation life stages compared to respective controls. San Diego kelps reared at 20°C performed better than all other treatments that experienced the MHW condition, with similar next generation life stage densities to controls that remained cultivated at a comfortable 12°C through the duration of the 68 day trial. Priming, or heat hardening, is commonly used in terrestrial cases to acclimate individuals to stressful drought, temperature, and salinity conditions[22]. In one study of priming of macroalgae (*Alaria esculenta*), gametophytes primed at 22°C (compared with 12°C) experienced enhanced survival under increased temperature and growth of the derived sporophyte stages[66]. Another study that cultivated kombu (*Saccharina japonica*) gametophytes at 22 - 24°C, resulted in increased heat-tolerance of the derived sporophytes by 2°C (as cited in [67]). The findings of my study contribute to this growing body of research, and suggest that rearing *M. pyrifera* at warmer temperatures may confer an stress memory-related advantage when deployed into warming oceans with increasing frequency and magnitude of MHWs, and provides an avenue forward for marine managers looking to future-proof restoration efforts against these acute thermal stressors. In better-studied terrestrial plant systems, specific epigenetic mechanisms such as alteration in DNA methylation, metabolite levels and histone modifications have been shown to confer improved stress tolerance to acclimatized individuals[22]. However, very few studies have looked at these mechanisms in early stage kelps and none use the iconic canopy-forming *M. pyrifera*[68]. A better understanding of the specific mechanisms underpinning kelp acclimatization and how they link to physiological, biochemical, metabolomic and morphological changes would initiate diverse opportunities to rear stress-tolerant *M. pyrifera* for restoration.

Further research on the constraints of acclimatization that explores tradeoffs between eco-physiological responses (e.g. growth, photosynthetic parameters, chlorophyll-a concentrations, enzyme activity) and competing kelp functions is needed to fully understand the potential impact of this

future proofing strategy, as cellular processes involved in temperature control also are critical to regulating responses to other conditions. One study[69] found that metabolic adjustments might aid Australasian kelp beds in enduring warmer waters, these adjustments also suppress canopy recovery from disturbances by reducing the ecological performance of kelp recruits. In other marine species, exposure to unfavorable thermal environments resulted in reduced reproductive condition, suggesting that priming may result in trade-offs and constraints lead to adverse effects on future generations[70]. The consequences of inaction and failure to proactively employ restoration strategies that improve future ecological resilience must also be considered.

Current avenues for future-proofing kelp restoration pursue beneficial outcomes of selective-breeding via genetic rescue, assisted gene flow, genetic manipulation and assisted expansion18. Selective-breeding approaches of tolerant individuals are underway in certain locations where climate-driven kelp loss is severe, such as in south-eastern Australia which has been warming four times faster than the global average[71,72]. Selective-breeding is common within kelp aquaculture settings to develop optimal cultivars for specific commercial applications (i.e. food, feed, bioenergy, fertilizer, and raw materials)[73], highlighting the potential for knowledge-sharing amongst kelp conservation and aquaculture sectors[23]. However, data concerning overall and adaptive genetic diversity and gene flow among kelp populations is generally insufficient or absent for most species, and thus the potential risks of applying these strategies (e.g., maladaptation and genetic pollution) remain a significant concern for the application of future-proofing interventions for kelp restoration at a global scale. Mechanisms underlying adaptation by acclimatization are often epigenetic, by which changes in expression do not result in permanent changes to DNA gene sequences rendering phenotypically plastic responses reversible or impermanent[74]. Therefore, acclimatization by priming of individuals used for restoration efforts in sub-lethal thermal conditions offers a more immediate future-proofing management option compared to more advanced genetic approaches. Furthermore, controlling kelp rearing temperature (achieved by utilizing cold rooms or water chillers), can be financially prohibitive and poses a challenge for kelp

culturing at scale. Rearing *M. pyrifera* at warmer temperatures that range between 16 - 20°C (conventionally taken as room temperature and sub-lethal for this species) will result in more energy- and cost- effective nursery facilities to support grow-out for restoration. Overall, these findings partly challenge the conventional practice of rearing kelps for restoration at cooler temperatures that are considered thermally optimal for the species, and suggest that acclimatization of *M. pyrifera* microstages to stressful thermal conditions during the nursery phase may bolster kelp restoration deployed into warming oceans.

