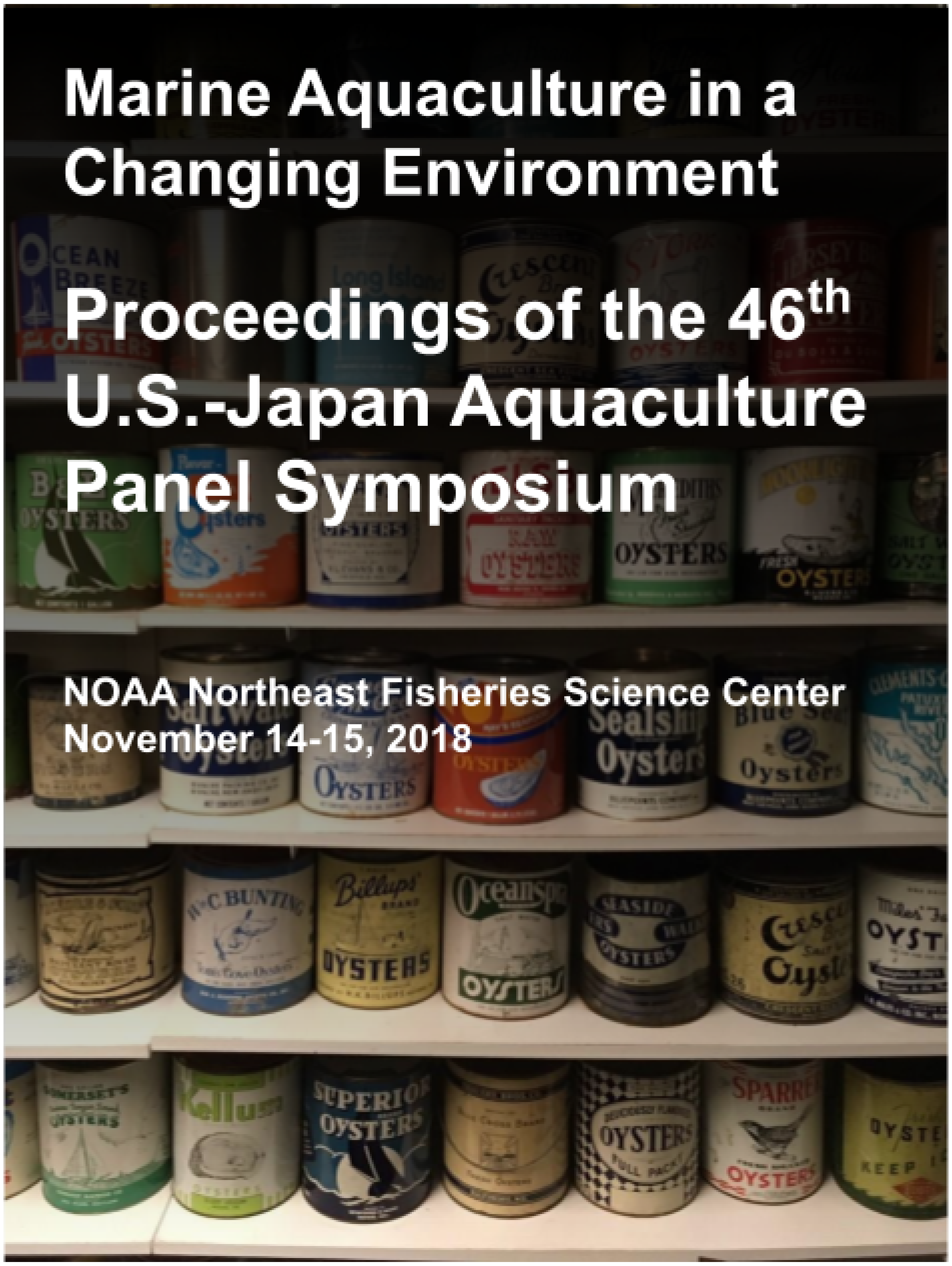
Marine Aquaculture in a Changing Environment

Proceedings of the 46th U.S.-Japan Aquaculture Panel Symposium

NOAA Northeast Fisheries Science Center
November 14-15, 2018



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Kristen Jabanoski[1], Clete Otoshi[2], Erick Sturm[3] and Mike Rust[2] (editors)

[1] Contractor with A.I.S., Inc.
NOAA National Marine Fisheries Service
Northeast Fisheries Science Center – Milford Laboratory
212 Rogers Avenue
Milford, CT 06460

[2] NOAA National Marine Fisheries Service Office of Aquaculture
1315 East-West Highway
Silver Spring, MD 20910

[3] Southwest Fisheries Science Center
Fisheries Ecology Division
110 McAllister Way
Santa Cruz, CA 95060

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Cover photo of historic canned oyster collection at Copps Island Oysters, LCC, in Norwalk, Connecticut. Photo credit: NOAA Fisheries

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Introduction

The impacts and interactions between environmental change and aquaculture are diverse and many are likely unknown. What we do know is that nutrient pollution is driving eutrophication and dead zones, ocean acidification is changing water chemistry and climate change is already influencing our food supply, fresh water availability, weather and way of life. Aquaculture will be impacted by, and can also impact, these environmental changes over various scales. Aquaculture of finfish, shellfish and seaweed have different threats, benefits and opportunities related to environmental change, but in the right location, and with the right management they can be mostly beneficial to the environment. Because of the significant aquaculture industry impacts caused by environmental change, and the potential for aquaculture to provide some mitigation, research on this topic was a priority shared by the Fisheries Research and Education Agency (FREA) of Japan, the U. S. National Oceanic and Atmospheric Administration (NOAA), and the U.S. Department of Agriculture. These Proceedings represent the second of three consecutive years of meetings where the theme is “Aquaculture in a Changing Environment”. This year, the research focused on the impacts to aquaculture production due to environmental change (e.g., ocean acidification impacts on shellfish aquaculture) and science to mitigate these impacts (counter measures). Proceedings from the previous Symposium were published by FREA (45th UJNR), where the focus was on the potential of aquaculture to mitigate impacts of environmental change (e.g., sequestering carbon, bioextraction of nutrients and CO_2, antacid, oxygen production). Looking forward, we will seek to explore science and technology that address these issues and will enable aquaculture to prosper in a changing environment and be a part of the solution to reducing the human impacts of environmental change.

Michael B Rust

Michael Rust
NOAA Fisheries Office of Aquaculture
United States Panel Chair

The influences of Environmental Changes on Japanese Nori Mariculture

Mahiko Abe[1*], Noboru Murase[1], Masahiro Kobayashi[2], Eiji Fujiyoshi[3], and Motoya Tamaki[3]

[1] National Fisheries University, FRA, Shimonoseki, Yamaguchi 759-6595, Japan
[2] National Research Institute of Fisheries Science, FRA, Yokohama, Kanagawa 236-8648, Japan
[3] Seikai National Fisheries Research Institute, FRA, Nagasaki, Nagasaki 851-2213, Japan

Corresponding author: abemahi@fish-u.ac.jp

Keywords: *Pyropia tenuipedalis*, breeding, cell selection, low salinity tolerance

ABSTRACT

Nori mariculture has a long history of over one hundred years and is one of the most important fisheries industries in Japan. Recently, nori production has been decreasing due to environmental changes, such as seawater temperature rise and low nutrient levels. As for the seawater temperature rise, the start of nori mariculture is delayed because of high water temperature staying above 23ºC in autumn, thereby shortening the season of nori mariculture. Spores start to be released when the water temperature decreases below 23ºC. Low nutrient levels in ambient waters cause bleaching of nori thalli, reducing the quality and price of nori. Currently, new culture species and strains of nori around the coasts of Japan are being explored. The thallus has a simple structure and cells within the thalli sometimes have mutations. Mutant cells may have the potential for tolerance to high water temperature and low nutrient level, as well as other favorable characteristics. More recently, shortening of the thalli is observed frequently. One of the causes is predation by herbivorous fishes (e.g. *Acanthopagrus schlegelii*, *Mugil cephalus cephalus*) and ducks (*Anas penelope*, *Anas acuta*). In Japan, it has been reported that herbivorous fishes cause the disappearance of marine forests, which is referred to as “iso-yake”. The duration of iso-yake caused by herbivorous fish has been prolonged because of increased water temperatures. While the activities of the iso-yake causing fish are reduced below 17ºC, the herbivorous fishes eating nori are active at low water temperatures. Even if some measures are taken to control herbivorous fish, birds would alternatively eat nori. It is urgently necessary to develop effective measures to control these herbivores.

INTRODUCTION

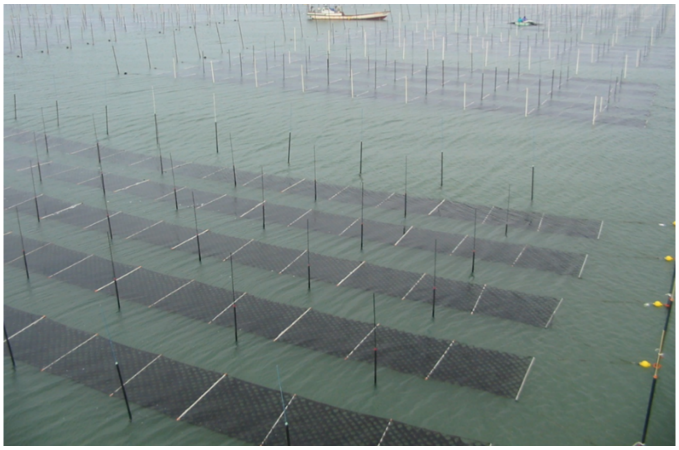

Figure 1. Photograph showing nori mariculture in Fukuoka Prefecture.

Nori mariculture has a long history and many farmers and researchers have developed innovative technologies which made nori mariculture one of the most important fisheries industries in Japan (Fig. 1). However, nori production has decreased due to environmental changes, such as seawater temperature rise and low nutrient levels. At present, nori mariculture of *Pyropia yezoensis* is mainly used and *P. tenera* is used in limited areas*. Culture strains have been repeatedly selected within *P. yezoensis* for a long time and, as a result, the strains have lost genetic diversity. Dissolved inorganic nitrogen (DIN) levels started to decrease (i.e. oligotrophication) in the 1980s in the western part of Japan, and in the 1990s in the eastern and central parts of the Seto Inland Sea (Abo and Yamamoto, 2019). After the late 1990s, disappearance of macroalgal beds, generally called “iso-yake” in Japanese, has spread along the coast of western Japan because of increasing sea water temperature (Yoshida et al., 2019). Nori production had the highest value in 2001 and has been decreasing until present. However, in the nori mariculture industry, it was not until recent years, when the production fell below the level of demand of 8 billion sheets, that the effects of oligotrophication and water temperature rise became an issue. Therefore, many researchers are searching earnestly for measures to address reduced nori mariculture production. In the present paper, we introduce the history, problems and solutions of nori mariculture.

Background on nori mariculture in Japan

In Japan, the use of nori has a long history of over two hundred years. In the Bunka-Bunsei period (1804 – 1830), Katsukawa Shunsen designed the ukiyo-e print of nori making (Miyata and Tomizuka, 1997). People during this era harvested nori growing in the wild. The procedure for nori making then is similar to that in the present, such as chopping, forming and drying, except that everything was done by hand. It is thought that sights of nori-making were common scenery in the Edo period (1603 – 1867). In the early 1900’s, nori mariculture began and was cultured on “sodahibi”, which are made of tree branches and set around the coast (Okamura, 1909). However, at that time, nobody knew where the spores came from, and nori was harvested, chopped and formed into sheets by hand like in the Edo period. In recent years, nori mariculture has changed. Dr. Kathleen Mary Drew Baker’s discovery of the microscopic conchocelis phase within the life cycle of nori (Drew 1949) made it possible for Japanese researchers to establish methods of maintaining different strains of nori. Nori mariculture in the present uses nets instead of “sodahibi” and is operated on larger scales using machinery for harvesting, chopping and drying of the nori. Nori mariculture has become one of the most important fisheries industries in Japan with many innovations.

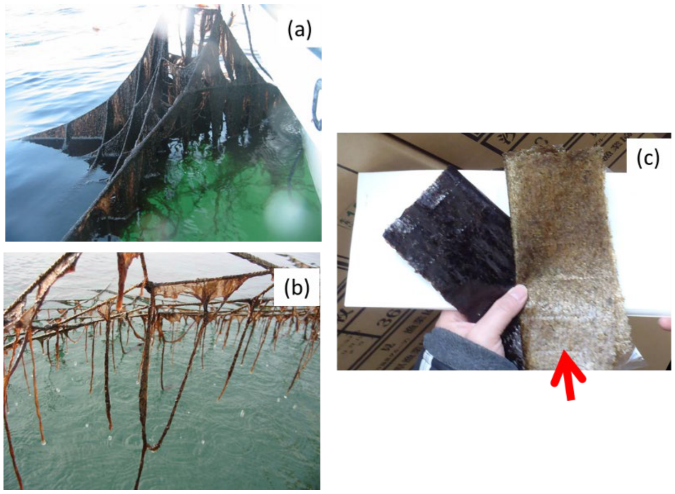

Figure 2. Photographs showing well grown nori thalli (a), bleached thalli (b) and products of well grown and bleached thalli (c). Red arrow shows product of bleached thalli.

Problems of nori mariculture

Nori mariculture is one of the largest fisheries industries in Japan. However, nori production has decreased for the past decade from 10 billion sheets in the 1990’s to below 8 billion sheets in recent years. Nori production further decreased to 6.3 billion sheets in 2018. The causes of the decrease in nori production are mainly seawater temperature rise and low nutrient levels.

Nori mariculture starts when the seawater temperature decreases below 23°C. The start of nori mariculture is delayed by increasing water temperatures, staying above 23°C in autumn, due to global warming. Nori mariculture used to begin in the first quarter of October in the 1980’s, but began in the second to the last quarter of October in the 2010’s. On the other hand, the timing of spring seawater temperature rise is unchanged, which results in a shortening of the culture period for nori. In Japan, the oligotrophication of coastal waters due to the regulation of wastewater discharge is becoming problematic. Low nutrient levels in the ambient water cause bleaching of nori thalli, reducing the quality and price of nori. Generally, nori thalli grow with a dark black color (Fig. 2-a, c), but in an oligotrophic environment, thalli grow with a light brown color (Fig. 2-b, c). Bleached thalli are 2 to 3 JPY cheaper per sheet than dark black thalli.

Potential Solutions to the Problems

Many researchers are trying to develop new breeding strains of nori with tolerances to environmental changes, such as high water temperature and low nutrient levels. In addition to conventional selective breeding, cell selection within thalli and cell fusion using protoplasts and mutation breeding with heavy ion beams are also carried out at several institutes (Sakaguchi 2011; Niwa et al., 2011). In China, pigment mutants using ^{60}Co-γ ray irradiation were isolated (Yan and Aruga, 2000). Field use of the strains obtained by cell fusion and mutation breeding is not yet permitted in Japan. However, strains obtained by cell selection within thalli have begun to be used in field culture (Sakaguchi, 2011; Abe et al., 2019). It is thought that cell selection within thalli is a useful technique for development of new strains and its use in the field has no legal restrictions so far. In addition, many researchers are exploring alternative culture species in the genus *Pyropia*. Strains of *P. yezoensis* used for mariculture have been screened by selective breeding for over 40 years, making genetic diversities of the strains poor. Moreover, breeding methods such as cell fusion and mutation mentioned above require special machines and techniques. Bangiaceae including the genus *Pyropia* contains 30 species, and many of them are used as local food resources. However, the distribution of nori species in many local areas is unclear. Different nori species growing in the wild have a wide variety of characteristics and they may be candidates for new culture species suited for different environmental conditions. In fact, *P. tenuipedalis* has been cultured for a few years on an experimental scale (Fig. 3).

Figure 3. Photograph showing experimental mariculture of new culture species Pyropia tenuipedalis.

Countermeasures for oligotrophication, fertilization, optimal management of wastewater, and plowing of the sea bottom have been attempted in several water bodies. Since nutrients are used not only by nori but also by phytoplankton, which could cause adverse effects to the environment, these methods must always be accompanied by a survey of the occurrence of phytoplankton (Mine, 2019). Moreover, development of polyculture techniques combining bivalve aquaculture (e.g. oyster and clams) with nori mariculture has been attempted. Bivalves feed on phytoplankton, which compete with nori for nutrients, and their excreta is supplied to nori as nutrients. Although these attempts are in the trial phase, environmental improvement utilizing biological functions is promising.

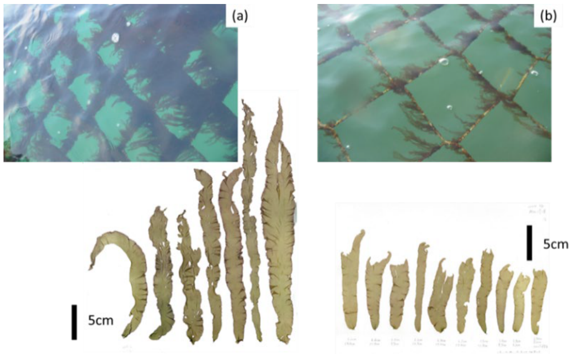

Figure 4. Photographs showing nori culture net with well grown thalli and the specimens (a) and culture net with shortened thalli and the specimens (b).

An emerging problem

In recent years, many incidents of shortening of nori thalli have been observed in Japan (Fig. 4). Until now, it was thought that one of the major causes of the shortening was low salinity of ambient water, based on the symptom of the cell damage (Kirita and Matsui, 1993). However, occurrences of the shortening have also been observed without low salinity conditions. Field observations at nori mariculture sites using video and time-lapse cameras have revealed another cause of the shortening: predation by herbivorous fishes (e.g. *Acanthopagrus schlegelii* and *Mugil cephalus cephalus*) and ducks (*Anas penelope* and *Anas acuta*). In Japan, it has been reported that herbivorous fishes (e.g. *Calotomus japonicus*, *Siganus fuscescens* and *Kyphosus bigibbus*) cause the disappearance of marine forests, which is referred to as "iso-yake". The duration of "iso-yake" caused by herbivorous fish has been getting longer because of seawater temperature rise because of global warming. The activities of fish causing "iso-yake" are reduced below 17ºC, allowing macroalgae to thrive in the winter. However, the fishes and ducks causing the shortening of nori can actively graze nori even below 17ºC. The seawater temperature typically ranges from 10ºC to 20ºC during nori culture season, and grazing pressure can cause an enormous loss of nori thalli. It is necessary to develop effective measures to lessen predation damage by these herbivorous fishes and ducks immediately.

* *Pyropia tenuipedalis*, *P. yezoensis* and *P. tenera* in this paper were replaced to *Neopyropia tenuipedalis*, *N. yezoensis* and *N. tenera* by Yang et al. (2020), respectively.

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Abe, M., M. Kobayashi, E. Fujiyoshi, M. Tamaki, N. Kikuchi, and N. Murase. 2013. Use of PCR- RFLP for discrimination of Japanese *Porphyra* and *Pyropia* species (Bangiales, Rhodophyta). J. Appl. Phycol. 25: 225-232. DOI. 10.1007/s10811-012-9856-7.

In this paper, the authors developed a PCR-RFLP method useful for discrimination of 16 Japanese and two non-Japanese *Porphyra* and *Pyropia* species. These species have simple morphological features, making morphological species identification difficult. The two primer sets on the mitochondrial DNA used in this study were able to amplify single fragments with PCR (partial mitochondrial DNA related to *ATP6* gene and *trnC – rns*). All 18 species were successfully distinguished using a combination of five restriction enzymes (*Taq*I, *Ssp*I, *Aci*I, *Cfr*13I and *Alu*I). It was concluded that PCR-RFLP analysis is a useful tool for discrimination of wild strains of *Porphyra* and *Pyropia* species for potential use in mariculture.

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Pyropia tenuipedalis is a new culture species of nori in Yamaguchi Prefecture, Japan. The habitat of this species is more brackish as compared with that of *P. yezoensis*, which is the common species in Japanese nori mariculture. In this paper, the authors investigated the relationship between salinity and growth of *P. tenuipedalis* and *P. yezoensis*. It was revealed that *P. tenuipedalis* tolerates lower salinity in comparison with *P. yezoensis*.

Abe, M., Y. Fujita, M. Kobayashi, E. Fujiyoshi, M. Tamaki, Y. Fukui, M. Satomi, and N. Murase. 2015. Effects of antibiotics on survival and growth for *Pyropia yezoensis* protoplasts. Aquacult. Sci. 63: 1-8.

The nori thallus sometimes has cell mutations. For the breeding of *Pyropia* species, it was necessary to develop an axenic cell culture method. In this paper, we investigated a way of isolating axenic protoplasts of *P. yezoensis* and the effects of twelve antibiotics on the survival and growth of the protoplasts. The axenic protoplasts were able to be isolated by a series of treatments with sodium citrate seawater. The growth rates of the protoplasts treated with FRM and ABPC were 1.1–2.7 times higher than that of the control, while PCG and SM suppressed growth. The authors succeeded in developing an axenic culture method for Pyropia species, but the survival, growth and morphogenesis of the cells in the axenic culture varied. Improvement of the methodological stability is necessary.

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Development of Environmentally Friendly, Cost-Effective and Nutritionally Balanced Alternative Protein Based Diets for High Value Marine Fish Culture in Recirculating Aquaculture Systems

Md Shah Alam*, Wade O. Watanabe, and Patrick C. Carroll

Center for Marine Science, Aquaculture Program, University of North Carolina Wilmington, 601 S. College Rd., Wilmington, NC-28403, USA, Tel. 910-962-2945

Corresponding author: alamm@uncw.edu

Keywords: Alternative protein sources, fish meal replacement, black sea bass, southern flounder, and cost-effective aquafeeds

ABSTRACT

With very little domestically grown seafood, along with increasing exploitation of wild stocks, the US annual seafood trade deficit now exceeds $15 billion. Economics, environmental issues and fish feed are factors vital to the sustainability of the aquaculture industry in the US and Japan, and these factors increase the pressure on aquaculturists to develop eco-friendly and cost-effective aquaculture practices. One of the challenges faced by the aquaculture industry is to prevent the pollution of water by artificial feed. Fish meal is the main protein source used in aquafeed and is made from small pelagic species of forage fish. Two nutrients in fish meal, nitrogen and phosphorus, have a great impact on the environment, and their release can result in eutrophication. Fish meal could also contain polychlorinated biphenyls (PCBs), dioxins, and other harmful chemicals, and become a vector of contamination in farm-raised finfish. Alternate protein sources such as terrestrial animal and plant protein sources can reduce the amount of wild fish used as protein, and potentially reduce the nutrient levels in effluent waste. One of the goals of our research at the University of North Carolina Wilmington Center for Marine Science is to develop sustainable marine finfish feed with reduced inclusion of fish meal in recirculating aquaculture systems, which are widely considered to be an "environmentally friendly" technology for producing farmed fish.

A series of experiments were conducted to test the effects of different dietary levels of soybean meal, ultra-low gossypol-based glandless cottonseed meal, and poultry meal in the diet of southern flounder and black sea bass reared in a recirculating aquaculture system for 8-10 weeks. Growth performance, feed utilization, digestibility, and the biochemical composition of fish tissues were evaluated.

Results suggest that about 68 and 39% fish meal protein could be replaced by soybean meal protein and 82 and 100% of fish meal protein could be replaced by poultry meal protein in the diet of black sea bass and southern flounder, respectively. Results also suggest that fish meal protein could be replaced up to 100 and 75% by glandless cottonseed meal protein in black sea bass and southern flounder diets, respectively. These findings reveal species-specific differences in effective substitution levels of alternative protein sources to fish meal protein, and provide a basis for developing environmentally- sound and cost-effective predominantly terrestrial plant and animal protein-based diets for high value marine fish culture in the USA and Japan.

INTRODUCTION

In terms of dietary composition, protein is the single largest and most expensive component in fish feed. In aquaculture feed, fish meal is a popular source of high-quality protein and highly digestible essential amino and fatty acids (Cho and Kim 2011). By replacing fish meal in aquaculture diets, alternate terrestrial plant and animal protein sources can lower feed costs, reduce the amount of wild fish used as protein, and potentially reduce the nutrient levels in effluent waste (Trushenski et al. 2006). However, for most species, there is a limit to how much fish meal can be replaced by alternative protein sources without negatively affecting fish growth and feed efficiency (Gatlin et al. 2007).

Soybean meal

Soybean meal is considered to be one of the most suitable ingredients for replacing fish meal in commercial fish feed. Compared with other plant protein sources, soybean meal has high protein content, very low carbohydrate and fiber, high digestibility, and a good amino acid profile (Hardy 2006; Gatlin et al. 2007). Soybean meal is used in fish feed not only because of its high protein content, but also owing to its worldwide availability.

Low-gossypol cottonseed meal

Cottonseed meal (CSM) has received limited attention as a potential fish meal replacement in aqua feeds; however, CSM protein is an increasingly attractive alternative to fish meal protein due to improved plant production and processing methods. Due to the large global production of cotton, as well as the large quantities of cottonseed by-products, CSM is much cheaper per unit of protein than fish meal and other fish meal replacements. An important constraint to the use of CSM in animal feeds is gossypol, a terpene-based secondary metabolite that has an important role in the cotton plant's defense mechanisms against pests and possibly diseases (Romano and Scheffler, 2008). The utilization of CSM as an ingredient in the feeds of animals and fish has been improved by reducing or eliminating gossypol through traditional genetic selection or through genetic modification (GMO). Regular high-gossypol CSM (R-CSM) protein has been used to replace fish meal protein at maximum levels of 35% in grass carp (Zheng et al., 2012) and 30% in parrotfish feeds (Lim and Lee, 2009). However, research on fish meal replacement by low-gossypol based CSM is very limited on marine fish species.

Poultry by-product meal

Terrestrial animal protein sources have several advantages, including a similar amino acid profile to fish meal, availability, and relatively low cost. Poultry by-product meal (PBM) is a protein source produced from waste and by-products of processed chickens, possibly including heads and feet, but excluding feathers and intestines. Like other animal- based protein feedstuffs, it has a high protein content, but can vary in compositional quality and lacks certain essential amino acids (Tacon et al. 2006). PBM has been used successfully to replace fish meal at high levels of dietary inclusion for a number of finfish species. In gilthead seabream, 50% of the fish meal protein was successfully replaced with PBM protein without a reduction in growth (Nengas et al. 1999). In juvenile red drum, 67% of the fish meal protein was replaced by PBM protein with no reduction in growth (Kureshy et al. 2000).

Black sea bass

Black sea bass *Centropristis striata* is a commercially important marine finfish species that commands a high market price. This species inhabits the coastal waters of the eastern USA, from the Gulf of Maine to Florida. The abundance of black sea bass along the U.S. East Coast has been declining since the 1950s (NOAA 2012; ASMFC 2016). Potential for limited market supplies and for higher prices of ocean caught black sea bass in the future are important economic incentives to investigate the feasibility of black sea bass production via aquaculture to help meet market demand (Watanabe 2011; Watanabe et al. 2016). Hatchery-raised black sea bass juveniles originating from captive wild- caught broodstock are routinely cultured through the market stage in a recirculating aquaculture system at the University of North Carolina Wilmington (UNCW) Aquaculture Facility (Watanabe et al. 2003; Watanabe 2011; Watanabe et al. 2021).

Southern Flounder

The southern flounder *Paralichthys lethostigma* is a flatfish in the family Paralichthyidae. It can be found in coastal waters from Albemarle Sound, North Carolina, through the South Atlantic states with the exception of South Florida. Southern flounder landings have declined, leading to more stringent fishery regulations and interest in culturing native flatfishes for stock enhancement or food fish production. The development of intensive culture methods for southern flounder in the southeastern U.S. is of great interest because of its euryhaline character and its status as a highly desirable food and recreational species and potential for commercial culture. Hatchery methodology for spawning and larval rearing is well investigated (Daniels and Watanabe, 2003; Watanabe et al., 2006; Wright- Moore et al., 2019).

MATERIALS AND METHODS

Experimental animals and system

Juvenile black sea bass and southern flounder were cultured from eggs supplied by photothermally conditioned captive broodstock held at the UNCW Center for Marine Science, Aquaculture Facility (Wrightsville Beach, NC). Adults were induced to spawn using luteinizing hormone-releasing hormone analogue (LHRHa) implants (Watanabe et al., 2003). Eggs were hatched and reared through juvenile stages in 150 L tanks using protocols established at UNCW (Watanabe, 2011; Carrier et al. 2011, Russo et al. 2017). All the experiments were conducted in a controlled environment laboratory consisting of twenty-four 75-L rectangular (76 x 32 x 43 cm) glass tanks, supported by a recirculating aquaculture system.

Table 1. Experimental designs.

Experimental fish	Tested alternatives	Replacement levels	References
Black sea bass	soybean meal	0-100%	Alam et al. 2012
Southern flounder	soybean meal	0-100%	Alam et al. 2011
Black sea bass	low-gossypol cottonseed meal	0-100%	Anderson et al. 2016
Southern flounder	low-gossypol cottonseed meal	0-100%	Alam et al. 2018
Black sea bass	poultry by product meal	0-100%	Dawson et al. 2018
Southern flounder	Poultry by product meal	0-100%	Dawson, 2012

Experimental diets and designs

A series of feeding trials were designed to test the substitution limits of soybean meal, CSM and PBM protein in replacement of menhaden fish meal protein (FMP) in the diet of juvenile black sea bass and southern flounder (Table 1) (Alam et al. 2011, 2012, 2018, Dawson, 2012, Dawson et al. 2018, Anderson et al. 2018).

Studies with soybean meal

Three experiments were conducted to determine the extent to which FMP can be replaced by solvent-extracted soybean meal protein (SBP) in the diet of juvenile black sea bass. In all experiments, diets were formulated to replace FMP by SBP from 0 to 100% and supplemented with squid meal, krill meal, and attractants (Alam et al. 2012). For southern flounder, two feeding experiments were conducted replacing FMP with SBP at 0, 10, 20, 30, 40, 50, 60 and 70% with or without supplemental L-methionine and L-lysine (Alam et al. 2011).

Studies with low-gossypol CSM

Eight diets were formulated for juvenile black sea bass to replace FMP by three CSM proteins: a CSM prepared from glandless cottonseed meal (GCSM, 50.4% crude protein, CP), a CSM that had been solvent extracted with acidic ethanol to remove the gossypol (SCSM, 53.8% CP), and a CSM prepared from regular (glanded) cottonseed (RCSM, 45% CP). Three diets replaced 50, 75 and 100% of FMP with GCSM, and three diets replaced 50, 75 and 100% of FMP with SCSM. One diet replaced 100% FMP with RCSM protein (Anderson et al. 2016). For southern flounder, CSM proteins from GCSM, genetically-modified low-gossypol seed (GMO-CSM, 56.0% CP) and R-CSM were evaluated to replace FMP. Six diets replaced 50, 75 and 100% FM protein with GCSM or GMO-CSM protein (Alam et al. 2018).

Studies with PBM

Two feeding trials were conducted to determine the maximum substitution limits of poultry by-product meal (PBM; 66% CP) protein for fish meal (Fish meal; 59% CP) protein in the diet of juvenile black sea bass and southern flounder. Eight diets were formulated for each experiment to replace FMP with feed-grade PBM protein at 0 (control), 40%, 50%, 60%, 70%, 80%, 90%, and 100% in black sea bass and southern flounder diets (Dawson et al. 2018, 2012).

All experimental test diets were prepared at the UNCW Aquaculture Facility as described by Alam et al. (2011, 2012, 2018) and stored in a freezer (-20 ^{o}C). All diets were isonitrogenous and isolipidic for each experiment. A control diet (0% SBM, PBM and CSM) was formulated with high FMP and other practical protein sources. L-methionine and L-lysine were supplemented to the diets to equal the control diet.

Feeding trials

Experimental diets were fed to triplicate groups of fish twice a day (0900 and 1500 h) to apparent satiation (i.e., as much as they can consume with minimal wastage) for six to ten weeks. Fish in individual tanks were lot weighed every two weeks to monitor weight gain throughout the experiment. Mortalities were also recorded for each tank throughout the experiment. Water quality was monitored throughout the experiment on a weekly basis and maintained at optimum levels (Watanabe et al. 2011). Temperature, salinity, dissolved oxygen, and pH were monitored using a multi-parameter probe (YSI, Yellow Springs, Ohio, USA). Ammonia and nitrite were monitored using a HACH spectrophotometer (DR 2010, Loveland, Colorado, USA).

Biochemical and statistical analysis

At the end of the feeding trial, five fish from each tank were collected for biochemical analysis to determine proximate composition (moisture, ash, lipid, and protein-AOAC, 2000) and fatty acid profiles of the whole bodies of the fish. Fatty acid analysis was conducted in triplicate as described in Dawson et al. (2018) and Alam et al. (2012). Total amino acid analysis of diets was conducted in duplicate (AAA Laboratories, Damascus, Oregon, USA). At the conclusion of the experiment, fish were reared for an additional 14 days on their treatment diets to determine the apparent digestibility coefficient (ADC) of crude protein in the experimental diets using 0.5% chromic oxide as inert marker. Chromic oxide content was determined using a spectrophotometer through a modified Furukawa and Tsukahara (1966) method. Statistical analysis was performed using the JMP 7 statistical software (SAS Institute Inc., ver. 13, Cary, North Carolina).

RESULTS AND DISCUSSION

Experiments with soybean meal protein (SBP)

No significant differences in body weight gain, feed efficiency, and survival were observed among fish fed diets replacing FMP with SBP at 0 to 70% (Alam et al. 2012). In addition, body weight gain was not significantly different in fish fed supplemental methionine and lysine in 70% SBP diets compared to fish fed 0% SBP diets. No significant differences were observed in whole-body n-3 polyunsaturated fatty acids among the treatments. Broken-line regression of the specific growth rate data suggested that the maximum level of FMP replacement with SBP in black sea bass diets was 67.6–68.4% with 7.5% squid meal and 5% krill meal in the diet (Fig. 1) and 57.2–58.0% without squid and krill meal supplementation (Alam et al. 2012). In black sea bass diets supplemented with squid meal, krill meal, and attractants, the maximum replacement level of FMP with SBP (67.6–68.4%) was higher than reported for other marine finfish species such as Japanese flounder (45%, Kikuchi 1999); gilthead seabream (45%, Martinez-llorens et al. 2008); cobia (50%, Zhou et al. 2005), and Atlantic cod (50%, Walker et al. 2010), but lower than that found for freshwater fishes such as common carp (100%, Viola et al. 1982) and Nile tilapia (100%, Deyab et al. 2002). In general, the fatty acid composition of the fish whole body reflected the composition of their diets. Linoleic acid (18:2[n-6]) was higher in the SBP diets as well as in the whole body of fish fed those diets. Studies on yellow perch (Kasper et al. 2007), pike perch (Schultz et al. 2005), and Japanese flounder (Lee et al. 2000) also found higher levels of linoleic acid in fish fed diets containing SBP or soybean oil. As SBP content was increased in the diets, fish oil was increased to compensate for the low lipid content of SBP, leading to higher n-3 PUFA levels in the SBP diets. All three feeding trials showed that black sea bass juveniles are able to utilize high levels of soybean meal in their diet without reducing growth performance.

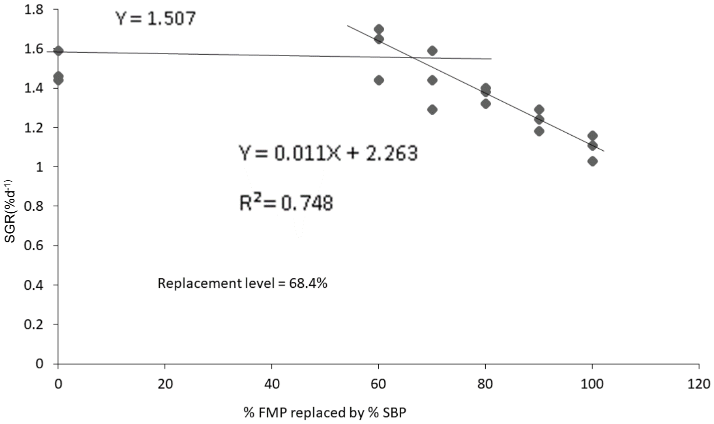

Figure 1. Broken-line regression analysis between specific growth rate (SGR) and % FMP replaced by % SBP with supplemental squid meal, krill meal and attractants in the diets of juvenile black sea bass (Alam et al. 2012).

In southern flounder, no significant differences were observed in body weight gain, specific growth rate, feed intake, feed conversion and protein efficiency ratios, and the whole-body proximate composition among fish fed diets replacing 0–40% of FMP with SBP (Alam et al. 2011). When compared with fish fed 0% SBP, weight gain decreased significantly for the fish fed more than 40% SBP. Broken-line regression showed that the optimum levels of FMP replacement with SBP in the diet of southern flounder without and with supplemental methionine and lysine were 35.1% and 38.9%, respectively, when diets contained 9% squid meal and 5% krill meal. Maximum replacement level of FMP with SBP without supplementing amino acids for southern flounder (40%) in this study was similar to what was found in cobia (40%; Zhou et al. 2005) and slightly lower than reported in the gilthead sea bream (45%; Martinez-Llorens et al. 2008). In both experiments (with or without amino acids), whole-body moisture, protein, and lipid contents were not affected by SBP replacement of FMP up to 40%, similar to what was reported in yellow croaker (Ai et al. 2006), rainbow trout (Bureau et al. 2000), and Indian major carp (Khan et al. 2003).

Experiments with glandless cottonseed meal (CSM)

Growth performance of black sea bass juveniles fed diets replacing between 50 and 100% of FMP protein with low-gossypol GCSM, or replacing 50 to 75% of FMP with low-gossypol SCSM protein was not impaired compared to fish fed a FMP-based control palatability of the RCSM diet was attributable to the anti-nutrient compound gossypol. No significant differences in survival, feed conversion ratio (FCR), protein efficiency ratio (PER), or whole body protein or lipid composition were observed among the fish fed the low-gossypol diets. Gossypol (25.9 mg/kg) was only detectable in the livers of fish fed the high- gossypol RCSM diet. Fish whole body essential amino acid compositions did not differ significantly among treatments. Whole body n-3 PUFAs decreased, while n − 6 PUFAs increased with increasing CSM protein in the diets. The apparent digestibility coefficient of protein was high (83.1– 87.1%) for all treatments (Anderson et al. 2016). For juvenile black sea bass, 75% FMP in the diet can be replaced with low-gossypol CSM protein prepared by solvent-extraction, and 100% of FM can be replaced with low-gossypol CSM protein prepared from glandless seed with no adverse effects on survival, growth or feed utilization. These results indicate that the CSM protein from glandless seed was utilized as efficiently as FMP by juvenile black sea bass. In comparison, low-gossypol CSM protein has been used to replace FMP at levels ranging from 20 to 100% for hybrid striped bass (Sullivan and Reigh, 1995), channel catfish (Li et al., 2008), rainbow trout (Lee et al., 2006), Florida pompano (Riche and Williams, 2010; Cook et al., 2016), white shrimp (Siccardi et al., 2012; Richardson et al., 2016) and black sea bass (Anderson et al., 2016).

For southern flounder, growth performance (final weight and percent weight gain) of juveniles fed diets replacing from 50 to 100% FMP with GCSM-, GMO-CSM, or R-CSM protein was not different from fish fed a control FMP-based diet (Figure 2; Alam et al. 2018). However, in the present study, replacing FMP with 100% GCSM, GMO or R-CSM appeared to lower growth performance (albeit not significantly), suggesting that longer-term studies are needed. After eight weeks of feeding, survival of fish ranged from 80 to 91%, with no treatment differences. Apparent protein digestibility of diets was significantly higher for the fish fed 75% and 100% GCSM and 100% GMO-CSM protein diets (83.5, 83.5 and 86.5%, respectively) compared with the control diet (79.4%) (Alam et al. 2018). Arginine levels in the diets increased as CSM was increased, consistent with the high arginine concentrations found in CSM. Liver gossypol was only detectable in fish fed the 100% R-CSM diet (37 µg/g). Replacing up to 75% FM protein by GCSM- or GMO-CSM protein did not affect whole body omega-3 PUFAs, or liver gossypol. The results suggest that up to 75% of fish meal protein may be replaced by GCSM or GMO-CSM protein in the diet of juvenile flounder without adverse effects on growth performance and body composition. In black sea bass juveniles, replacing 100% FM protein with GCSM protein did not alter growth performance or survival when diets were supplemented with lysine (Anderson et al., 2016), results similar to what was found in southern flounder in the present study. These findings suggest that CSM protein, regardless of source, was more digestible than menhaden FM in juvenile southern flounder.

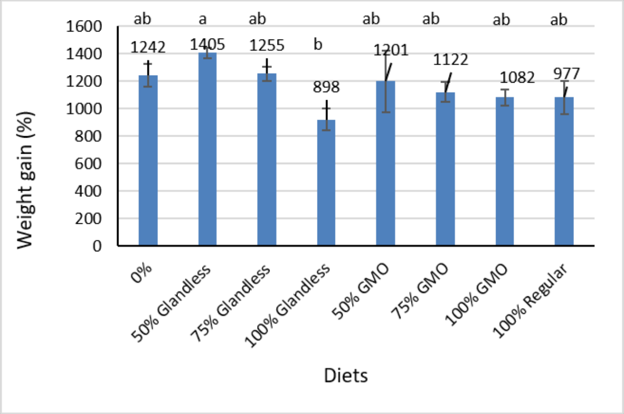

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Figure 2. Effects of replacing FMP with CSM protein of different sources in southern flounder diets: Glandless, GMO = genetically modified (glandless), and Regular (glanded). A control fish meal-based diet (0% CSM) was also tested. Bars represent percent body weight gain of juvenile flounder after 8 weeks of feeding. Values are mean ± SEM of triplicate tanks.Means with different letters differ significantly (Alam et al. 2018).

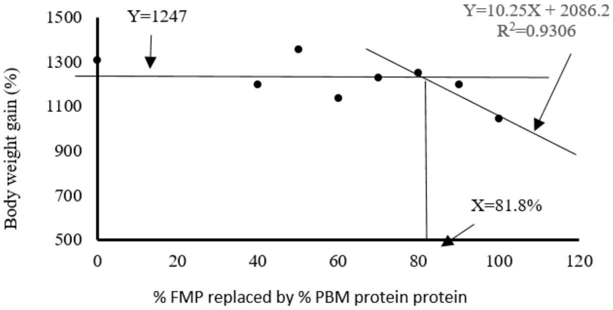

Figure 3. Broken-line regression analysis between body weight gain and % FMP replacement by PBM protein in the diets of juvenile black sea bass.