Priming of *M. pyrifera* through stressful rearing environments can result in induced mortality and selection of pre-adapted genotypes, which may explain the observed positive effects from the primed gametophyte to the derived next generation life stages of kelp[75]. To avoid incorrectly attributing priming to thermal stressors to acclimatization, it is critical to examine genetic evidence for both priming and selection processes. One approach is to establish connections between positive priming effects and changes in epigenetic traits that are distinct from genetic shifts due to mortality induced selection[76,77]. Another approach is to test for outlier loci that have become dominant under positive selection[78]. The establishment of strong evidence for priming memories relies on the availability of annotated genomes[75], which remain scarce for marine macrophytes and do not include *M. pyrifera* despite the species high ecological and commercial relevance. In this study, tissue samples were collected to capture the genetic variability at the start of the rearing treatments and post-MHW, and can provide crucial evidence of whether these responses are a result of priming or selection processes.

Despite the broad distribution of *M. pyrifera* along strong temperature gradients, kelp adaptive capacity to complex thermal conditions is poorly understood and remains a critical knowledge gap for marine managers. Climate-driven threats pose an imminent threat to the persistence of kelp ecosystems and the ecosystem services they provide valued at USD $500 billion per year[2]. Since climate-driven stressors cannot be directly ameliorated in the short-term, innovative and immediate solutions are needed

to boost ecological resilience. Only recently has research highlighted the importance of species- and ecotype-specific approaches in the nursery phase to ensure successful kelp aquaculture outcomes in new regions of cultivation[79], highlighting a need for further studies of thermal adaptation that encompass molecular, organellar, cellular, and whole‑organism responses. Effective kelp restoration practices that employ a portfolio of strategies — including adaptation and acclimatization processes — may have a better chance of establishing populations that will survive and thrive in future conditions of ocean warming.

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Table 3.1. Rationale for experimental temperature treatments.

Temperature	Rationale
12°C	Standard thermal temperature for aquaculture, winter sea-surface temperature for Santa Cruz population
16°C	Summer sea-surface temperature of Santa Cruz population, winter sea-surface temperature of San Diego population
20°C	Summer sea-surface temperature of San Diego population, 4°C heatwave of Santa Cruz population
24°C	Upper limit of gametophyte survival[38], heatwave of San Diego population and near maximum sea-surface temperature of San Diego population