Experiments with poultry by-product meal (PBM)

For black sea bass, no significant differences in body weight gain were observed in fish fed the 40–90% PBM protein diets compared with the control diet. However, body weight gain of fish fed the 100% PBM protein diet was significantly lower than in the control group. Regression analysis with body weight gain indicated that PBM protein can replace FMP in black sea bass diets at levels as high as 81.8%, with no reduction in fish growth performance (Fig 3) (Dawson et al. 2018). For fish fed diets with up to 90% PBM protein, feed conversion and protein efficiency ratios were not significantly different from fish fed a control 100% FM-protein-based diet (Dawson et al. 2018). Apparent digestibility coefficients of dietary protein remained high (81.6– 87.0%) under all levels of FMP replacement with PBM protein. After the feeding trial, whole body and muscle protein content and the concentrations of whole body n-3 polyunsaturated fatty acids showed no significant differences among the treatments at FM protein replacement levels up to 90%. For the marine finfish totoaba, PBM protein was a good source of nutrients in juvenile diets at a FMP replacement level of 67%, whereas fish fed a 100% PBM protein diet showed the slowest growth and highest mortality (Zapata et al. 2016). In the marine fish, Florida pompano, growth performance was unaffected when PBM protein replaced 67% of the FMP in the diet (Riche 2015); however, growth performance was reduced when PBM protein replaced 100% of the fish meal protein (Rossi and Davis 2012). The results demonstrated that FMP can be replaced by feed-grade PBM protein in juvenile black sea bass diets at levels as high as 81.8% without adversely affecting survival, growth, feed utilization, fish biochemical composition, or ADC of protein or lipid. Poultry byproduct meal is a highly effective protein source for alternative protein-based feed formulation for black sea bass. Juvenile southern flounder fed diets with PBM protein in substitution for FMP at levels up to 100% showed comparable growth (i.e., fish weight and percent weight gain) to fish fed the control FMP diet (Dawson, 2012). Many marine carnivorous species such as cobia (Zhou et al., 2011) and humpback grouper (Shapawi et al., 2007) have been reported to show no differences in growth when fed diets with substantial levels of PBM (60 and 75%, respectively) in replacement of FMP, but reduced growth when FMP was completely replaced with a 100% feed- grade PBM diet (Shapawi et al., 2007). Feed utilization (feed intake, FCR, and PER) in juvenile southern flounder fed diets replacing FMP with up to 100% PBM protein was not significantly different from fish fed a control fish meal diet (Dawson, 2012). This suggests that palatability, digestibility, and assimilation of feed were not compromised by substitution of PBM protein for FMP in the diet at any replacement level (Dawson 2013). These results are very similar to those reported in African catfish (Abdel-Warith et al., 2001) and sunshine bass (Webster et al., 1999). Whole body moisture content of juvenile southern flounder was not affected by level of PBM protein replacement for FMP. Dietary fatty acid composition was reflected in the whole body fatty acid profile (Dawson 2012). The ADC of protein by southern flounder was not affected by PBM protein substitution for FMP at levels of up to 100%, suggesting PBM protein is as digestible as FMP in juvenile southern flounder. In conclusion, PBM protein completely replaced FM protein in the diet of juvenile southern flounder without adverse effects on growth or feed utilization, indicating an exceptional ability of this species to digest and to assimilate PBM protein.

CONCLUSION

The results demonstrate that juvenile black sea bass and southern flounder are able to utilize high levels of soybean meal, low-gossypol CSM, and PBM protein in their diet without reducing growth performance. About 68 and 39% fish meal protein could be replaced by soybean meal, 82 and 100% of fish meal protein could be replaced by poultry meal in the diet and 100 and 75% fish meal protein could be replaced by glandless cottonseed meal protein in the diet of black sea bass and southern flounder, respectively. These findings provide a basis for developing environmentally-sound and cost-effective predominantly terrestrial plant and animal protein-based diets for high value marine fish culture.

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One of the biggest operational expenses in marine recirculating aquaculture systems (RAS) is high quality feed. Traditionally, fishmeal (FM) is used as the main source of protein in marine fish diets due to its high protein content, amino acid profile, digestibility, and palatability. Unfortunately, as the aquaculture industry continues to grow it is creating a higher demand for FM worldwide. Limited supplies along with increasing demand are causing the price of FM to continually increase. In an aim to investigate the replacement of FM by alternative protein sources in southern flounder diets, glandless cottonseed meal was tested at our University of North Carolina (UNCW)-Aquaculture Facility. Cottonseed meal (CSM) proteins from genetically-improved (glandless) low-gossypol seed (GI-CSM, 52.1% crude protein, CP), genetically-modified low-gossypol seed (GMO-CSM, 56.0% CP) and from an untreated regular (glanded) seed (R-CSM

49.9% CP) were evaluated to replace fish meal (FM) protein (59.5% CP) in juvenile southern flounder *Paralichthys lethostigma* diets. The results suggest that up to 75% of fish meal protein may be replaced by GI- or GMO-CSM protein in the diet of juvenile flounder without adverse effects on growth performance and body composition. Cottonseed meal is a potentially cost- effective alternative plant protein source for use in aquafeeds. These ultra-low gossypol cottonseed flour proteins could be inexpensive protein sources for the commercial culture of southern flounder and other finfish species.

Alam, M.S., W.O. Watanabe, K.B. Sullivan, T.C. Rezek, and P.J. Seaton. 2012. Replacement of menhaden fish meal protein by solvent extracted soybean meal protein in the diet of juvenile black sea bass *Centropristis striata* supplemented with or without squid meal, krill meal, methionine and lysine. North American Journal of Aquaculture 74:2, 251-265.

Three experiments were conducted at our UNCW-Aquaculture Facility to determine the extent to which menhaden fish meal protein (FMP) can be replaced by solvent-extracted soybean meal protein (SBP) in the diet of juvenile black sea bass *Centropristis striata*. Diets were formulated replacing FMP by SBP at 0, 10, 20, 30, 40, 50, and 60% (experiment 1) and 0, 60, 70, 80, 90, and 100% (experiment 2), with supplementation with squid meal, krill meal, and attractants in both experiments. Experiment 3 was designed to replace FMP by SBP at 40, 50, 60, 70, and 80% without supplemental squid and krill meal and at 60% and 70% with supplemental methionine and lysine. Diets were fed twice daily to triplicate groups of fish (*N* = 15 per group) in 75-L tanks containing recirculating seawater. Fish were fed for 6, 10, and 8 weeks in experiments 1, 2, and 3, respectively. No significant differences in body weight gain, feed efficiency, and survival were observed among treatments in experiment 1. In experiment 2, no significant differences in percent weight gain were observed among fish fed diets replacing FMP at 0, 60, and 70%. In experiment 3, body weight gain was not significantly different for fish fed supplemental methionine and lysine in 70% SBP diets compared with fish fed 0% SBP diets. No significant differences were observed in whole-body n-3 polyunsaturated fatty acids among treatments in experiment 2. Broken-line regression of the specific growth rate data suggested that the maximum level of FMP replacement with SBP in black sea bass diets was 67.6–68.4% with 75 g/kg squid meal and 50 g/kg krill meal in the diet and 57.2–58.0% without squid and krill meal supplementation.

Dawson, M.R., M.S. Alam, W.O. Watanabe, and P. Carroll. 2018. Evaluation of poultry by-product meal as an alternative to fish meal in the diet of juvenile black sea bass reared in a recirculating aquaculture system. North American Journal of Aquaculture 80:74–87.

Feeding trial was conducted to determine the maximum substitution of limits of poultry by-product meal (PBM) protein for fish meal (FM) protein in the diet of juvenile black sea bass *Centropristis striata* (family Serranidae). Eight iso-nitrogenous (44% CP) and iso-lipidic (13%, crude lipid) diets were formulated to replace FM protein with PBM protein at 0 (control), 40, 50, 60, 70, 80, 90, and 100% in black sea bass diets. Diets were fed twice daily to triplicate groups of juveniles to apparent satiation for 8 weeks in a recirculating aquaculture system. Final survival was excellent (95-100%) in all diet treatments, with no significant differences. No significant differences on body weight gain were observed in fish fed the 40-90% PBM protein diets compared to the control diet.

However, body weight gain of fish fed the 100% PBM protein diet was significantly lower than in the control group. Regression analysis with body weight gain indicated that PBM protein can replace FM protein in black sea bass diets at levels as high as 81.8%, with no reduction in fish growth performance. For fish fed diets with up to 90% PBM protein, feed conversion and protein

efficiency ratios were not significantly different from fish fed a control 100% FM protein-based diet. Apparent digestibility coefficients of dietary protein remained high (81.6-87.0%) under all levels of FM replacement with PBM protein. After the feeding trial, whole body and muscle protein content and the concentrations of whole body n-3 PUFAs showed no significant differences among the treatments at FM protein replacement levels up to 90%. PBM is a promising alternative protein source for sustainable diet development in black sea bass.

Seaweed Aquaculture in a Warming Environment—Building Resilience with *Saccharina angustissima* (Laminariales, Phaeophyceae)

Simona Augyte* and Charles Yarish

Department of Ecology & Evolutionary Biology, University of Connecticut, 1 University Place, Stamford, CT 06901-2315, USA

Corresponding author: simona.augye@uconn.edu

Keywords: aquaculture, climate change, kelp, *Saccharina angustissima*

ABSTRACT

Locally sourced, high quality sea vegetables, in particular kelp, are sparking consumer interest and demand in the New England region, USA. The narrow-bladed sugar kelp*, Saccharina angustissima*, with one narrow (1.6 ± 0.7 cm wide) and one long (average 1.8 ± 0.96 m, up to 4.4 m) blade is morphologically and genetically distinct from *Saccharina latissima*, which grows much wider (20-30 cm) and is usually shorter (up to 3.5 m) in length. In the intertidal, *S. angustissima* is adapted to withstand extreme hydrodynamic forces an order of magnitude higher than the subtidal populations of sugar kelp that are usually found in more sheltered locations. It is restricted in distribution in mid-coastal Maine and occurs in the low intertidal, exposed to high ocean swells. In collaboration with kelp farmers from *Maine Fresh Sea Farms* and *Maine Coast Sea Vegetables,* we successfully domesticated this kelp, growing it to full maturity on longlines to be used commercially for its desirable culinary traits. Based on harvest results from two growing seasons, biomass yields of this kelp were up to 24.1 (±6.3) kg m^{-1} of line with a plant density of 400 plants m^{-1} of line. The phenotypic characteristics that make this kelp exceptionally adapted to extreme sea conditions were preserved at the open-water farm sites. Experiments with the microscopic gametophyte stages of *S. angustissima* showed that it has the capacity to withstand some temperature fluctuations. Overall, this domesticated kelp species has great potential as an economically valuable sea vegetable in the Gulf of Maine. Nevertheless, because of its restricted geographic distribution, care must be taken to protect the donor population. Although additional work needs to be done to bring this kelp to market, growing it also offers a suite of ecosystem services, including nutrient bioextraction and temporary habitat formation for sea animals.

INTRODUCTION

Global aquaculture production continues to increase with wild fisheries stagnating. Sustainable cultivation can be a significant contribution for food security. Seaweeds, including those incorporated into integrated multi-trophic aquaculture (IMTA) and nutrient bioextraction, contribute to food and feed production. Currently, on a global scale, seaweed production is valued at 11 billion USD (FAO 2018) with 95% of cultivation taking place in developing countries where it provides income to millions of families. The global industry is dominated by a few main seaweed species with a predominance of *Saccharina japonica*, *Undaria pinnafida, Pyropia/Porphyra spp., Gracilaria* spp., *Eucheuma/Kappaphycus spp.* (Kim et al. 2015; Buschmann et al., 2017). Seaweed aquaculture is also gaining interest in the US and Europe. In New England, USA, kelp farming is developing as an environmentally and economically sustainable aquaculture activity that is revitalizing the working waterfronts by providing economic opportunities that are ecologically sound (Augyte et al, 2017; Yarish et al. 2017).

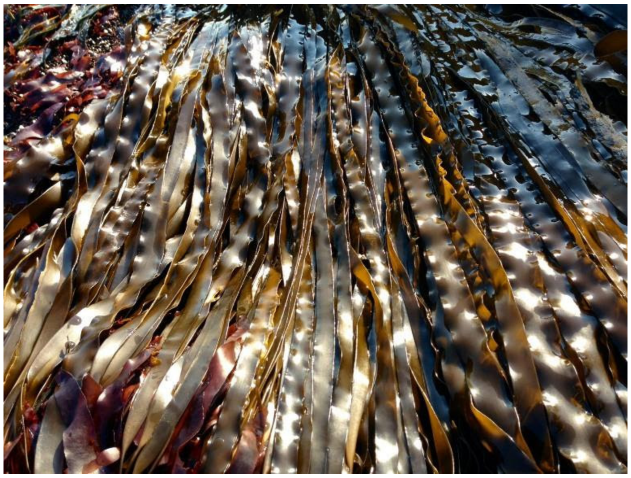

Figure 1. Saccharina angustissima bed in the field showing long and narrow morphology.

The rare kelp, *Saccharina angustissima* (F.S. Collins) Augyte, Yarish & Neefus, is an endemic narrow-bladed species found on localized and exposed islands and promontories on the contiguous mid-coastal areas of Maine, USA (Figure 1, Mathieson et al. 2008, Augyte et al. 2018). *S. angustissima*, has one blade that is very narrow (1.6 ± 0.7 cm wide) and long (average 1.8 ± 0.96 m, up to 4.4 m) that is morphologically and genetically distinct from *Saccharina latissima*, which grows much wider (20-30 cm) and is usually shorter (up to 3.5 m) in length (Augyte et al. 2018). Many studies on kelps have identified phenotypic plasticity as a driver of the great range of morphological variation seen in thallus shapes as well as macroalgal production and physiology when exposed to various environmental parameters, specifically strong wave exposure (Hurd 2000, Blanchette et al. 2002, Fowler-Walker et al. 2006). Transplant experiments of kelp (in the order Laminariales) from exposed environments with rapid water movement to protected ones with slow flow, often result in blade morphology changing from narrow, thick, flat blades to wider, thinner, and more undulate blades (Koehl 2008). The research conducted here using both genetics and sea farming allowed us to test the assumption that this unique kelp had a genetic basis for its morphology and was not just phenotypically plastic based on the exposed conditions of where it was found. It was also unknown if this species could be domesticated using traditional kelp cultivation methods.

MATERIALS AND METHODS

Reproductive material was collected in Harpswell, Maine, USA, following low tides in October-November when the kelp was at peak sorus production. Following standard cultivation protocols, kelp was grown in nursery conditions at the UCONN Stamford Marine Biotechnology Laboratory on seedstring before being outplanted at two open-water sea farms in Maine. Sporophytes of the common sugar kelp, *S. latissima*, with parental meiospores obtained from subtidal populations from Casco Bay, Maine, were grown alongside the *S. angustissima* sporophytes to test how environment affects morphology. The wave energy at both farm sites was low compared to what the kelp population experiences in the field. The resulting lengths and widths of the two morphologies were compared at harvest. The kelp was grown to maturity on long-lines for 6 months. In the lab, experiments were run to expose the microscopic gametophyte stages to different temperature and light levels to better understand the ecophysiological tolerance of these life stages.

RESULTS

The results of the common garden experiments at two sea farms revealed that the sporophyte *S. angustissima* blade retained its length to width ratio and did not become wide like the sporophytes of *S. latissima* (Figure 2). However, some characters, including thickness of the blade, were lost, while ruffles formed on blade edges.

Figure 2. Saccharina angustissima from the sea farm in Sorrento, Maine, USA. (Photo courtesy of Sarah Redmond).

These results confirmed that environmental cues alone were not wholly responsible for the unique morphology in *S. angustissima* and suggested a genetic basis. Peak meristematic growth rates for blade length occurred from March through April at 2.85 (± 0.34) cm day^{-1}. The kelp was harvested from May through June with yields of up to 17 (± 4.4) kg m^{-1} of line and plant density of 330 plants m^{-1} of line at the Bristol farm and yields of 13.3 (± 6.2) kg wet weight m^{-1} line and a plant density of 400 plants m^{-1} of line at the Sorrento farm. Second season yields at Sorrento were on average 24.1 (±6.3) kg m^{-1} of line.

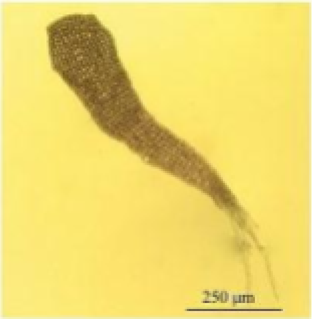

Figure 3. Saccharina angustissima juvenile sporophyte grown in lab conditions.

The microscopic gametophytes and juvenile sporophytes grew best at temperatures of 8-13°C at the lowest irradiance of 10-µmol photons m^{-2} s^{-1} (Augyte et al. *Accepted*). Light had a significant effect on both male and female gametophyte grown only at the higher temperatures. Temperatures of 8-15°C and irradiance levels of 10- 100 µmol photons m^{-2} s^{-1} were conditions for the highest juvenile sporophyte growth (Figure 3). Finally, calculations estimating the nutrient bioextraction capability of the cultivated *S. angustissima* kelp harvested in June reveal N removal of 88.7 kg ha^{-1} and C removal of 1666.7 kg ha^{-1} (Augyte et al. 2017).

DISCUSSION

Both farms grew significantly narrower blades of *S. angustissima* than of the sugar kelp, *S. latissima*. Common garden experiments with the two morphotypes identified trait stability for length and width, while blade ruffles and thickness varied with the environment. Overall, this unique kelp form has emergent potential as a crop for the Gulf of Maine, with several sea farmers growing it for commercial purposes. In terms of microscopic stages, high light intensity effects might pose stress on *S. angustissima*, although, as an intertidal species, it could also be better adapted to temperature and light extremes than its subtidal counterpart, *S. latissima*.

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Augyte, S., C. Yarish, S. Redmond, and J.K. Kim. 2017. Cultivation of a morphologically distinct strain of the sugar kelp, *Saccharina latissima forma angustissima*, from coastal Maine, USA, with implications for ecosystem services. Journal of Applied Phycology 29: 4.10.1007/s10811-017-1102-x.

Consumer interest and demand for North Atlantic sourced sea vegetables drives opportunities for aquaculture development in the northeast USA. The unique morphology and desirable culinary traits of the wild narrow-bladed kelp, *Saccharina latissima* forma *angustissima*, were successfully translated into a cultivated crop on two geographically distinct open-water farms in Maine, USA. Environmental conditions, growth, and tissue analysis were quantified. Peak meristematic growth rates for blade length occurred from March through April at 2.85 (±0.34) cm day^{-1}. The kelp was harvested from May through June with yields of up to 17 (±4.4) kg m^{-1} of line and plant density of 330 plants m^{-1} of line at the Bristol farm and yields of 13.3 (±6.2) kg wet weight m^{-1} line and a plant density of 400 plants m^{-1} of line at the Sorrento farm. Second season yields at Sorrento were on average 24.1 (±6.3) kg m^{-1} of line. Both farms grew significantly narrower blades of f. *angustissima* than of the sugar kelp, *S. latissima*. Common garden experiments with the two morphotypes identified trait stability for length and width, while blade ruffles and thick-ness varied with the environment. Calculations estimating the nutrient bioextraction capability of the cultivated f. *angustissima* kelp harvested in June reveal N removal of 88.7 kg ha^{-1} and C removal of 1666.7 kg ha^{-1} (combined farm site averages). Overall, this unique kelp form has potential as a new aquaculture crop for the Gulf of Maine while providing several coastal ecosystem services.

Augyte, S., L. Lewis, S. Lin, C.D. Neefus, and C. Yarish. 2018. Speciation in the extremely exposed intertidal: the case of *Saccharina angustissima* (Collins) Augyte, Yarish & Neefus *comb. nov. et stat. nov.* (Laminariales, Phaeophyceae). Phycologia 57: 100-112.

Saccharina latissima is a perennial kelp with a circumboreal distribution from the North Pacific to the North Atlantic coasts. Our study clarified the taxonomy of the morphologically distinct *Saccharina latissima* forma *angustissima* (Collins) A. Mathieson from the low intertidal zone on exposed islands and ledges of Casco Bay, Maine, USA. To identify genetic divergence between the two morphotypes, *S. latissima* and *S. latissima* f. *angustissima*, we used a multilocus phylogenetic approach. Genetic analysis suggested low divergence between the two forms. However, there was as much or more genetic divergence between *S. latissima* and *S. latissima* f. *angustissima* as there were between other taxonomically accepted species of *Saccharina*. To investigate sexual compatibility between the two forms, we made reciprocal crosses of the gametophytes and observed sporophyte formation. All crosses were successfully grown to the juvenile sporophyte stage, suggesting that the two are reproductively compatible *in vitro*. It is unknown if the two populations freely hybridize in the field. Last, we compared wave action, the ecological factor most likely driving the unique morphology, at exposed sites with *S. latissima* f. *angustissima* and protected sites with *S. latissima*. The mean wave force at the exposed site was over 30 times higher in magnitude than at the protected site respectively, during the summer. The significant differences in morphology, the lack of specimens with intermediate morphologies, and the results of a common garden experiment suggest that the morphological differences in *S. latissima* f. *angustissima* are heritable with a genetic basis. Therefore, on the basis of our molecular evidence coupled with ecological studies, we are elevating *S. latissima* f. *angustissima* (Collins) A. Mathieson to specific rank as *S. angustissima* (Collins) Augyte, Yarish & Neefus comb. nov. & stat. nov

J. K. Kim, G.P. Kraemer, and C. Yarish. 2015. Use of sugar kelp aquaculture in Long Island Sound and the Bronx River Estuary for nutrient extraction. Marine Ecology Progress Series 531:155-166.

The present study revealed an even higher nutrient bioextraction capacity in the cold-water species *Saccharina latissima* at three sites - the mouth of the Bronx River Estuary (Bronx, NY; BRE), western Long Island Sound (Fairfield, CT; WLIS), and central Long Island Sound (Branford, CT; CLIS), during winter and spring of the 2012−2013 growing season. These sites differ in temperature (BRE > CLIS > WIS), salinity (BRE < WLIS = CLIS) and nutrients (BRE >> WLIS = CLIS). It was estimated that *S. latissima* could remove up to 180, 67 and 38 kg N ha^{-1} at BRE, WLIS and CLIS respectively, in a hypothetical kelp farm system with 1.5 m spacing between longlines. In the same hypothetical kelp farm system, the estimated carbon sequestration values are 1350 (BRE),1800 (WLIS) and 1100 (CLIS) kg C ha^{-1}. The potential monetary values of N sequestration by the sugar kelp are up to $1600 (BRE), $760 (WLIS) and $430 (CLIS) ha^{-1}, if incorporated into the State of Connecticut Nitrogen Credit Trading Program and a carbon-pricing scheme. The potential economic values of C sequestration are $30−300 (BRE), $40−400 (WLIS), and $24−240 (CLIS) ha^{-1}. These results suggest that seaweed aquaculture is a useful technique for nutrient bioextraction in urbanized coastal waters, such as LIS and BRE. Alternation of the warm- and cold- water species would maximize nutrient bioextraction and augment other ecosystem services, producing economic benefits for the region while helping to manage non-source eutrophication.