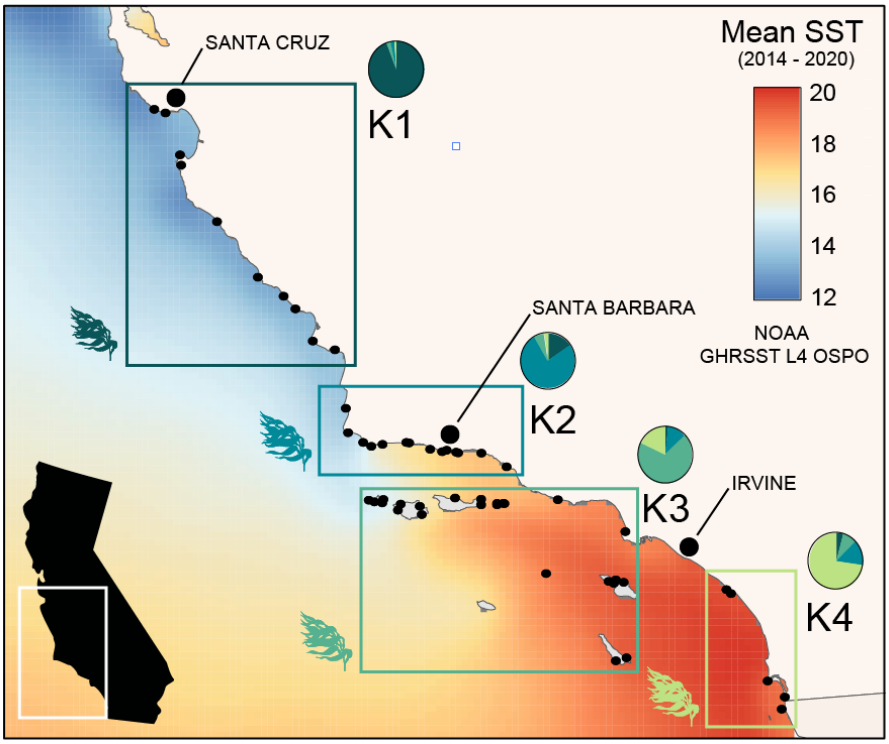

Figure 3.1. Genetic continuity and geographic clusters of *Macrocystis* in California overlaid on average sea surface temperature (SST) data from June 2014 to May 2020. Black filled circles represent sampling locations obtained from Johansson et al. (2015) and are enclosed by colored boxes that represent the predominant genetic cluster (K1-K4 regions). Pie charts represent the average *Macrocystis* genotype for each region. Mean SST was generated using datasets from the National Oceanic and Atmospheric Administration's Office of Satellite and Product Operations (OSPO). Map generated using R v6.3.

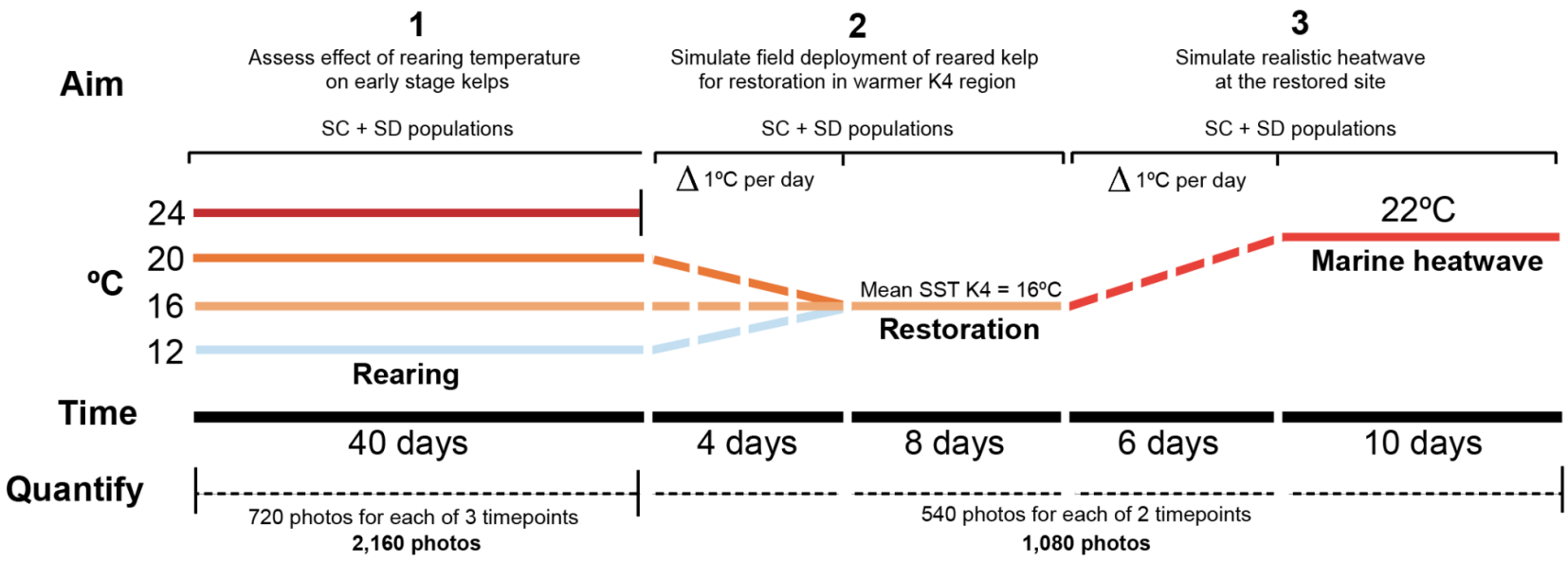

Figure 3.2. Illustration depicting Santa Cruz and San Diego thermal 'treatment' conditions and rationales for Aims 1 - 3.

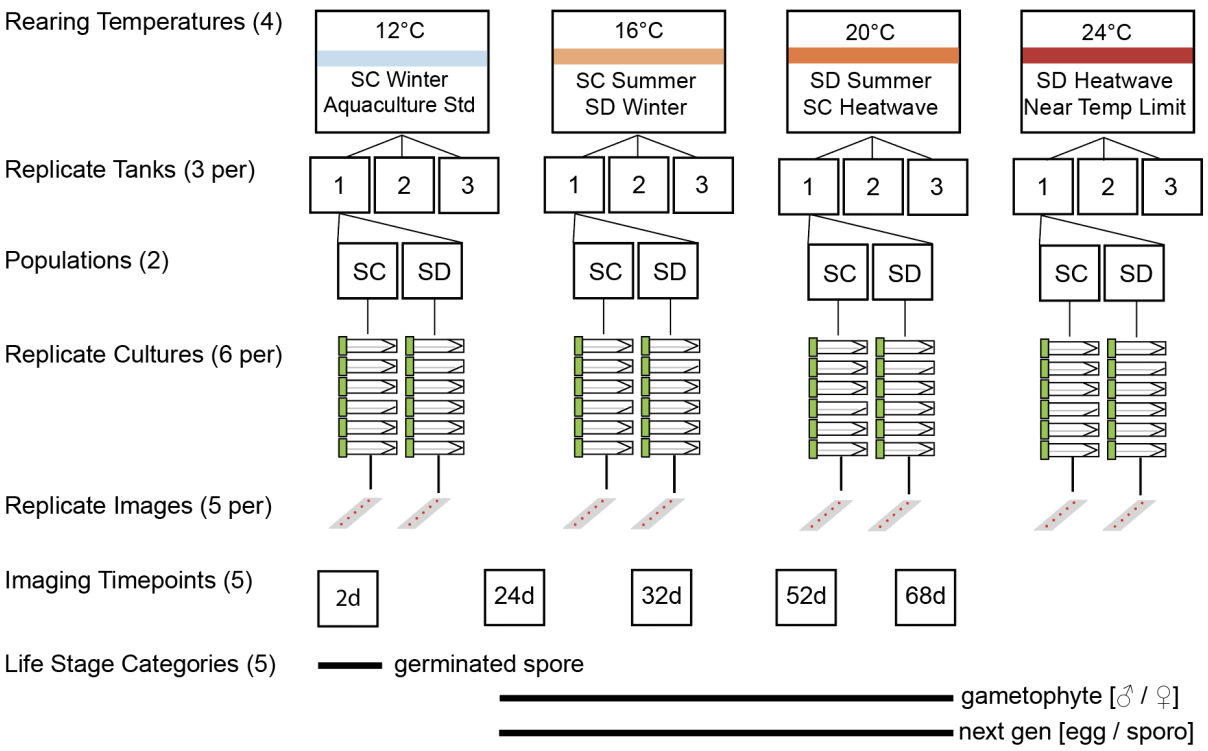

Figure 3.3. Illustration depicting experimental design for **Aim 1** (4 thermal conditions x 3 replicate tanks x 2 populations (Santa Cruz [SC] and San Diego [SD]) x 6 slide replicates x 5 fixed fields = 720 images x 3 time points), resulting in a total of 2,160 images.