Scallop Farming in Maine, a 20-year Overview

Hugh S. Cowperthwaite[1*], Dana L. Morse[2], and Melissa L. Britsch[3]

[1] Coastal Enterprises, Inc. 30 Federal Street, Brunswick, ME, 04011, USA
[2] Maine Sea Grant College Program, and University of Maine Cooperative Extension. Darling Marine Center, 193 Clark's Cove Road, Walpole, Maine, 04573, USA
[3] University of Maine, Darling Marine Center, 193 Clark's Cove Road, Walpole, ME 04573, USA

Corresponding author: hugh.cowperthwaite@ceimaine.org

Keywords: scallop, aquaculture, Maine, Aomori

ABSTRACT

Climate change has profoundly impacted capture fisheries in the Gulf of Maine. Notably, the largest fishery in the United States is in flux: landings of lobster (*Homarus americanus*) have shifted northeast in recent years. These challenges – which are not unique to Maine and are also experienced by Japanese fisheries - have caused great uncertainty among fishermen in Maine. Consequently, some are turning to aquaculture to compliment fishing incomes. Maine's fishing and aquaculture industries have benefited from exchanges with Japan's well-established scallop industry, and we continue to learn directly from the Japanese. Coastal Enterprises Inc. (CEI), Maine Sea Grant (MESG) and others are working to introduce commercially-viable scallop aquaculture into the state of Maine, building on a 20- year-old foundation of expertise. Maine's sister-state relationship with Aomori Prefecture is central to this process, with an origin dating to the wreck of the Bath-built Cheseborough, which foundered off the coast of Aomori in 1889. Currently, several projects are being carried out in Maine, developing the culture sector for scallops in coastal waters. An aquaculture cooperative in Maine focused on scallops is now in place. Optimization of spat collection has been ongoing work over several years. Biotoxin monitoring in scallop tissues enables some producers to access the live market. A dedicated license for scallop spat collection is underway. Altogether, fishermen, farmers, economic development groups, scientists and regulators are engaged in pursuit of developing Maine's scallop aquaculture industry. These collaborations - combined with expertise from Japanese colleagues - are a powerful aggregation of expertise to solve the many problems involved. Ex-vessel value for Maine scallops has risen for over a decade, though state and federal landings have increased, reflecting the value that consumers place on Maine scallops specifically; and that trend is expected to continue (Hale Group 2016). Trials by CEI and collaborators indicate that the Japanese production machinery appears to work well, and growth rates from ongoing studies and older works appear to fall within acceptable limits, often in excess of 25mm/year shell height (Cowperthwaite, unpublished; Morse, unpublished; Davidson et al. 2014; Pottle and Hastings 2001). Lastly, all indications point to a growout period between two and three years from spat (seed) to commercially viable harvest size. Aquaculture practitioners in Maine desire to incorporate Japanese expertise so the industry can ramp up effectively and provide new sources of protein. We seek ongoing advice with lease site set-up and husbandry, growout techniques, biofouling and predators, equipment and machinery, handling, processing, food safety and packaging. This innovative and unique project offers a one-of-a-kind exchange experience that is

helping to further strengthen ties between the United States and Japan. The introduction of Japanese technical knowledge and equipment to our Maine scallop industry is the first of its kind in the United States and is groundbreaking for the American aquaculture community.

Maine/Aomori relationship

Groups from Maine visited Aomori, Japan in 1999, 2010 and 2016 with the primary focus on scallop aquaculture. Specific topics included: spat collection, growout techniques, product development, and environmental interactions. Aomori is the northernmost prefecture on the Japanese mainland of Honshu (just south of Hokkaido) and includes some of the most productive scallop grounds in all of Japan.

Aomori has a population of ~1.5M people and when overlaid on the US (from a geographic standpoint) it's at the same latitude as the state of New York. There are several similarities Aomori shares with Maine, including rural communities with natural resource and tourism-based economies encompassing fishing, farming and forestry.

Maine has had a longstanding connection with Aomori; one that began with a shipwreck. On October 31, 1889, the Maine-built Cheseborough, was caught by a typhoon off the coast of the Aomori Prefecture. The ship was destroyed and only four of the (23 member) crew survived. In 1994, a Sister City agreement was signed between Maine & Aomori to remember and recognize the tragedy as an event that brought our cultures together, and eventually a sister state agreement was signed. Over the years there have been several cultural, education and trade exchange programs. This laid the foundation for the opportunity to learn about their scallop industry (Fig.1).

Figure 1. The Cheseborough, built in Bath, Maine, and lost off the coast of Aomori Prefecture, Japan, October 31, 1889.

Following is a timeline of some important events in the development of scallop culture in Maine. In many cases, the scientific questions being asked are direct outputs from technology transfer from Japan.

1999

The delegation from Maine that visited Aomori in May of 1999 was an important event in the advancement of scallop farming and stock enhancement the US. Thirteen individuals from industry, science, management and education travelled for a week to Aomori Prefecture, where stops at processors, fishermen's cooperatives, and scientific offices provided the US delegates with critical first-hand information about spat collection and growout of scallops. Equipped with this experience, there were a number of subsequent outreach efforts all along the coast, to introduce the materials and methods of seed collection to Maine's fishermen. Importantly, such events also involved scientists and regulators, which allowed the licensing process for such work to proceed smoothly, and to assist other scientists in joining the overall pursuit of diversifying the scallop industry in Maine. Another principal outcome was the publication of a report that covered the major findings of the trip, entitled: The culture of Japanese scallops (*Patinopecten yessoensis*) (Beal et al., 1999).

2000-2003

In the years immediately after the 1999 delegation, there were upward of 100 Maine fishermen who participated in scallop spat collection, along virtually the entire Maine coast, and several reseeding efforts - mostly of the informal kind - were undertaken. Of the quantifiable efforts that occurred, re-seeding success was undemonstrated

(Schick and Feindel, 2005). Given this lack of clear proof that reseeding worked, most fishermen eventually stopped participating in spat collection. However, this period introduced spat collection to a wide segment of fishermen in Maine, and the processes and terminology involved became much more familiar territory for fishermen. In addition, spat collection provided a mechanism by which the early life stages of scallops became more clear, as well as the interplay between spawning populations, oceanographic conditions, substrate and larval behavior. Regular communications and interactions with Canadian scientists, fishermen and gear suppliers also grew during this time; all of which became resources for Maine participants. As a result, those in Maine involved in scallop spat collection and aquaculture became much more connected to efforts elsewhere, and in essence greatly broadened their professional networks.

In 2001, the consulting company Gardiner Pinfold completed a document entitled: Development Potential of the Maine Scallop Industry. The report included the potential for the development of an aquaculture industry, and identified farm production as an area of growth, though with caveats on sale of whole products and the size of the market.

2001 also saw a report from Tom Pottle and his collaborators on a demonstration project in Cobscook Bay (Pottle and Hastings, 2001). The project examined growth rates and density in caged scallops and was notable in that it provided a roadmap for the testing needed to sell whole scallops. This roadmap and regulatory process was invaluable for efforts that came later.

2004-2010

The mid- to late-2000's saw relatively low activity in scallop reseeding and aquaculture, although involvement in spat collection continued each year with a small group of fishermen. Scallops were added to the species allowed within the Limited Purpose Aquaculture License (LPA) in 2008. This was important because the LPA was an easy way for new entrants to experiment with aquaculture in Maine's coastal waters, and it opened the door for small trials. Consequently, familiarity with aquaculture increased for many fishermen, and the passing of time allowed fishermen to better understand the opportunities and mechanics of aquaculture, and not to see the new industry as merely a threat to their existing businesses.

2011-2019

Between 2011 and 2019, there were several significant events in both the relationship between Maine and Aomori, and the development of scallop farming in Maine.

Figure 2. 2016 Maine Delegation (front row) at the Aomori Prefecture Welcome Reception.

2016

At the invitation of Shingo Mimura (Governor of Aomori Prefecture), Coastal Enterprises, Inc. (CEI), the Friends of Aomori (FOA), and Maine Sea Grant assembled a ten-member group of shellfish aquaculture industry professionals from Maine to visit Aomori, Japan, in the fall of 2016. The purpose of the visit was to expose Maine aquaculturists to Aomori's well-established scallop aquaculture industry experts for an intense one-week tech transfer exchange trip to help advance the development of Maine's scallop aquaculture industry. The Japanese are recognized world leaders in aquaculture and through the existing relationships Maine has with Aomori *(*Friends of Aomori, Maine-Aomori Sister-State Advisory Council) we strengthened our relationship with Aomori prefecture staff and private businesses through aquaculture. (Fig. 2)

Figure 3. Location and map Aomori Prefecture, Japan.

Our visit focused on Mutsu Bay, which is surrounded by Aomori Prefecture. The bay experiences three foot (1 m) tides and encompasses ~650 square miles (1683 ~km^2). Tsugaru Strait is to the north, the Sea of Japan to the west, and the Pacific Ocean to the east. (Fig 3)

Aomori Agenda

Our agenda included visits to learn about scallop processing, the Fisheries Research Institute for a scientific and regulatory overview, a few different fishing cooperatives, observing aquaculture gear on land, a few trips out on scallop farms to witness ear-hung scallops being washed and stocking densities being reduced in pearl nets. Lastly, we spent two days on land viewing several demonstrations of specialized scallop aquaculture machinery including: scallop graders, an age pin automatic pin setter, scallop drilling machine, and scallop cleaning machines. We learned about the use of various equipment installed on scallop vessels to accommodate long line culture, as well as specific gear used in the water including: spat collection gear, pearl nets, lantern nets and ear hanging gear.

Mutsu Bay

A typical long line (in Mutsu Bay) is 200m (~650

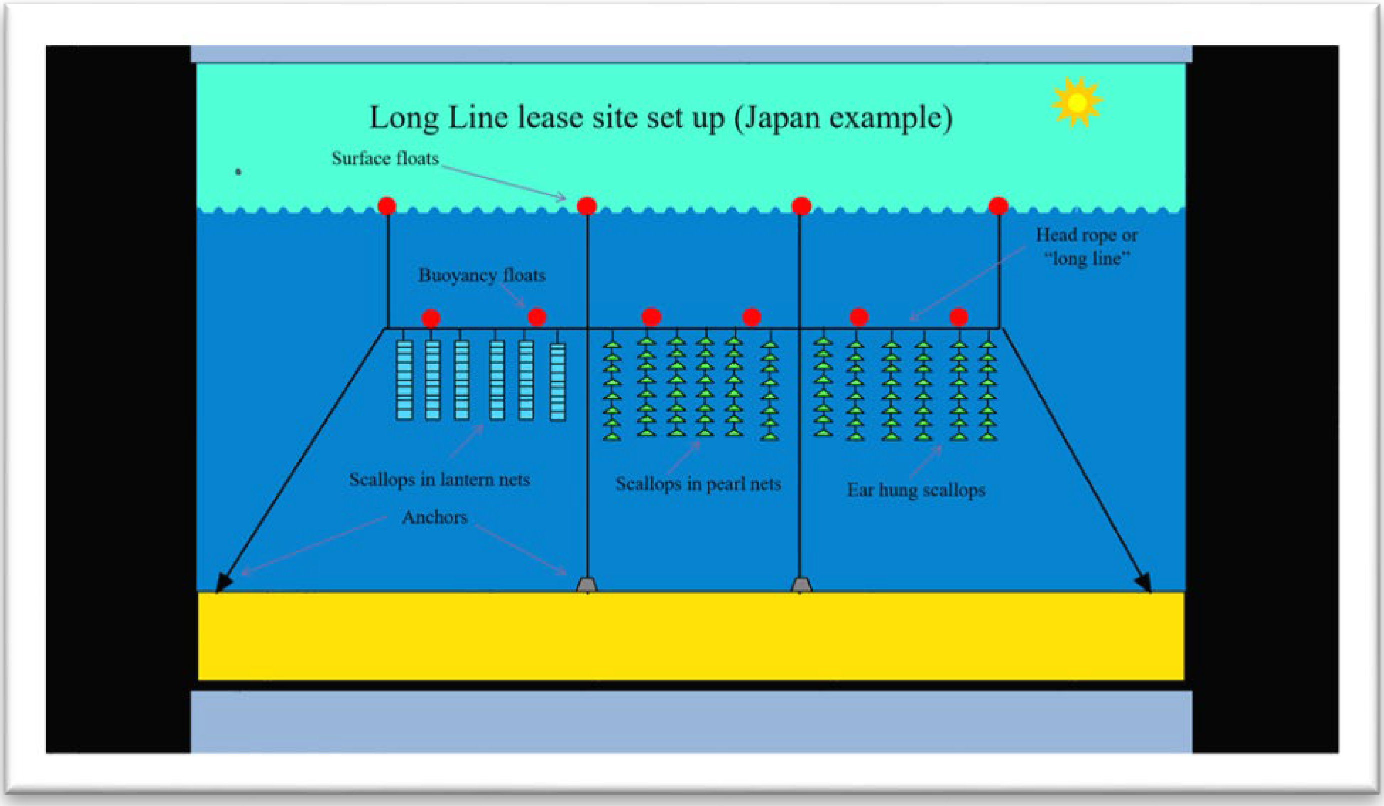

Figure 4. Schematic of a typical longline.

Figure 5. The principal steps of Japanese scallop culture, and the times of year at which they occur.

feet). Farms are sited in water that is typically at least ~70 feet (23 m) deep or greater. All stages of collection and growout can occur on the same line including: spat collection bags, pearl nets, lantern nets and the ear-hanging technique. Each long line is held taut with tensioning buoys and suspended ~10-20 feet below the surface of the water. Dropper lines with ear hung scallops or nets are kept ~10 feet (3m) off the bottom to avoid predators.

Anchors weigh between 130-220 lbs. (59-100 kg) and each long line has three anchors on each end (Fig 4). A long line is lowered and raised at a single point along the line to tend to the gear. The line is then secured on the bow and stern of the vessel with automated star wheels, which allows the vessel to slowly work its way along the line. Biofouling is partially mitigated by raising and lowering the line in the water column at different times of the year. High water temperatures are also a reason to lower the line to cooler waters.

Scallop Culture in Mutsu Bay

The basic process of scallop culture in Mutsu Bay is illustrated along a 12-month timeline. Spat collection bags are set in April-May. Aomori Prefecture and fishing co-ops collectively monitor environmental conditions in the bay to best predict approximate settlement and when to set bags.

In July and August juveniles are transferred out of spat collection bags and put into pearl nets ~80-100 animals per net chamber. In October, the juveniles are then transferred into clean pearl nets and the stocking density is reduced to ~15-20 animals per net chamber. In February and March, the scallops are then transferred to either lantern nets or the ear hanging technique for further growout. At the beginning of the second year, some half-mature scallops are harvested and sent to market, others are grown out for the remainder of year two and some for a third year for the largest meats. (Fig 5)

Commercial Efforts in Maine

The effort to test Japanese scallop equipment in Maine with Atlantic Sea Scallops was led by CEI. CEI has been working with two commercial farms: Bangs Island Mussel and Pine Point Oyster Co. This involved drilling and pinning on ear hanging lines to determine: how the shells hold up to drilling, the optimum time to drill, and to track mortality. Several months later we returned to the ear hung scallops to run them through the wash machine to clean off the biofouling organisms. Observations were made about the organisms encountered, the fragility of shells going through the machine, pins breaking off, mortality, cleaning frequency and growth rates. We've also been tracking labor costs and market price paid at final sale of the scallops.

Spat bag materials: 2016-2018

Two seasons of spat collection experiments were undertaken to examine the efficacy of collection substrate on numbers of scallops retained, size frequency, and presence of other settlers. The rationale behind the experiments was that the standard substrate - Netron - has grown very expensive in recent years. We undertook two seasons of experiments to compare Netron to less expensive options.

In September and October of 2016, four lines of spat collectors were deployed in the coastal waters of Maine, approximately three miles southeast of Cape Elizabeth, in approximately 50m of water. Each line supported 12 spat collectors; four at each depth of approximately 40m off bottom, 30 m off bottom, and 24 m off bottom. Each level contained one collector filled with the following substrates: Netron (serving as the control), and one each of Industrial Nettings 1/6" (OV-7100), ¼" (OV-7822), ½" (OV-1581). Each collector contained approximately 21 ft^2 of substrate. Collectors were hauled in June and July of 2017, and comparisons were made via ANOVA for the number of scallops retained, their size frequency, and the numbers of other bivalve settlers in the bags, by mesh type and depth. Depth significantly affected the number of scallops and other bivalves per collector (ANOVA, $p < 0.001$ & $p = 0.027$), but not scallop shell height (ANOVA, $p = 0.91$).

Additionally, the number of mussels was significantly higher near the surface of the water (ANOVA, $p = 0.001$, Table 1). The mean number of scallops per collector (reported as the average, ± the standard error) was highest at mid depths with ¼" mesh (12293 ± 2671). The mean number of all other bivalves per collector was highest at mid depths (1084 ± 150), and the mean number of mussels per collector was highest near the surface of the water column (393 ± 94). Mussels were analyzed separately because they can cause substantial damage to scallop spat. The mean scallop shell height across all depths was 9.41 ± 0.29 mm (Fig 1).

Retrieval date had no effect on the mean number of scallops per collector (t-test, $p = 0.24$) nor the mean shell height of scallops (t-test, $p = 0.49$). Retrieval date affected the mean number of other bivalves per collector (t-test, $p = 0.004$), but because scallops grow slowly, the two-week difference in collection date did not have a large effect and we treated all data equally, with four replicates of each factor.

Two spat bags were lost during the experiment: one at the surface with ¼" mesh and one at mid depth with ½" mesh. Notably, blue mussels (*Mytilus edulis*) were observed to settle preferentially on the bags nearest the surface (Fig 6).

Table 1. Summary of effects of mesh type and depth on scallop catches in spat collectors, 2016-2017.

		Depth above seafloor (total depth 150')		
		High (132')	**Mid (108')**	**Low (84')**
Mesh Type	**1/2"**	7171 ± 2068	6947 ± 735	3773 ± 1537
	1/4"	10980 ± 2803	12293 ± 2671	4620 ± 931
	1/6"	7798 ± 1366	8626 ± 1009	3923 ± 1001
	Netron	6771 ± 1155	7525 ± 871	4294 ± 1409

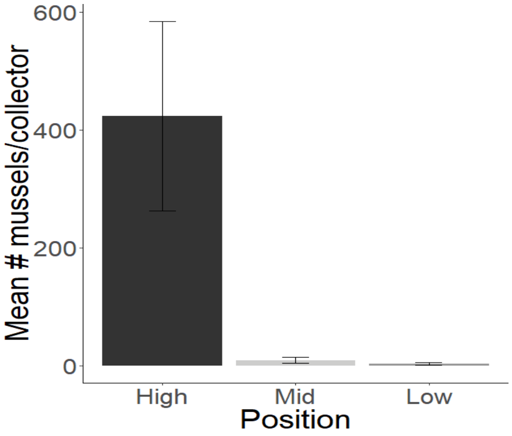

Figure 6. Mussel settlement on spat collectors by depth, 2016-2017.

Given the results of the 2016-2017 experiment, we repeated the experiment, with slightly different variables. Collectors were set in the same locations, and set in Sept and October of 2017, and retrieved in June and July of 2018. We repeated the same depths as previously, but our substrate types changed, to include: Netron (control), OV-7822 (as in 2016/2017), and two similar agricultural nettings - Tenax Plurima and Tenax R-9. All bags contained approximately 21 ft^2 of netting.

Although the data are not completely analyzed to date, we examined the same variables of depth and mesh type on the catches of scallop spat, size frequency and the presence of other settling bivalves. Data thus far indicates that for this site, the number of scallops collected goes down with greater set depth and that the OV-7822 appears to perform as well as the more expensive Netron (Table 2). In coming months, we will analyze the data using ANOVA, to test for statistical differences.

Table 2. Mean number of scallops retained in collectors by depth and by substrate type, 2017-2018.

		Depth above sea floor (total depth = 150')			
		High (132')	**Mid (108')**	**Low (84')**	**Mean by Material**
Mesh Type	**Netron**	7594.64	3668.57	3726.00	4966.40
	¼" (OV7822)	7038.57	5255.83	1831.67	4709.69
	Plurima	2879.03	2326.25	2495.50	2566.93
	R9	4499.36	5251.25	2862.50	4237.70
	Mean by depth	**5502.90**	**4150.48**	**2728.92**	

Exploring the feasibility of whole, live scallop markets

Maine fishermen and scallop farmers have shown interest in pursuing markets for live and whole scallops, such as indicated by the Pottle and Hastings project (2001) and the Gardiner-Pinfold report (2001), but biotoxins have presented a persistent threat to exploring this opportunity. Specifically, saxitoxin and domoic acid are differentially present in scallop tissues, and reside principally in the digestive gland and the mantle, where they can persist for weeks and months. Moreover, as the scallop metabolizes these compounds, they may transform from low-toxicity forms to high-toxicity forms over time, even in the absence of a harmful algal bloom (Bricelj and Shumway, 1998). Therefore, any meaningful effort will need to include a rigorous testing program to ensure the safety of any product other than adductor muscles.

Such work has been ongoing since May of 2017, on three sites along the coast of Maine. In brief, the testing and communications mechanisms created during the Pottle and Hastings project have been revisited and updated and have allowed whole scallops to go to market in each winter between 2017 and 2019. We have documented that toxicity will vary strongly by site, and that regular testing can provide the necessary confidence in product safety for regulators, producers and consumers. While testing using the mandated High-Performance Liquid Chromatography (HPLC) method is expensive, it has been helpful in supporting the market exploration of whole and live scallops, and has provided producers with additional income, while adding valuable information about the requirements of scale to be able to afford testing costs.

ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Beal, B.F., M. Hastings, W. Hopkins, S. Inches, D. Morse, K. Porter, T. Pottle, S. Rappaport, L. Taylor, and J. Trenholm. 1999. The culture of Japanese scallops (*Patinopecten yessoensis*). Report of the Maine delegation to Aomori Prefecture, Japan, May 14-21, 1999. The Maine Aquaculture Innovation Center, 49p.

This report summarizes the findings of the delegation from Maine to Aomori Prefecture in 1999. It reviews the history of the scallop aquaculture industry and fishery in Japan, and the technologies, management, science and to some degree the products and processing that have supported the development of this sector. In particular, there is attention paid to the spat collection process for scallops, underlaid by reviewing the biology of *Patinopecten yessoensis*, and the subsequent steps and equipment used in scallop farming in Aomori.

Bricelj. M. and S.E. Shumway. 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics and biotransformation. IN: Reviews in Fisheries Science 6(4): 315-383.

This paper is a critical review of the biotoxin dynamics of several species of bivalve molluscs, including the sea scallop, *Placopecten magellanicus*. Specifically, the paper reviews the global distribution, sources of variation in toxicity, anatomical partitioning, metabolism and detoxification kinetics of toxins important in Paralytic Shellfish Poisoning (PSP). As it relates to the scallop aquaculture effort in Maine, the paper provides details showing that scallops can rapidly toxify, that toxins are spread differentially between tissues within the scallop, and that toxins can take an extended time (weeks or months) to be fully metabolized. Moreover, the paper notes that toxins change form during metabolism, and can go from less-toxic forms to more-toxic. The practical implication is that even in the absence of a toxic algal bloom, a scallop can test above regulatory limits, through this biotransformation process.

Davidson, L.A., R. Nowlan, and M. Niles. 2014. Open ocean sea scallop (*Placopecten magellanicus*) trials in Chaleur Bay: Comparing culture gears and husbandry practices. Canadian Technical Report of Fisheries and Aquatic Sciences 3121. 26p.

This report examined the use of several different gear types in raising sea scallops; some were suspended and some on the seabed. The main evaluation was a comparison of alternate gears versus the traditional lantern net. Lantern nets have low unit cost and good performance, but high labor cost for maintenance. The study identified cost and growth tradeoffs for the different methods used, and recommended cost analyses at given sites for the methods in question. Additionally, the study identified spat collection in the region as being high enough to support ongoing activities.

Gardiner-Pinfold Consulting Economists, Ltd. 2001. Development Potential of the Maine Scallop Industry. Report to the Maine Dept. of Marine Resources. 60p.

This report examines the status of the scallop resource in Maine, and mechanisms by which the resource can be supported to gain maximum value. They address details of the product relative to the market, an examination of the wild fishery and its structure, and the possibility of cultured scallops.

Hale Group. 2016. Farmed Shellfish Market Analysis. A report to the Gulf of Maine Research Institute, Portland, ME. 65p.

The analyses for farmed product in this study addressed eastern oysters, blue mussels and sea scallops. Authors summarized the large domestic market for scallop products in the US, and the landings for US vessels as well as imports. They project that supply over the next 15 years - the time of the planning horizon - will stay even or perhaps decline somewhat, and that Maine-produced scallops command a price premium. They also note that the industry is still in early phases of development, and much needs to be done to capture the market opportunity.

Pottle, T.J. and M. Hastings. 2001. Sea Scallop Demonstration Project, Final Programmatic Report. Submitted to National Fish and Wildlife Foundation. 10p, with appendices.

This project brought forth important processes for the sale of whole scallops in Maine, and tested culture gear and density in a scientific setting. A purpose-built vessel was constructed for the project, and growth was found to be acceptable at all densities tested. A protocol and Memorandum of Understanding was developed with the Maine Dept. of Marine Resources as a prerequisite for permission to sell whole scallop products. Bi-weekly testing of scallop tissues revealed that regulatory limits for saxitoxin and domoic acid were not exceeded over the duration of the study.

Schick, D. and S. Feindel. 2005. Maine scallop fishery: monitoring and enhancement. Final report to the Northeast Consortium. Award #013-138-2051-30. 73p.

This project created and refined a methodology for evaluating scallop enhancement efforts in coastal Maine, paired with information about the fishing fleet, and the spatial patterns of the scallop resource in terms of size, meat yield, relative abundance, recruitment and associated fauna. A GIS database of suitable spat collection areas was created, and spat collection field trials were undertaken. Field deployment of collected spat followed. Collections were generally positive, with some spat collectors containing over 10,000 individuals, although collections varied widely by location. Results of re-seeding efforts were mixed, with an admission that more complete observation of re-seeded areas was warranted.

The Influence of Climate and Environment on the Spawning, Condition, and Larval Set of Naturalized Pacific Oysters *Crassostrea gigas* in a U.S. West Coast Estuary

Brett R. Dumbauld[1*], Jennifer L. Ruesink[2], Alan J. Trimble[2], and Zachary Forster[3]

[1] Agricultural Research Service, U.S. Dept. of Agriculture, Hatfield Marine Science Center, Newport, OR 97365, USA

[2] Department of Biology, University of Washington, Seattle, WA 98195, USA
[3] Washington Dept. of Fish and Wildlife, Ocean Park, WA. 98640, USA

Corresponding author: brett.dumbauld@usda.gov

Keywords: Pacific oyster, condition index, Willapa Bay, spawning, climate

ABSTRACT

The Pacific oyster *Crassostrea gigas* was introduced to the US west coast in the early 1900's where it replaced the native oyster *Ostrea lurida* and has become the mainstay for the shellfish aquaculture industry. Pacific oysters only regularly spawned and became "naturalized" in several discrete estuarine locations where conditions allowed for both adult oyster conditioning and spawning and larval survival, retention and settlement. The shellfish industry relied on "natural" set at these locations and/or continued to import seed from Japan until the advent of hatchery technology in the late 1970's. Since that time, the industry has gradually shifted to hatchery production of oyster larvae, with large operators establishing their own in-house hatcheries and smaller companies incorporating remote setting facilities and purchasing product from hatcheries that specialize in producing late stage eyed larvae. Most operators continued to take advantage of "wild" set until a prolonged set of failures in larval production termed the seed crisis occurred in both hatcheries and in these estuaries like Willapa Bay, Washington, where wild set was and continues to be a feature of management on state established oyster reserves. The failures at hatcheries have been clearly linked to water chemistry and ocean acidification, where aragonite saturation state is reduced and larval oysters are unable to deposit shell, but evidence for direct effects of carbonate chemistry in estuaries like Willapa Bay is more equivocal due to complex interactions with other factors. Variables like temperature, salinity, and phytoplankton as food, as well as other sources of larval mortality, are still related to the proximity of the ocean and cold upwelled water, but have been less studied in this estuary. Some of these factors also influence gametogenesis and spawning in adult oysters. We summarize historical records for spawning and setting in this naturalized Pacific oyster population, including a long term record of oyster condition. We propose a new effort to evaluate some of these additional factors and compare these records with data from locations in Japan, where these oysters were originally sourced, and other locations with similar climate records where Pacific oysters have been introduced. This effort will help understand and potentially mitigate for the effects of anthropogenic changes to these systems and an uncertain future climate.

INTRODUCTION

The Pacific oyster *Crassostrea gigas* was introduced to the US west coast in the early 1900's where it replaced the native oyster *Ostrea lurida* and has become the mainstay for the shellfish aquaculture industry (Lindsay and Simons 1997; Robinson 1997; Shaw 1997; Steele 1964). Pacific oysters only regularly spawned and became "naturalized" in several discrete estuarine locations where conditions allowed for both adult oyster conditioning and spawning and larval survival, retention and settlement (Chapman and Esveldt 1943; Quayle 1988). The shellfish industry relied on "wild" set at these locations with the most consistent supply of US seed oysters produced in Dabob Bay in Washington state and/or continued to import seed from Japan until the development of consistent hatchery technology in the late 1970's (Chew 1984; Hi Im et al. 1976; Langmo and Clark 1979). Since that time, the industry has gradually shifted to hatchery production of oyster larvae, with large operators establishing their own in-house hatcheries and smaller companies incorporating remote setting facilities and purchasing product from hatcheries that specialize in producing late stage eyed larvae (Nosho and Chew 1991).Most operators continued to take advantage of "wild" set until a prolonged set of failures in larval production termed the seed crisis occurred in both hatcheries and in these estuaries like Willapa Bay, Washington (Hales et al. 2017). Wild set, however, continues to be an important feature of management of Pacific oysters on state established oyster reserves in Willapa Bay (Dumbauld et al. 2011).

Due at least in part to the longstanding importance of the oyster industry in Washington state, a relatively substantial effort was devoted to understanding or at least monitoring and forecasting oyster spawning, larval abundance, and larval set, with water chemistry laboratories established at both the Washington Department of Fish and Wildlife (then Fisheries) field stations located on Willapa Bay (Nahcotta) and Dabob Bay (Pt. Whitney) (Lindsay et al. 1959; Packer and Mathews 1980). In addition to monitoring water chemistry and oyster larvae during the summer spawning season, a long term monitoring program was undertaken to examine the condition or "fatness" of adult oysters (Westley 1959; Westley 1961; Westley 1964), with sampling in Willapa Bay beginning in 1955 and continuing with some gaps through present day.

Recent failures of Pacific oyster larval production at US West Coast shellfish hatcheries have been clearly linked to water chemistry and ocean acidification, where aragonite saturation state is reduced and larval oysters are unable to deposit shell (Barton et al. 2015). Evidence for direct effects of carbonate chemistry on larvae and wild set Pacific oysters in estuaries like Willapa Bay, however, is more equivocal due to complex interactions with other factors (Hales et al. 2017; Ruesink et al. 2018). Upwelled oceanic water, which already has a low aragonite saturation state, enters this coastal estuary during summer months (Banas et al. 2007; Roegner et al. 2002), and while this low aragonite saturation state has been enhanced by recent atmospheric CO_2 contributions (Hales et al 2017), other riverine drivers influence this chemistry up estuary (Ruesink et al. 2018). Variables like temperature, salinity, and phytoplankton as food have also been shown to vary along the estuarine gradient (Ruesink et al. 2003; Ruesink et al. 2015) and potentially influence gametogenesis and spawning in adult oysters. This estuarine gradient is well known to the shellfish growers, in part as the fattening line in Willapa Bay (Banas et al. 2007; Dumbauld et al. 2011). Oyster growers typically plant or harvest juvenile seed oysters that are grown south of this imaginary line (from just North of Nahcotta to just north of the Nemah River on the East side, Fig. 1) and transplant these oysters to better "fattening" beds located north of this line for eventual harvest. This line also serves as a rough boundary for the locations of successful spawning and setting of Pacific oysters in this estuary, with the majority of naturally set oysters occurring south of the line (Dumbauld et al. 2011; Ruesink et al. 2018). Thus older oysters north of the line have a high condition index or meat to volume ratio while those south of the line a much lower condition, in part because they spawn during the summer season and release gametes.

Condition index has been relatively extensively evaluated as a tool for examining the effects of environment on oysters and other bivalves elsewhere (Dutertre et al. 2009; Filgueira et al. 2013; Rheault and Rice 1996; Soniat et al. 1989), and the Willapa Bay record was previously examined as well (Schoener and Tufts 1987; Schumacker 1999; Strickland and Ebbesmeyer 1995). We are unaware, however, of any temporal records like this that now exceed six decades. We characterize spatial trends that are related to the slightly less complete record for spawning and setting of this naturalized Pacific oyster population in this estuary, and propose a new examination of the relationship between condition and similar records of ocean climate in the Northeast Pacific.

MATERIALS AND METHODS

While all measures of bivalve condition relate some estimate of meat biomass to the volume of the shell cavity, multiple methods have been used to make these assessments (Abbe and Albright 2003; Crosby and Gale 1990; Lawrence and Scott 1982). Condition of oysters in Willapa Bay from 1954 – 1998 was measured using the Westley method (Westley 1961) which uses Archimedes principle to obtain the shell cavity volume and is calculated as follows:

$$CI = \frac{\text{Dry body wt x 100}}{[\text{whole wt. in air} - \text{whole wt. in water}] - [\text{shell wt. in air} - \text{shell wt. in water}]}$$

Oysters were pooled to make these assessments so no estimate of error was obtained for these monthly values. After a detailed comparison with other methods, a consistent relationship was established between conditions collected using this technique and the gravimetric method, which is much easier to conduct on individual oysters and less prone to error (Schumacker 1999). Thus, the gravimetric technique was adopted and all values from 1999 – present were calculated as follows:

$$CI = \frac{\text{Dry body wt x 100}}{[\text{whole wt.}] - [\text{dry shell wt.}]}$$

Pre 1999 values are converted to gravimetric equivalents using the relationship developed by Schumacker (1999): Gravimetric = Westley/1.180 - 0.905 (n= 504, r^2 =0.9126). Twenty oysters were sampled monthly at each of four sites in most years from 1954 to present (Stony Pt., Nahcotta, Oysterville, and Stackpole; Fig. 1). Expanded surveys at six additional sites were conducted by WDFW biologists from 1969-1978, with four of those notably located south of the "fattening line" described above, near the south end of the estuary (Refuge, Bear River, Smokey Hollow and Naselle, Fig. 1). We used these data to examine spatial and annual temporal patterns in condition index values and group data (selecting portions of the gametogenic cycle = months) to average and obtain annual means and better examine the longer 60 year record. Some simple univariate analysis (ANCOVA with month as a co-variate) were conducted to test for differences by site. We calculated and examined the long term anomaly = deviation from the mean for each of these records. Finally, we examined two similar long term records of environmental data from the Eastern North Pacific: the El-Nino Southern Oscillation (ENSO; data accessed at https://www.ncdc.noaa.gov/teleconnections/enso/indicators/soi/) and the Pacific Decadal Oscillation (PDO; data accessed at http://research.jisao.washington.edu/pdo/PDO.latest) and calculated similar anomalies to visually compare with the oyster condition index record and evaluated these relationships with simple multiple regression. We also used the condition index data from one site (Nahcotta) where oysters appear to spawn on a regular basis (e.g. condition drops dramatically between consecutive months) to determine whether these climate anomalies were correlated with the month(s) during which this apparent spawning occurred. Finally, we calculated a similar anomaly for the less continuous long term record of oyster larval set in Willapa Bay, collected by WDFW biologists and ourselves over time for comparison. We expect the mostly visual examinations presented here to instruct new quantitative time series analyses like those undertaken by previous authors where time lags and processes can be incorporated and further assessed (Schoener and Tufts 1987).

RESULTS AND DISCUSSION

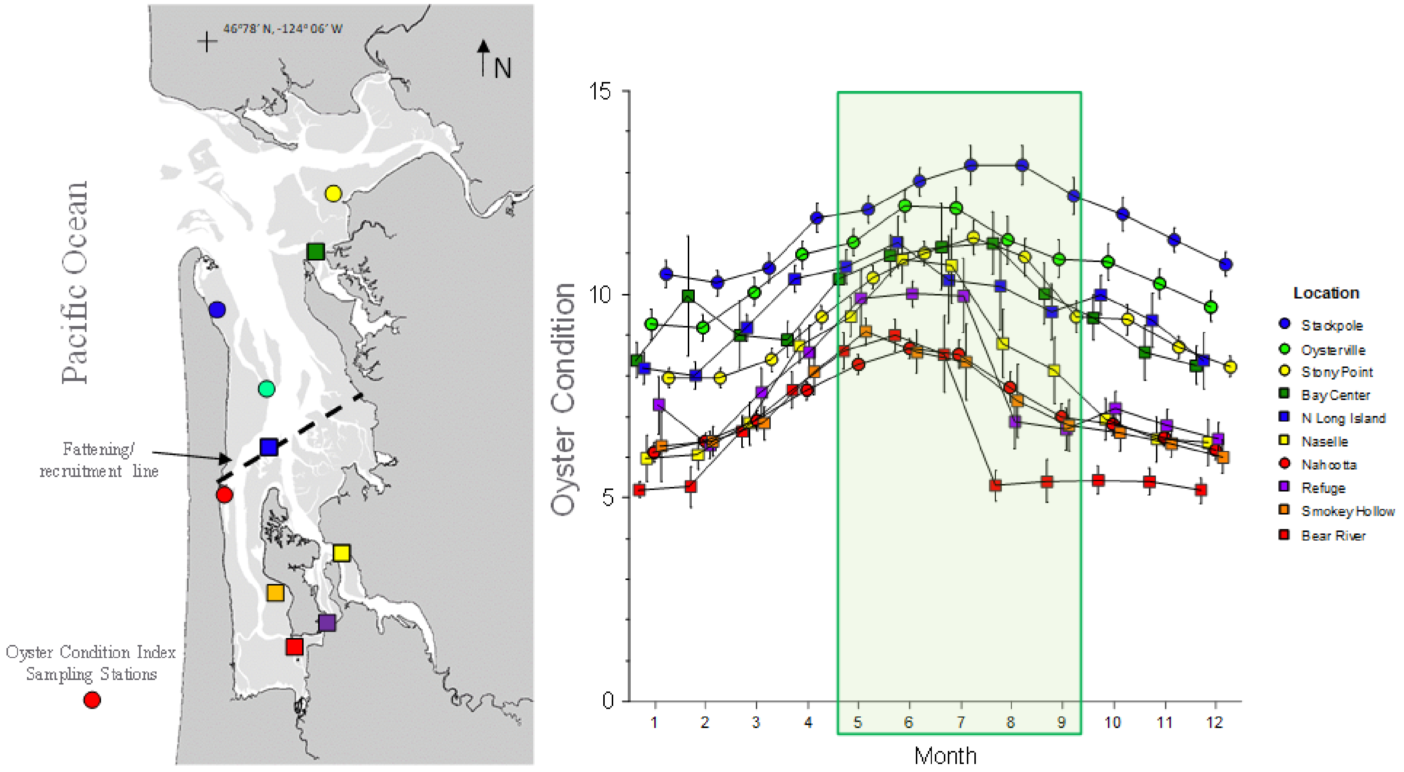

Figure 1. Average oyster condition at 10 sites in Willapa Bay, Washington, monitored from 1968-1978 (bars = SE's). Note estuarine gradient with higher values near the estuary mouth and lower values and seasonal drop during summer at sites south of an estuarine fattening line during late spring and summer (May – Sept).

The seasonal pattern of oyster condition was similar, but average condition varied by site over the 10 year interval with data from 10 sites in Willapa Bay (1968-1979, Fig. 1B). As might be expected, oyster condition increased from seasonal lows in January and February to highest values in summer (June – August), and then declined again over fall months. This seasonal cycle has been well documented, and is related to changes in the reproductive cycle and quantity of associated nutrients including carbohydrates, proteins and minerals in the tissues and loss of meat weight when/if the gametes are spawned at numerous locations for both *C. virginica* (Abbe and Albright 2003; Austin et al. 1993; Galtsoff 1964; Medcof and Needler 1941; Rainer and Mann 1992; Rheault and Rice 1996; Soniat et al. 1989) in the US as well as *C. gigas* in Asia and multiple locations worldwide where it has been introduced (Chavez-Villalba et al. 2003; Enriquez-Diaz et al. 2009; Kang et al. 2000; Mason and Nell 1995; Ubertini et al. 2017). While condition was significantly different amongst years (ANCOVA, $F_{1,9} = 29.7$, $p < 0.001$), it was highest at sites near the estuary mouth and north of the "fattening" line (Stackpole, Oysterville, Stony Point, and Bay Center) where it also fluctuated less, especially during the summer season. Oyster condition was significantly lower at the southern end of the estuary (Nahcotta, Smokey Hollow, Bear River, Naselle, and Refuge; ANCOVA, Site $F_{1,8} = 406.1$, $p < 0.001$) where there was often a more dramatic drop during summer, presumably due to spawning events at these sites. This type of regional variation has also been well studied and related to environmental parameters like temperature, salinity and food (phytoplankton or chlorophyll) that influence oyster growth and reproduction (Brown and Hartwick 1988; Dutertre et al. 2009; Gasmi et al. 2017). We suspect that both temperature and food are the key factors in Willapa Bay, but that water residence time is also important. A north to south gradient in chlorophyll a, phytoplankton production, and phytoplankton species composition has been observed in Willapa Bay that is driven by intrusion of oceanic water into the estuary during spring and summer months (Newton and Horner 2003; Roegner et al. 2002; Ruesink et al. 2003). When a non-conservative tracer for Chl-a was added to a detailed oceanographic model created for this estuary (Banas et al. 2007), results suggested that there was a large difference in water residence time between areas north of a mid- estuary line, such that benthic grazers, like oysters south of this line, would not experience these intrusions of oceanic phytoplankton as often and potentially be subject to drawdowns of this production by grazers north of the line. Growers have recognized this feature for some time and traditionally plant small seed oysters to grow to harvest size and/or place shell on their beds to encourage natural settlement of these oysters south of this line and then transplant them to areas north of the line before marketing them.

Based on the observed seasonal cycle, we divided the data for the extended 1954-2017 time series into two separate time segments that reflect oyster physiology and ocean estuary conditions instead of calendar year: 1) winter and early spring months (October through April, termed "winter") during which oysters resorb gametes and accumulate glycogen and other reserves and 2) late spring and summer (May through September, termed "summer") during which oysters produce gametes and potentially spawn. The average condition during winter at the four sites measured over this longer time frame was highest at Stackpole (11.02 ± 0.13) and lowest at Nahcotta (6.64 ± 0.09), and fluctuated over time – declining on average from 1954 – 1997 and increasing since then (Table 1, Fig. 2).

Table 1. Average oyster condition index (±SE) for winter and summer months at each site. These values represent the entire time series (1954-2017) for the first four stations, but only the shorter period (1968- 1978) for the latter six sites.