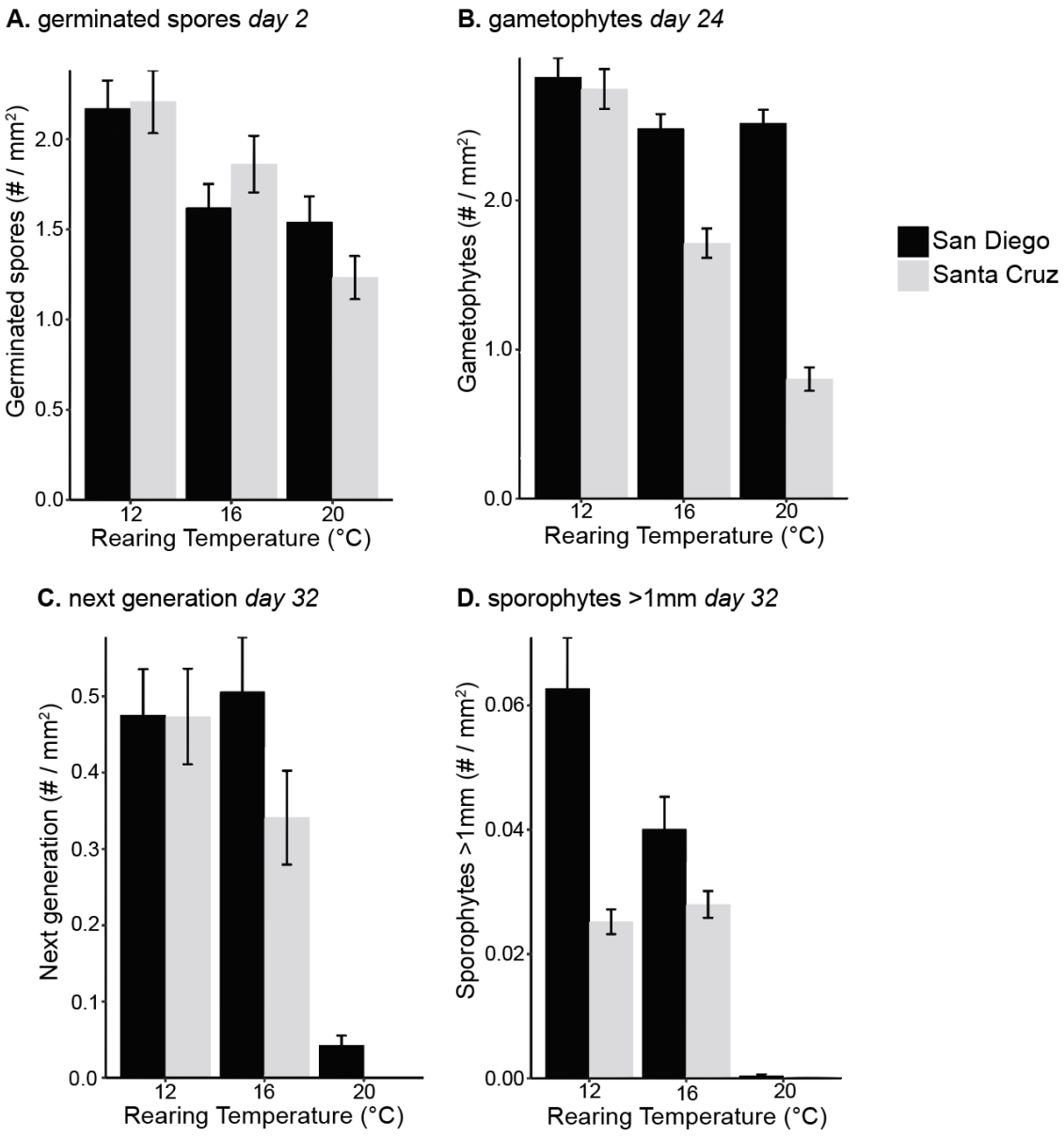

Figure 3.4. Life stages observed for San Diego and Santa Cruz populations of origin reared at constant thermal conditions of 12, 16, and 20 °C. San Diego indicated in black and Santa Cruz indicated in gray. (A) Germinated spores at day 2 (N = 107 samples, 535 observations). (B) Gametophytes at day 24 (N = 108 samples, 540 observations). (C) Next-generation at day 32 (N = 105 samples, 525 observations). (D) Sporophytes > 1mm in length at day 32 (N = 108 samples, 108 observations). Error bars, mean ± 1 SE.

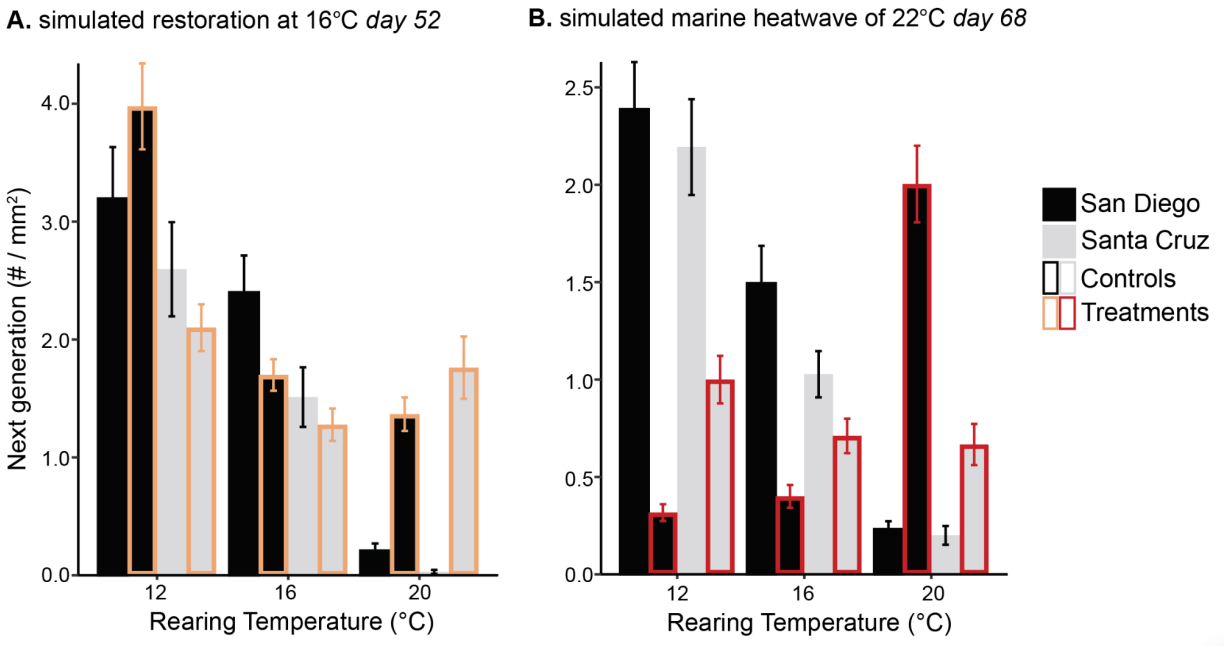

Figure 3.5. Quantification of next generation life stages observed for San Diego and Santa Cruz populations of origin reared 12, 16, and 20 °C following (A) a simulated restoration at 16°C (N = 108 samples, 540 observations), and (B) a simulated heatwave of 22°C (N = 107 samples, 535 observations). San Diego indicated in black and Santa Cruz indicated in gray, 'control' thermal conditions indicated by black/gray outlines, 'treatment' thermal conditions for a simulated restoration at 16°C indicated by orange outlines and 'treatment' thermal conditions for a simulated heatwave of 22°C indicated by red outlines. Error bars, mean ± 1 SE.

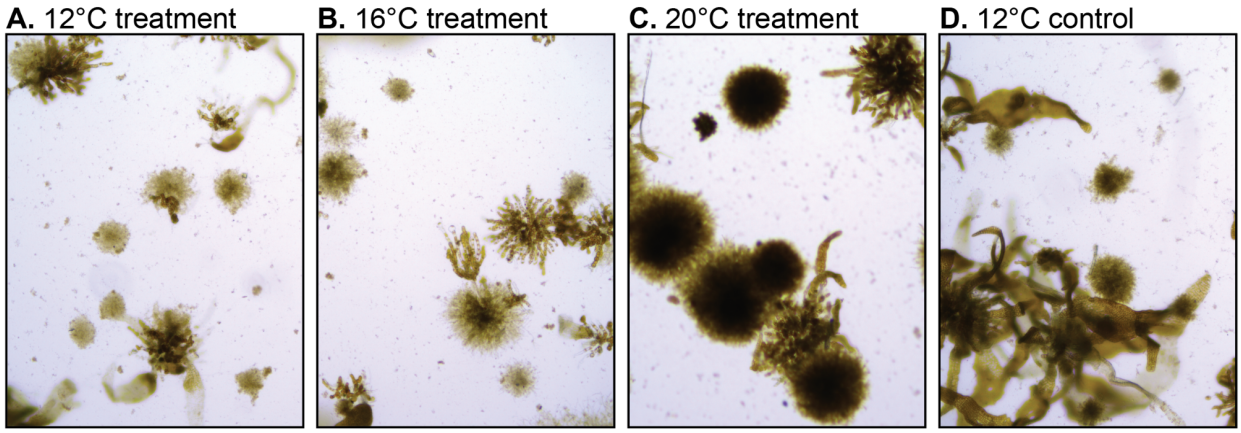

Figure 3.6. (A - C) Examples of microscopy photos after the simulation of a marine heatwave for San Diego kelps reared at 12°C, 16°C and 20°C, respectively. (D) Example of microscopy photo of 'control' sample that remained at 12°C.