Site	Winter CI (Oct-April)	Summer CI (May-Sept)
Stackpole	11.02 (0.13), n = 355	12.70 (0.18), n = 237
Oysterville	10.02 (0.14), n = 314	11.57 (0.21), n = 213
Stony Point	8.56 (0.11), n = 392	10.67 (0.17), n = 266
Nahcotta	6.64 (0.09), n = 391	8.06 (0.14), n = 269
Bay Center	8.91 (0.28), n= 67	10.74 (0.31), n = 48
North Long Island	9.08 (0.20), n = 62	10.42 (0.35), n = 47
Naselle	6.76 (0.21), n = 69	9.55 (0.34), n = 47
Smokey Hollow	6.66 (0.20), n = 67	8.01 (0.32), n = 47
Refuge	7.18 (0.21), n = 67	8.63 (0.35), n = 48
Bear River	5.83 (0.17), n = 69	7.32 (0.33), n = 48

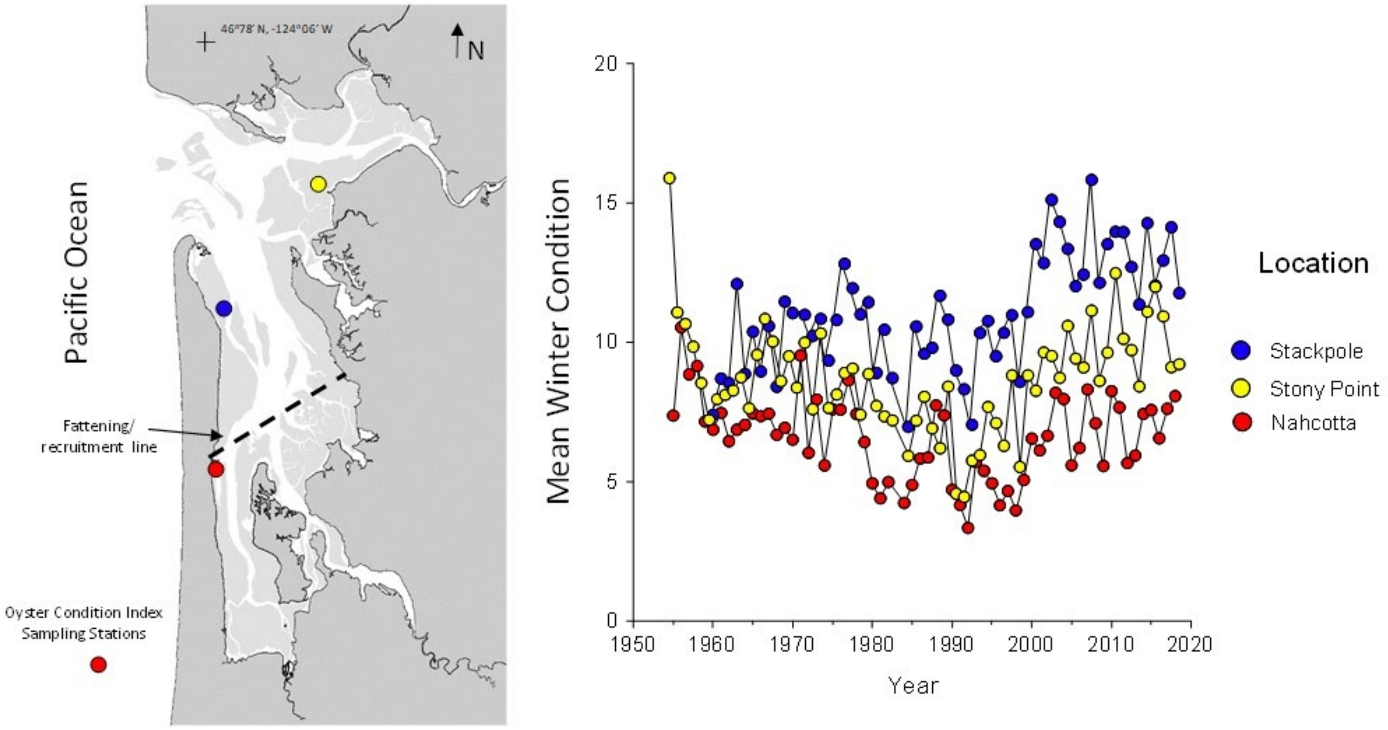

Figure 2. Average winter (Oct through April) oyster condition index at 3 sites in Willapa Bay from 1954 - 2017. Note distinct differences between sites, but similar fluctuations and long term trends with a decline from 1954 – 1997 and subsequent increase.

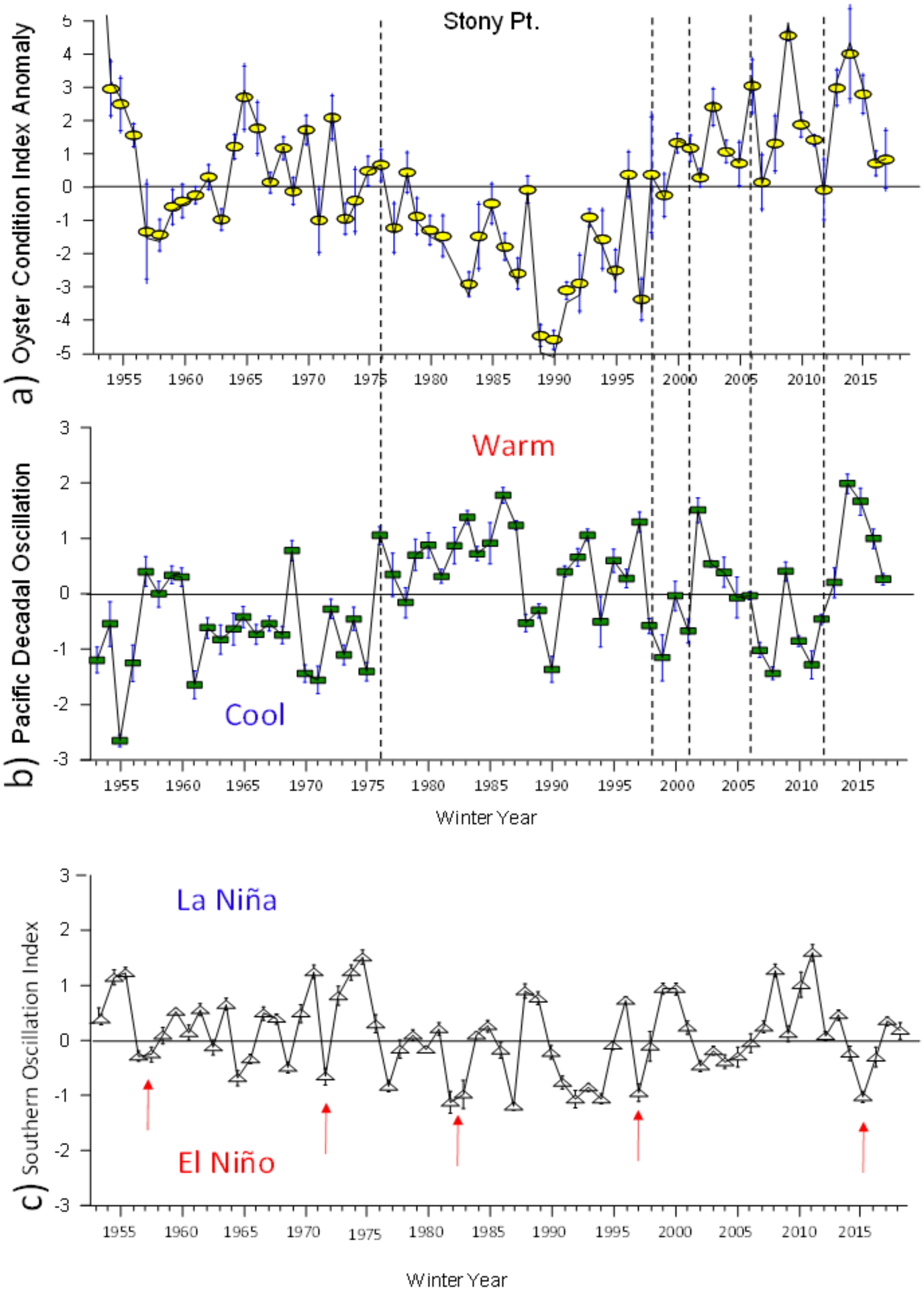

Figure 3. a) Oyster condition index anomaly at Stony Pt. site for winter and spring months (October through April) compared with b) Pacific Decadal Oscillation anomaly and c) Southern Oscillation Index anomaly for the same period. Note the axis has been inverted for the PDO and shifts are noted with vertical dashed lines and warmest El Niño events with red arrows.

Summer values were higher but more variable, especially at sites closer to the southern end of the estuary, where rapid declines during these months indicated that the oysters likely spawned. Since the most complete data records were for the Stony Pt.

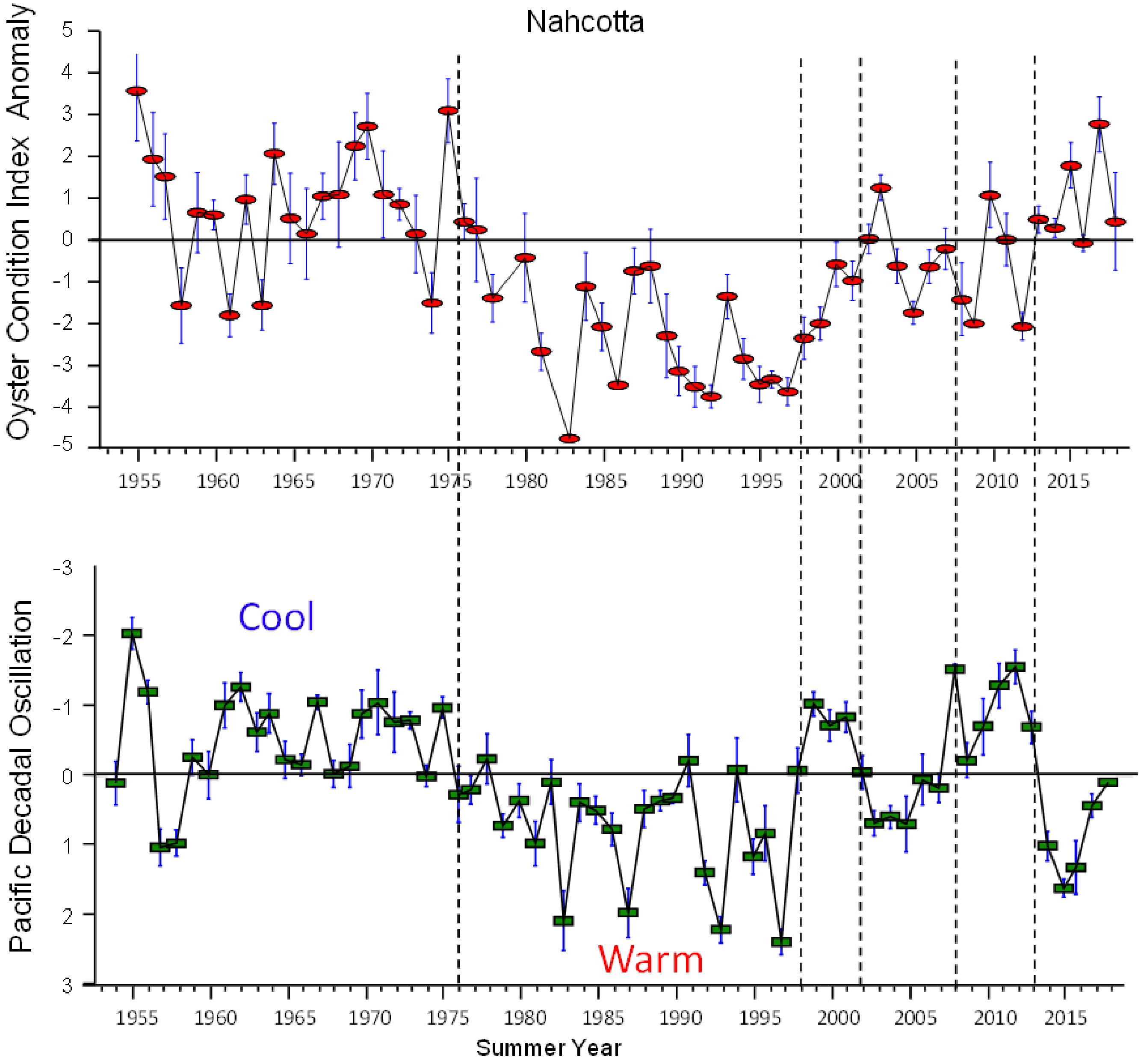

Figure 4. Oyster condition index anomaly at Nahcotta during summer months (May through September, top) compared with Pacific Decadal Oscillation anomaly for this same period (bottom). The axis for the PDO has been inverted and shifts are noted with vertical dashed lines.

and Nahcotta sites, we used these records to calculate and evaluate the long term anomalies in condition and compare them with several indices of nearshore ocean climate. A general relationship was apparent between oyster condition and the Pacific Decadal Oscillation (PDO), with negative anomalies corresponding with warm phases and positive condition anomalies with cool phases (Fig. 3), but a breakdown in the strength of this pattern occurred after about 1997. A general visual relationship was also evident between the winter oyster condition anomaly and the southern oscillation index (SOI) anomaly, with positive SOI values (and El Niño events) corresponding to lower condition and negative SOI (La Niña events) corresponding with positive condition (Fig. 3). Though individual events, including those after 1997, seem to be correlated, no statistical relationship emerged. Schoener and Tufts (1987) found little relationship with sea surface temperature when examining the same data set, but a significant relationship with sea level height that best fit a phase lag with changes in sea level height preceding those in condition by several months. They documented the lowest condition index on record to that point in time corresponding with the 1982-1983 El Niño. That low value has now been surpassed by two even more negative condition anomalies, one of which corresponds with the even stronger 1997 El Niño. The previous declining trend in oyster condition in Willapa Bay has also been correlated to the Pacific Northwest Index (a combined climate index derived from three more terrestrial parameters including temperature and precipitation) and the decline in Columbia River discharge (Strickland and Ebbesmeyer 1995), but we suspect the recent rebound in condition would not track at least the latter.

Table 2. Results of simple multiple regression relating the long term record of oyster condition index anomaly at two sites to similar records for the Pacific Decadal Oscillation (PDO) and Southern Oscillation Index (SOI). Results from two separate models for summer and winter averages are shown.

	Estimate	SE	df	F	P
Model			3	10.75	< 0.001
Summer PDO	-0.97	0.19	1	25.49	< 0.001
Summer SOI	0.01	0.28	1	0.002	0.97
Site (Stony Pt.)	-0.05	0.24	1	0.05	0.81
Error/Total			122/ 125		
Winter PDO	-0.12	0.22	3	2.48	0.12
Winter SOI	0.35	0.18	1	0.45	0.50
Site (Stony Pt.)	-0.06	0.20	1	0.09	0.76
Error/Total			123/ 126		

The relationship between PDO and the oyster condition anomaly was significantly negative in summer (Fig. 4, Table 2). Years when a significant drop in condition occurred during these summer months, signaling a spawning event, were clearly evident at the Nahcotta site, and we further separated this data into those years occurring during warm (PDO > 0) and cool phases (PDO< 0). Spawning occurred during both phases, yet during warm phases of the PDO, condition was significantly higher in years that these events occurred than when they did not (Fig. 5, ANCOVA , $F_{1,165} = 11.12$, $p = 0.001$), while there was no difference during the cool phase (ANCOVA, $F_{1,180} = 1.78$, $p = 0.18$). In those years when these spawning events occurred, they often occurred earlier (June and July) during warm phase years and later (August) during cool phase years. This result suggests that oceanic conditions may affect the seasonal gametogenic cycle prior to spawning. More broadly, multiple triggers including temperature, but also food and even disturbance events, can be associated with spawning (Dutertre et al. 2009; Ubertini et al. 2017).

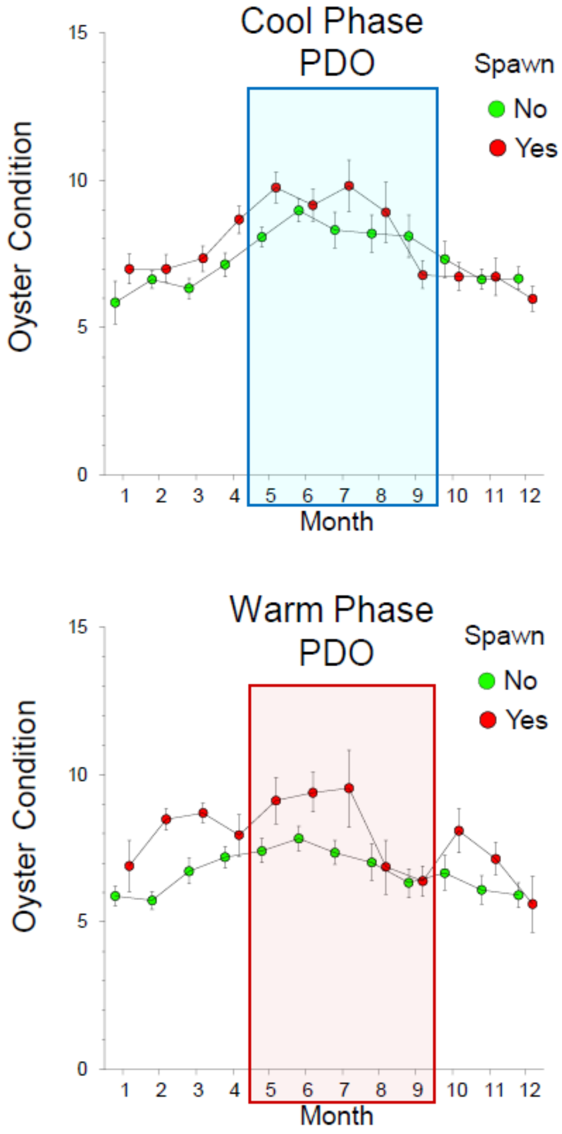

Figure 5. Average monthly oyster condition index at Nahcotta site for years where the average PDO anomaly in the summer (May through September) was negative (top) and positive (bottom). Years with a visually distinct drop in condition (presumably due to spawning) are contrasted with years when this was not apparent. Note the apparent seasonal difference in timing of spawn events with more and potentially earlier spawning events occurring during warm phase of the PDO.

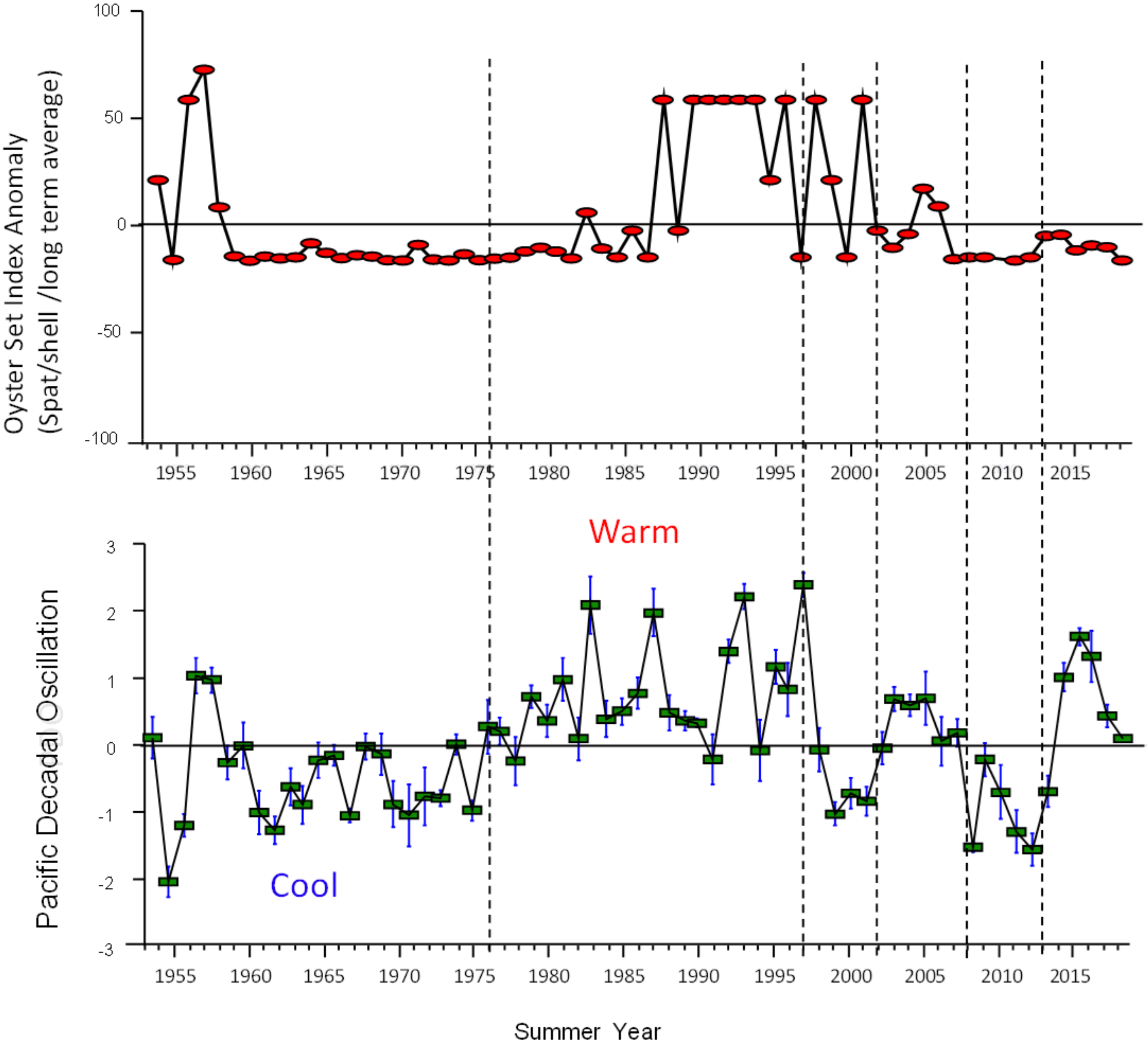

Figure 6. Anomaly of oyster sets (counts of oyster spat per shell) on seasonal shell strings deployed at three sites in Willapa Bay (top) compared with the Pacific Decadal Oscillation anomaly for summer months (May – October; bottom) with shifts noted by dashed lines.

There appears to be a positive relationship between successful spatfall resulting from these spawning events and the PDO with larger spatfalls occurring during warm phases of the PDO (Fig. 6). While it may be that earlier or multiple spawning events are simply more likely to result in successful spatfall in this estuary, due to the typical three week period of larval development during which other factors (e.g. water retention, vertical migration, wind and weather) are involved, this relationship requires further investigation. The PDO has been related to numerous other biological endpoints, from zooplankton to coho salmon (Malick et al. 2015; Menge et al. 2008; Shanks 2013). This included oyster condition in one of the original characterizations (Hare and Mantua 2000), but defining the mechanism for this interaction with nearshore ocean conditions remains a useful research avenue. The PDO has recently also been related to phytoplankton community structure (Du et al. 2015) with cool phases being connected to a diatom rich community and warm phase communities dominated by dinoflagellates. These authors have also shown that a similar community was present during the recent presence of an anomalous warm water mass (referred to as the blob, Bond et al. 2015) in the nearshore coastal ocean off of the US Pacific Coast and that the chl a signature and overall biomass remained high. This could explain the breakdown we observed in the relationship between the PDO anomaly and oyster condition at about the same point in 2013, but more evidence should be gathered to test this hypothesis, and we propose that a new effort to evaluate some of these additional factors be initiated. This could include a comparison with data from locations in Japan where these oysters were originally sourced and similar long-term records for other locations where Pacific oysters have been introduced and long term records are in place, in order to understand and potentially mitigate the effects of anthropogenic changes to these systems and an uncertain future climate.

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ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Barton, A., G.G. Waldbusser, R.A. Feely, S.B. Weisberg, J.A. Newton, B. Hales, S. Cudd, B. Eudeline, C.J. Langdon, I. Jefferds, T. King, A. Suhrbier, and K. McLaughlin. 2015. Impacts of Coastal Acidification on the Pacific Northwest Shellfish Industry and Adaptation Strategies Implemented in Response. Oceanography 28: 146-159.

This is the most recent review of the history and science underpinning the effects of changing seawater chemistry on bivalve shellfish larvae and the impacts that have already taken place to the commercial shellfish aquaculture industry on the

US West Coast. Multiple authors contributed to this review, which addresses a broad audience, but covers the leading research on direct effects to bivalve larvae, as well as monitoring seawater conditions and adapting to these changes.

Dumbauld, B.R., J.L. Ruesink, A.C. Trimble, and B.E. Kauffman. 2011. The Willapa Bay oyster reserves in Washington State: Fishery collapse, creating a sustainable replacement, and the potential for habitat conservation and restoration. Journal of Shellfish Research 30: 71-83.

The authors review the history of the state oyster reserves in Willapa Bay, Washington, where Pacific oysters were introduced in 1928 and one of a few locations on the US West Coast where a naturalized population became established. They review the record of larval set in this estuary and attempt to link this to broad scale oceanographic conditions (but see Hales et al 2017 and Ruesink et al. 2018 below for a more detailed discussion of water chemistry within this estuary).

Hales, B., A. Suhrbier, G.G. Waldbusser, R.A. Feely, and J.A. Newton. 2017. The carbonate chemistry of the "Fattening Line," Willapa Bay, 2011-2014. Estuaries and Coasts 40: 173-186.

The authors present detailed data on seawater chemistry (especially PCO_2 and aragonite saturation state) for Willapa Bay, Washington, where Pacific oysters have been the mainstay of the oyster aquaculture industry for almost 100 years and there is a long term record of spawning and setting. They reconstruct this record for a longer historical period and their data suggests that recent conditions provide a smaller window of optimal conditions (low aragonite saturation state and warm enough temperatures for oyster spawning) than occurred historically. While they did not sample larvae (see Ruesink et al 2018 below) and therefore can't confirm effects, they substantiate the complexity of measuring these effects and attributing them to a single cause in an estuary where conditions are variable.

Ruesink, J.L., A. Sarich, and A.C. Trimble. 2018. Similar oyster reproduction across estuarine regions differing in carbonate chemistry. Ices Journal of Marine Science 75: 340-350.

These authors measured seawater chemistry in Willapa Bay, Washington, but unlike Hales et al (2017), they also present simultaneously collected data on four cohorts of Pacific oyster larvae that were collected over three summers. The southern end of Willapa Bay has two arms which create distinctly different characteristics because one is much more affected by riverine conditions that cause reduced aragonite saturation relative to the other. Oyster settlement differed greatly between cohorts, but they did not find differences they could attribute to this different water chemistry, and instead found thermal conditions were perhaps more important.

Weisberg, S.B., N. Bednarsek, R.A. Feely, F. Chan, A.B. Boehm, M. Sutula, J.L. Ruesink, B. Hales, J.L. Largier, and J.A. Newton. 2016. Water quality criteria for an acidifying ocean: Challenges and opportunities for improvement. Ocean & Coastal Management 126: 31-41.

This review represents a call for developing ecologically relevant water quality criteria for acidification and augmenting coastal water quality monitoring efforts to characterize this at appropriate temporal and spatial scales. Historical data for oyster larval recruitment in Willapa Bay is one of two case examples presented that demonstrate the challenges involved in relating biological endpoints to appropriate water chemistry criteria in estuaries where conditions are variable.

A Review of the Potential of Macroalgae Feedstuffs to Reduce Methane Emissions from Ruminant Livestock

Luke Gardner[1,2*], Scott Hamilton [2], Michael Graham[2], Stacey Gunter[3], and Kenneth Kalscheur[4]

[1] California Sea Grant, Scripps Institution of Oceanography, 9500 Gilman Drive, Dept. 0232 La Jolla, CA 92093
[2] Moss Landing Marine Laboratories, 8272 Moss Landing Rd., Moss Landing, CA 95039
[3] United States Department of Agriculture, Agricultural Research Service, Southern Plains Range Research Station, 2000 18th Street, Woodward, OK 73801
[4] United States Department of Agriculture, Agricultural Research Service, U. S. Dairy Forage Research Center, Dairy Forage Research Unit. 1925 Linden Drive, Madison, WI 53706

Corresponding author: lgardner@ucsd.edu

Keywords: seaweed, climate change, methane, aquaculture, livestock

ABSTRACT

To address climate change in California beyond carbon-based gases, the state has recently passed legislation to reduce short-lived climate pollutants. In particular, the state aims for a 40% methane reduction by 2030. Several reductions are expected to come through existing technologies associated with landfills and energy producers. However, the state's largest contributor to methane production is cattle, specifically 1.4 million resident dairy cows and one million beef cattle. Methods to reduce gases produced from cattle have largely been unexplored in California. However, recent research in Australia has shown that some species of sub-tropical seaweed can reduce methane production from cattle by up to 99% when used as a feed additive at 2% inclusion rates of organic matter. Temperate species have not yet been assessed for methane reducing properties, but many of the species present in the California Current Ecosystem are closely related to these sub- tropical species. The nutrient-rich waters off the Californian coast are ideal for the development of seaweed aquaculture and this project offers an excellent opportunity to address the underdevelopment of aquaculture in California. Seaweed aquaculture's low environmental impact, coupled with the potential to mitigate climate change impacts and bio-remediate coastal eutrophication zones make this area of research a promising mitigation solution for climate change. Research is required to assess different species of local seaweed for total gas production and digestibility when added to cattle diets. Methane reducing candidate species also need to be assessed for optimal culture conditions and scalability to be used as a feed additive for ruminant livestock.

INTRODUCTION

Seaweed has been harvested by humans for more than 1700 years and has been used for a variety of purposes, including food, animal feed, and numerous industrial uses over this history. However, it is only in the last few decades that seaweed aquaculture has begun to develop. At last census, approximately 27 million tons of seaweed is cultivated per year, representing a quarter of the total aquaculture production worldwide (FAO, 2016). The vast majority of seaweed is produced in China and Indonesia, generating a combined total of 23 million tons. Cultivation of seaweed in the US is nascent and does not yet generate enough products to be quantifiable on a global scale. This imbalance is largely due to the traditional and common use of seaweed in Asian countries. However, a US market has begun to emerge based on powerful consumer preferences towards organic and sustainable products. The sustainable benefits of seaweed aquaculture are undeniable; it requires zero additional nutrient inputs for growth and does not displace terrestrial crops for arable land or require fresh water, representing an alternative biomass for nutrition, compounds and energy in the future.

Furthermore, seaweed aquaculture can provide a myriad of ecosystem benefits including: oxygenation and uptake of nutrients by enhancing primary production (Vásquez et al., 2014); bioremediation of eutrophic zones, reducing harmful algal blooms; creating food, habitat and refuge for a diverse array of fish and invertebrates, thereby increasing biodiversity (Almanza and Buschmann, 2013); and mitigation of greenhouse-gas emissions by acting as a CO_2 sink (Chung et al., 2011) and most recently by reduction of methane production from livestock (Li et al., 2018a; Maia et al., 2016).

Release of greenhouse gases into the atmosphere is a major environmental, social, and economic threat worldwide. Methane is a particularly potent greenhouse gas that is 25 times more efficient at trapping heat in the atmosphere than carbon dioxide (Solomon et al., 2007). It is well documented that ruminant livestock contribute significantly to the global release of methane via digestion. Methane is emitted from livestock because of methanogenesis (a byproduct) in the forestomach of ruminants; a similar process occurs in the decomposition of manure. The United States is the fourth largest cattle producer in the world and cattle contribute 26% of the total U.S. methane emission (EPA, 2018). Dairy cattle are the largest methane producers among livestock and produce between 73 and 182 kg of methane per cow annually (Dong et al., 2006). California is the dairy industry's most productive state and has 1.4 million dairy cows that account for the majority of the enteric methane emissions. Consequently, California recently enacted legislation in 2016 mandating a 40% reduction from present day methane emissions by 2030 (2016).

Several technologies and strategies currently exist to mitigate methane production from sources like landfills, yet solutions for the dairy industry are less developed. Dairy digesters, specialized structures used to capture methane generated from decomposing manure, are slowly being implemented on farms in California. However, this only accounts for about a quarter of the methane emitted from dairy cattle with the vast majority being released via burps as a result of enteric fermentation (EPA, 2018; Powers et al., 2014). Enteric methane is difficult to mitigate because it involves the natural process of a cow's digestion. However, research on dietary strategies have shown promise (Hristov et al., 2015; Lee et al., 2017; Maia et al., 2016). Some dietary practices that have been shown to reduce methane include the addition of ionophores, fats, the use of high quality forages, and the increased use of grains (Caro et al., 2016). These nutritional strategies reduce methane through the manipulation of the ruminal environment, inhibition of methanogens and protozoa, or by redirection of hydrogen ions away from methanogens. Relatively new mitigation options have been investigated and include the addition of such additives as probiotics, acetogens, bacteriocins, organic acids, and plant extracts (i.e. condensed tannins) (Martin et al., 2010). More recently, researchers abroad have begun to investigate the effect of sub-tropical seaweed as feed additives for ruminants, discovering that a red macroalga called *Asparagopsis taxiformis* can reduce methane production by 99% at less than 2% inclusion in vitro and produced up to a 81% reduction in vivo sheep fed at 3% inclusion rates (Li et al., 2018a; Machado et al., 2016). The mode by which *A. taxiformis* reduces methane production is still unclear, but most recent research attributes the reductions to the bioaccumulation of halogenated methane analogues produced as algal secondary metabolites (Machado et al., 2018). The advantages of seaweed over other dietary manipulations is that seaweed has shown the most significant methane reductions with minimal declines in digestibility (Li et al., 2018b; Maia et al., 2016), and seaweed production does not compete with terrestrial food production systems.

While an assessment of enteric methane production has been done for a number of tropical species (Machado et al., 2014), nothing is known about the methane reduction properties of temperate macroalgae. This presents an excellent opportunity for the development of the seaweed aquaculture industry in California, in temperate coastal waters that are highly productive compared to tropical latitudes, and thus provide superior conditions for seaweed culture.

Scalability: In concert with identification of methane reducing seaweed species, it is prudent to theoretically consider the scalability of seaweed production. The United States has the world's largest marine exclusive economic zone, an area equivalent to the total land area of all 50 states. The U.S. Department of Energy estimates that the U.S. has suitable conditions and geography for producing approximately 500 million tons (DW) of seaweed (ARPA-E, 2017). California has the third largest coastline in the U.S., with state waters encompassing approximately 13,688 km^2. Based on a series of assumptions, including an average seaweed production value of 1,604 t km^2 $year^{-1}$ (DW) (Xiao et al., 2017) and a dairy cow consuming 9,855 $kg^{-1}year^{-1}$ (DW), a total of 140,000 t (DW) seaweed $year^{-1}$ would be required to feed the state's 1.4 million dairy cows at a 1% inclusion rate. Using these assumptions, a total of 86 km^2, or 0.6% of state waters, is all that would be required to culture the necessary seaweed volume to achieve approximately a 50% or more reduction of methane emissions from California's 1.4 million dairy cattle, the biggest methane contributor in the state. While this estimation is based on many assumptions, a total of 0.6% of state waters potentially being used for seaweed aquaculture seems relatively tolerable, especially when considering the likely secondary ecosystem benefits resulting from seaweed cultivation. California has the potential to utilize this resource to build and grow the nascent seaweed aquaculture sector. Growing seaweed for livestock feed offers a unique opportunity to circumvent many challenges associated with terrestrial production systems, while also mitigating methane emissions. Such a large potential local market could help develop vital infrastructure, skills, and distribution systems for seaweed aquaculture, if realized.

Potential benefits: The potential economic benefit of this research to California's aquaculture industry could be vast considering the immense value of the state's dairy and beef industry at $21 billion dollars annually. Beyond this, the nation's combined cattle industry is worth $78.2 billion, of which most cattle are fed formulated diets for a significant period of their life. Development and production of feed additives in California that could be exported and supplemented to cattle diets across the United States would be extremely beneficial to California's entire economy. Additionally, the culture of seaweeds for such purposes in California could help develop nascent aquaculture industries providing alternative economic growth for coastal communities that are significantly dependent mostly on commercial fisheries. Outside the potential economic value of this research, the state would also stand to benefit environmentally by reduction of methane and absorbing agricultural nutrient nonpoint source pollutants as they are incorporated into seaweed biomass. California is home to a significant number of coastally drained waterbodies that are impaired due to high levels of nutrients coming from irrigated agriculture (Anderson et al., 2003). Sixteen of these eutrophic water bodies drain into the Monterey Bay where Moss Landing Marine Laboratories is located. Seaweed aquaculture has been shown to be effective in bioremediation of coastal eutrophic areas (He et al., 2008). Development of seaweed aquaculture could aid in reducing the impact of eutrophication by absorbing nutrients from the water and incorporating them into seaweed biomass. This biomass could then be harvested for a variety of uses, including the recycling of these nutrients back into agricultural production as a feed ingredient.

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ANNOTATED BIBLIOGRAPHY OF KEY WORKS

Machado, L., M. Magnusson, N.A. Paul, R. de Nys, and N. Tomkins. 2014. Effects of Marine and Freshwater Macroalgae on In Vitro Total Gas and Methane Production. PLOS ONE. 9, e85289.

The authors for the first time assess an array of different sub-tropical seaweeds for methane reducing properties in cattle using in vitro techniques. They find that a red seaweed species called *Asparagopsis taxiformis* is the most effective seaweed tested, reducing methane production by up to 99%.

Maia, M. R., A.J. Fonseca, H.M. Oliveira, C.

Mendonca, and A.R. Cabrita. 2016. The Potential Role of Seaweeds in the Natural Manipulation of Rumen Fermentation and Methane Production. Sci Rep. 6, 32321.

This research is important in that it assesses the effect of the basal feed source (substrate) on methane reduction for a number of different seaweed species. The authors find that there is a significant effect on methane reduction based on whether the basal feed is either grass or corn, highlighting the need to further examine the seaweed interaction with different cattle feeds.

Li, X., H.C. Norman, R.D. Kinley, M. Laurence, M. Wilmot, H. Bender, R. de Nys, and N. Tomkins. 2018. *Asparagopsis taxiformis* decreases enteric methane production from sheep. Animal Production Science. 58, 681-688.

The authors for the first time assess the effect of seaweed on methane reduction using a whole animal experimental design. The article reports that sheep fed a 3% diet of seaweed *Asparagopsis taxiformis* reduce their methane emissions by 81% without any decrease in weight gain compared to the control treatment.

Condition Index and Fecundity of Manila Clam *Ruditapes philippinarum* Related to Habitat Environment

Natsuki Hasegawa[1*], Toshie Matsumoto[1], Satoshi Watanabe[1], Kazuhiro Hanyu[2], Dai Miyawaki[3], Ryogen Nambu[4], and Masami Hamaguchi[5]

[1] National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, 422-1 Nakatsuhamaura, Minamiise, Mie, 516-0193, Japan, [2] Suzuka Branch, Mie Prefecture Fisheries Research Institute, Suzuka, Mie, 510-0243, Japan
[3] Aichi Fisheries Research Institute, Gamagoori,
Aichi, 443-0021, Japan
[4] National Research Institute of Fisheries Engineering, Japan Fisheries Research and Education Agency, 7620-7, Hasaki, Kamisu, Ibaraki, 314-0408, Japan
[5] National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, 2-17-5, Maruishi, Hatsukaichi, Hiroshima, 739-0452, Japan
* Present address: Kushiro Field Station, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, 116 Katsurakoi, Kushiro, Hokkaido, 085-0802, Japan

Corresponding author: hasena@fra.affrc.go.jp

Keywords: *Ruditapes philippinarum*, Manila clam, condition index, fecundity, habitat

ABSTRACT

Understanding reproductive traits is important for maintenance or recovery of Manila clam *Ruditapes philippinarum* fisheries production, which has dropped considerably in Japan over the past half century. Relationships between condition index and number of eggs (fecundity) of the Manilla clam were assessed at several clam habitats in Ise Bay, Japan, during spring and autumn spawning seasons in 2015. Condition index was calculated from the shell size and soft body wet weight. Fecundity was quantified using an indirect enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody specific to a Manilla clam yolk protein. Land elevation (i.e. subtidal or intertidal), association with river input (river mouth or not), closeness of the shore, and chlorophyll *a* concentration in the water column were the habitat parameters analyzed in this study. A correspondence analysis revealed that the condition index during both spawning seasons was positively correlated (*r* = +0.48 to +0.80) with the 1st and 2nd correspondence analysis scores on various habitat parameters. On the other hand, the fecundity, which is summed for each spawning season (three sampling days) and normalized to 30 mm shell length, was negatively correlated with the 2nd habitat score (spring: *r* = -0.64, autumn: *r* = -0.80). These results show that the nutritional condition as determined by the condition index is not the sole factor affecting fecundity of the clam. Actually, the fecundity was high in the intertidal zones and enclosed river mouth areas, whereas the condition index was higher in the subtidal zones and open non-river mouth areas. The information obtained about asari clam reproduction may be useful for stock enhancement by improving reproduction in the populations. This study was conducted as part of a research project for recovery of asari populations by the Fisheries Agency of Japan.

INTRODUCTION

Manila clam, *Ruditapes philippinarum*, alias name short neck clam, or asari in Japanese, is an important bivalve species for commercial and recreational fisheries in Japan. According to the statistics released by the Ministry of Agriculture, Forestry and Fisheries of Japan, fisheries production of suspended and bottom culture of bivalve species, such as scallop and oyster, are slightly decreasing or staying level in Japan (Fig. 1). However, capture fisheries production of Manila clam has dropped considerably in Japan over the past half century (Matsukawa et al., 2008). The production peaked at 170,000 tons in 1983 and went down to 9,000 tons in 2016, which is a 95% decrease. The Fisheries Agency of Japan and local governments have undertaken some projects for clam stock enhancement, but the decreasing trend is continuing.

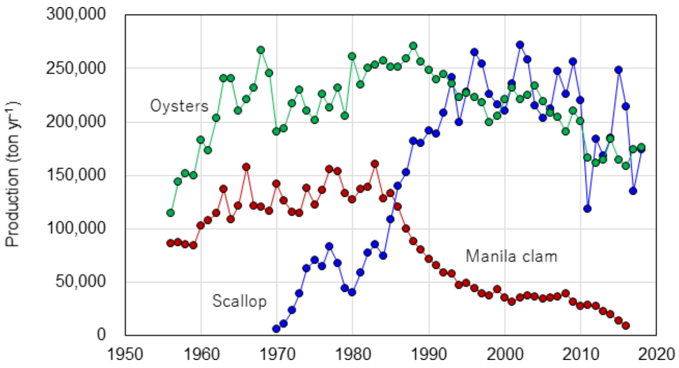

Figure 1. Fisheries production of bivalve species: Manila clam, Japanese scallop and oysters, from the statistics released by the Ministry of Agriculture, Forestry and Fisheries, Japan. The production of Manila clam includes a small amount of purplish Washington clam, Saxidomus purpurata, production.

We need more basic knowledge on the population dynamics to improve or create new countermeasures to mitigate the population decline of the clam. Reproductive traits may be important for executing stock enhancement programs for the clam.

One of the most important reproductive traits to be studied is variation in fecundity (the number of eggs) of the clam associated with environmental factors. Some previous studies reported that individual fecundity is simply proportional to the clam's body weight; in other words, larger clams spawn more eggs than smaller clams (Toba & Miyama 1994) and robust clams have more fecundity than thin clams (Park et al. 2006, Hasegawa et al. 2014). Therefore, individual fecundity depends on the body weight and the nutritional condition, and fecundity per unit body weight is relatively constant among clams of different sizes. These statements sound intuitively true, however, these results were obtained for clams collected within the same habitat or cultured under similar rearing conditions except food level (abundance). Variation in the clam's growth depending on the habitat was evaluated in various studies such as Kakino (1996). The effects of various environmental factors on clam fecundity should be further studied.

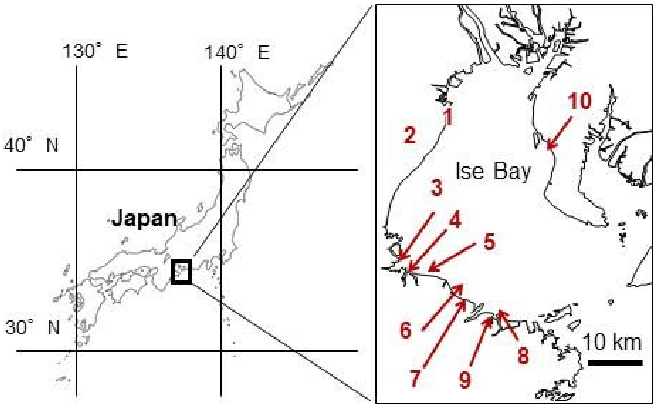

Figure 2. Study sites in Ise Bay, Japan. The ID 1 on the map represents the Manila clam, Ruditapes philippinarum, habitat in Kusu, Yokkaichi (2: Shiroko, Suzuka, 3: Miwatari, Matsusaka, 4: Kushida, Matsusaka, 5: Higashikurobe, Matsusaka, 6: Offshore-Muramatsu, Ise, 7: Intertidal-Muramatsu, Ise, 8: Kawaguchi flat, Ise, 9: Isshikimae flat, Ise, 10: Kosugaya, Tokoname).

Table 1. Clam habitat environmental parameters in this study.

Habitat ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Land elevation	inter	sub	sub	sub	sub	sub	inter	sub	inter	inter
River input	none	River mouth	River mouth	River mouth	none	none	none	River mouth	River mouth	none
Chl. *a* µg L^{-1}	12.8	6.9	7.6	4.9	6.1	6.8	6.8	4.0	3.2	8.7
Openness of the shore	150°	0°	50°	0°	150°	210°	210°	0°	10°	180°

In Japanese coastal areas, Manila clams can be found in various habitats. In Ise Bay, the clams are distributed in habitats ranging from intertidal to subtidal, exceeding 10 m depth, and from river mouth to non-river offshore areas (Hanyu et al. 2017). River mouth areas have large salinity fluctuations, while non-river areas typically have stable and high salinity water, ranging from closed and calm areas to more open areas under the influence of strong wave action, and from poor to good food availability. Therefore, we need to evaluate the reproductive capacity of the clam under a wide variety of environmental conditions.

MATERIALS AND METHODS

Study area and environmental characteristics of habitat

Condition index and the fecundity was assessed in Manila clams collected from 10 locations in Ise Bay, central Japan, in 2015. The environmental characteristics of the studied clam habitats are shown in Table 1. Here we focused on the land elevation, river water input, chlorophyll *a* level as an index of food abundance, and openness of the shore to offshore water. Chlorophyll fluorescence at around 1-m depth from the sea surface was measured by a CTD (Rinko profiler, JFE Advantech Co. Ltd., or DS5, Hydrolab Co. Ltd.) and chlorophyll *a* concentration was estimated from these at each clam collection site. The mean chlorophyll *a* concentration in 2015 (n = 4 to 20) was used as the chlorophyll *a* level at each habitat. The openness was expressed as the angle between two lines connecting the habitat and two points on the coastline 1 km away from the habitat (i.e. a modified method of that described by Ruuskanen et al. 1999).

Measurement of condition index and fecundity of the clams

Manila clams were collected approximately monthly for monitoring of nutritional condition and fecundity from 10 habitats in Ise Bay between April and November 2015. Approximately 10–30 clams with a shell length (SL) of 16–51 mm (mean 31.2 mm) were haphazardly collected. After measuring the shell length, shell height (SH), shell width (SW), and the total wet weight, the clams were dissected, and soft body tissue wet weight was measured. The soft body tissue of female clams (identified by the presence of oocyte in cut out tissue) was stored at -80°C for fecundity assessment. Condition index (CI) of the clams was calculated as follows;

$$CI = \frac{Soft\ tissue\ weight\ (g\ WW)}{SL\ (mm)\ x\ SH\ (mm) x\ SW\ (mm)}\ x\ 10^5$$

The number of eggs in the whole soft body tissue of individual clams was determined using an indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies specific to Manila clam yolk protein following Park and Choi (2004). Frozen whole tissue was freeze dried and homogenized using a sonicator in 10–15 ml buffer (20 mM Tris-HCl, 150 mM NaCl, pH7.5) containing a protease inhibitor cocktail (#04080-11 for General use; Nacalai Tesque Inc., Kyoto, Japan). The homogenate supernatant was separated by centrifugation (15,000×g for 20 min at 4°C) and stored at -80°C until analysis. The supernatants were diluted 10-100 times with 0.5% BSA-PBS buffer before ELISA. A 100 µl sample was added to a 96-well polystyrene microplate and incubated at 4°C overnight. After incubation, the plate was washed twice with PBS containing 0.05% detergent (Tween20; Promega Co. Ltd., Madison, WI, USA); 250 µl of 1% BSA and 5% skim milk in SBB was added as a blocking agent. After incubation at room temperature for 1 hour and washing 5 times, the monoclonal antibody (Hamaguchi and Usuki, 2006) was added to 100-µl aliquots in each well and incubated at room temperature for 2 hours. After washing 5 times, a 100-µl aliquot of polyclonal goat anti-mouse immunoglobulins peroxidase conjugate (diluted 1: 4000) was added to each well. Following incubation at room temperature for 2 hours, 100 µl of substrate- chromogen containing 3,3′,5,5′-tetramethyl-benzidine (TMB+; Dako Co. Ltd., Glostrup, Denmark) was added as a color agent. Finally, 50 µl of 2-N sulfuric acid was added as the stop solution. The optical density of the end product was measured at 450 nm using a microplate reader. The tissue homogenate supernatant of the clams and the yolk protein content per egg obtained by Hasegawa et al. (2014) was used for calculation of the number of eggs. Clam fecundity was represented as the number of eggs per unit of soft body weight (1 g WW).

In south-western parts of Japan including Ise Bay, the clam has two spawning seasons: spring and autumn, and the clam spawns repeatedly during each spawning season (Matsumoto et al. 2014). Therefore, as the representative fecundity in each spawning season, we summed the egg number that was normalized to the size of the clam, 30 mm SL (for removing the effect of size), for three months in each spawning season. However, the histological observation of the gonadal development showed that small numbers of Manila clam in Ise Bay were sexually undifferentiated even during the spawning seasons (Matsumoto et al. 2014). Therefore, the sum of the egg number might be overestimated in our study.

Analysis of the relationships between Manila clam and habitat

To relate the condition index to habitat parameters, and fecundity to habitat parameters, correspondence analysis was performed using the computing environment R (R Development Core Team, 2017). Habitat parameters were summarized in two-dimensional graphical form (1st and 2nd axis). Correlation analysis was conducted between condition index or fecundity and habitat scores of these two axes in each spawning season. As the representative fecundity in each of the two spawning seasons, the egg number was summed and normalized to 30 mm SL for three months. The mean value of condition index for three months in each spawning season was used as the representative nutritional condition of the clam.

RESULTS AND DISCUSSION

Fecundity and condition index of the clam had seasonal and spatial variations (Fig. 3). Condition index and fecundity were higher in spring and autumn, coinciding with the spawning season as reported by Matsumoto et al. (2014). However, there were large variations in each month. These variations imply that there are spatial variations in condition index and fecundity among the habitats, although the variation of fecundity among individuals was also large. Correspondence analysis revealed that canonical correlations of the 1st and 2nd axis were 0.48 and 0.31, respectively, and the cumulative contribution ratio up to the 2nd axis was over 78%. The habitat could be summarized by these two axes. By summarizing the set of habitat data in a two- dimensional graphical plot, the 10 habitats were separated into 4 groups (Fig 4). Habitats in non-river areas and open shore with strong wave action had positive mean scores of the 1st axis (right side) and food availability is considered to be higher than that in the river mouth and closed habitats. Moreover, the river mouth and intertidal habitats had a negative score of the 2nd axis (below part) and the variation of environmental conditions, such as salinity and temperature, are considered to be larger than those in subtidal and non-river habitats. Therefore, it is considered that the 1st axis is related to food availability and the 2nd axis is related to the magnitude of environmental stress.

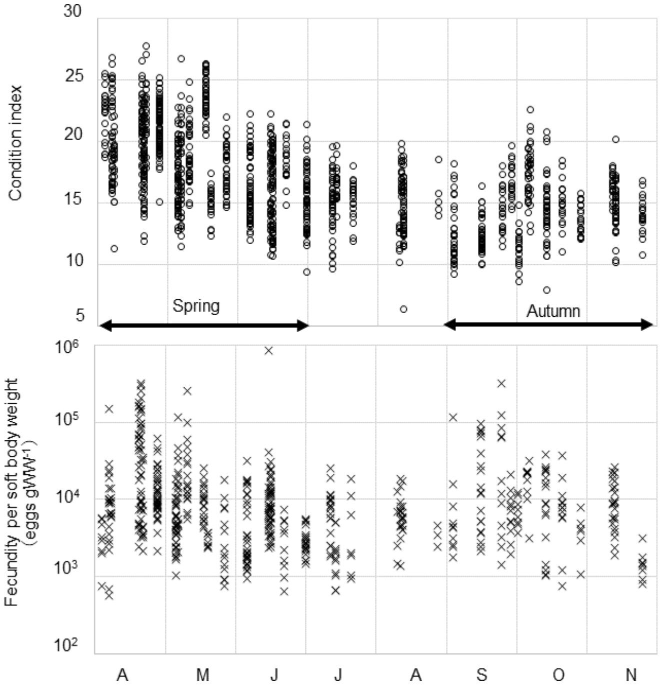

Figure 3. Seasonal variations in condition index (a) and fecundity (b) of Manila clam, Ruditapes philippinarum, during 2015 in Ise Bay, Japan.

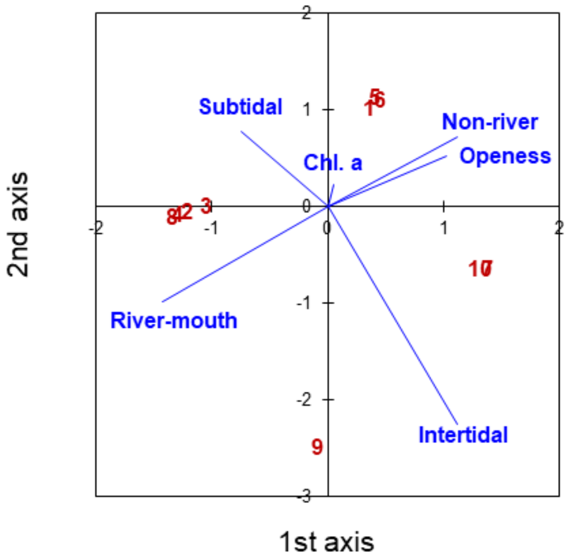

Figure 4. Two-dimensional graphical plot t of Manila clam, Ruditapes philippinarum, habitats and habitat parameters in Ise Bay, Japan by correspondence analysis. The ID 1 on plot represents the Manila clam habitat in Kusu, Yokkaichi (2: Shiroko, Suzuka, 3: Miwatari, Matsusaka, 4: Kushida, Matsusaka, 5: Higashikurobe, Matsusaka, 6: Offshore-Muramatsu, Ise, 7: Intertidal-Muramatsu, Ise, 8: Kawaguchi flat, Ise, 9: Isshikimae flat, Ise, 10: Kosugaya, Tokoname).

Relationships between habitat scores of the 1st and 2nd axis and condition index or fecundity in two spawning seasons were different between the condition index and fecundity and between spawning seasons (Fig. 5). In both spring and autumn spawning seasons, the average condition index of the 10 habitats was positively correlated with the habitat scores of the 1st axis (*r* = +0.48 in spring and +0.79 in autumn) and the 2nd axis (*r* = +0.80 in spring and +0.24 in autumn). On the other hand, fecundity during the two spawning seasons negatively correlated with the habitat score of the 2nd axis (*r* =-0.64 in spring and -0.80 in autumn). There was only a limited relationship between fecundity and habitat scores of the 2nd axis (*r* =

+0.07 in spring and +0.11 in autumn). Different relationships between condition index or fecundity to habitat scores show that the nutritional condition as determined by the condition index is not the sole factor affecting the fecundity of the clam, contrary to the results in previous studies (Hasegawa et al. 2018).

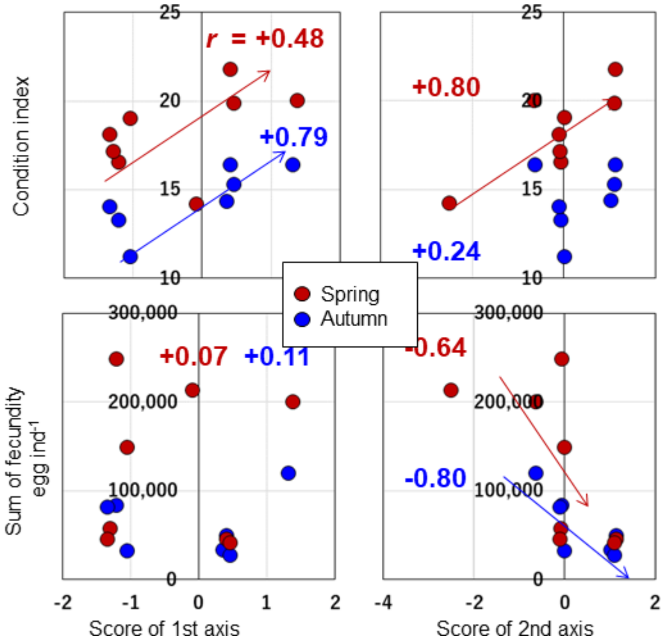

Figure 5. Relationships between condition index or fecundity of Manila clam, Ruditapes philippinarum, and the score of 1st or 2nd axis of correspondence analysis.

Moreover, if the 1st and the 2nd axes are related to food availability and magnitude of environmental stress, respectively, condition index of the clam, which is positively correlated with the 1st axis, is considered to reflect the food availability. These relationships may be intuitively acceptable. Otherwise, fecundity, which is negatively correlated with the 2nd axis, is considered to reflect the magnitude of environmental stress. The fecundity was higher in the intertidal zones and river mouth areas than in subtidal and non-river (offshore) areas. This trend may be explained by clams exposed to stressful and hazardous habitats exerting more energy to reproduction than those under mild and reliable environmental conditions.

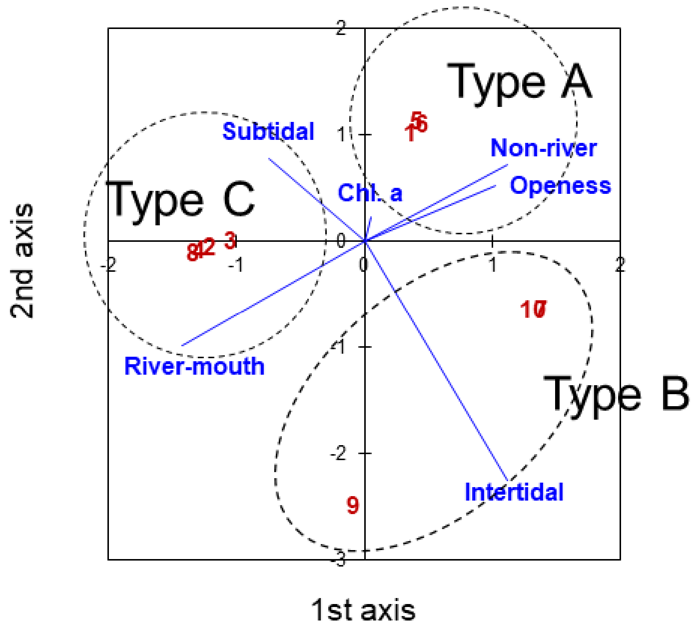

Figure 6. Habitat types of Manila clam, Ruditapes philippinarum, on two-dimensional graphical plot by correspondence analysis.

In conclusion, the nutritional condition as determined by the condition index is not the sole factor affecting the fecundity of the clam. The condition index was higher in the subtidal zones and open non-river mouth areas, where food availability was good. On the other hand, the fecundity was high in the intertidal and enclosed river mouth areas, which are stressful and life-threatening to the clams. These findings on clam reproductive traits may be useful in improving the efficiency of stock enhancement programs by maximizing the reproductive potential of the clam populations. For example, a habitat categorized as type A in Fig. 6 can be considered as a useful fishing ground in which the clam nutritional condition is good therefore, survival rate and growth are also good but the fecundity per body weight is not high. In habitat type A, enforcement of catch size regulation may improve the reproductive contribution through increasing the number of large clams. Habitat type B has ideal conditions for the clam to thrive because both nutritional condition and fecundity are high. Creation of this type of habitat may be helpful for sustainable fishing of the clam. The clam in habitat type C has a short life with high contribution to reproduction because fecundity per unit body weight is high. Improvement of food availability in this type of habitat may increase the reproductive contribution of the clam.

ACKNOWLEDGEMENTS

We are grateful to C. Hiro and K. Seko for assistance with analysis. Also, the members of Kaiyo Engineering Co., Ltd, Diving Techno Co., Ltd and Fisheries Cooperative Association around Ise Bay for assistance with field research. This study was supported by the Ministry of Agriculture, Forest, and Fisheries and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Toba, M. and Y. Miyama. 1994. Relationship of size to gonadal maturation and spawning in artificially conditioned Manila clams. Nippon Suisan Gakkaishi, 60: 173-178.

In this study, the authors observed sexual maturation and measured the number of eggs released per spawned female subjected to thermal spawning stimuli in breeding experiments for evaluating the size dependent batch fecundity of asari clam. Smaller clams at 0 yr old (groups A and B) produced as many eggs per soft tissue weight as larger clams at ≥ 1 yr (groups D and E). No significant differences in egg diameter, percent fertilization, and larval survival from straight-hinge to pediveliger stage were observed among the clam size groups.

Kakino, J. 1996. Growth and survival of Japanese littleneck clam *Ruditapes philippinarum* in cage test related to the sharpness index. Nippon Suisan Gakkaishi, 62: 376-383.

The author evaluated the variation in asari clam growth in a tidal flat in Tokyo Bay, Japan, by using the “sharpness index” defined as width / length of shell. The author concluded that juvenile clams offshore grew faster than those that were shoreside, but the juvenile survival rate in the intermediate zone of the flat was higher than those in the offshore and shoreside areas. Juveniles having a high sharpness index tended to show a high survival rate. At the same age, the juveniles with a high sharpness index grew faster than or equal to those with a low sharpness index

Ocean Acidification Experiment in Seagrass-Oyster Ecosystems Using a Novel System to Conduct In-Situ CO_2 Enrichment

Masakazu Hori[1*], Tomohiro Kuwae[2], and Masahiro Nakaoka[3]

[1] National Research Institute of Fisheries and Environment of Inland Sea, Japan Fisheries Research and Education Agency, Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan
[2] Port and Airport Research Institute, National Institute of Maritime, Port and Aviation Technology, Nagase, Yokosuka 239-0826, Japan
[3]Akkeshi marine station, Field Science center for Northern Biosphere, Hokkaido University, Aikkapu, Akkeshi, Hokkaido 088-1113, Japan

Corresponding author: mhori@affrc.go.jp

Keywords: Oyster aquaculture, *Zostera marina*, Ocean acidification, Free ocean CO_2 enrichment, Non-environmental risk deterrents

ABSTRACT

Coastal ecosystems are under the influence of global environmental changes that threaten their ecological status and the sustainability of seafood production. Seagrass beds, which are widely distributed along coastlines around the world, are highly important ecosystems, offering various types of ecosystem services to human beings. Seagrass beds are important for environmental change mitigation and adaptation measures, via carbon storage, acidification mitigation, water quality improvement, as well as providing a nursery for coastal marine organisms. Therefore, seagrass beds are essential for harmonization of coastal fisheries and aquaculture with environmental conservation, resulting in the sustainable use of ecosystem services. Here we developed a novel system of in-situ manipulation of CO_2 and nutrient enrichment to demonstrate the function of seagrass in mitigating environmental changes. The system is called the Free-Ocean Real-Time Experimental System (FORTES). In addition, we developed non-environmental risk deterrents for invertebrates made from natural algae, specializing in arthropod and molluscan species (PCT/JP2018/038204). Two types of deterrents were made: liquid and dry-powdered chemicals. The liquid deterrent can be manipulated by FORTES. For instance, the deterrent against the arthropod species prevents settlement and recruitment of sessile crustaceans such as barnacles at low concentrations and also removes shrimps and amphipods from the target area at higher concentrations. Since the algal species used as a deterrent is also taken as an oral herbal medicine in Asian countries, the deterrent is safe for humans.

Recently, there has been a focus on the interaction between oyster aquaculture and seagrass vegetation as a common practice to achieve better coastal management with the framework of the sustainable development goals (SDGs) in a changing environment. Oysters have been grown using the original oyster–seagrass interactions, including facilitation of spat survival rate, and especially trophic support from eelgrass beds to oysters over an extended period of time. This is typically regarded as the indigenous and local knowledge (ILK) of Japanese oyster aquaculture. Using the FORTES, we can demonstrate not only the direct effects of environmental changes such as pCO_2 increase/pH decrease, eutrophication and agrochemical inputs on oysters, but also the mitigative effects of seagrass beds, which benefit oyster aquaculture.

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Campbell, J.E. and J.W. Fourqurean. 2013. Effects of in situ CO_2 enrichment on the structural and chemical characteristics of the seagrass *Thalassia testudinum*. Mar. Biol. 160: 1465-1475.

Seagrasses commonly display carbon-limited photosynthetic rates. Thus, increases in atmospheric pCO_2, and consequentially oceanic $CO_2(aq)$ concentrations, may prove beneficial. While addressed in mesocosms, these hypotheses have not been tested in the field with manipulative experimentation. This study examines the effects of in situ $CO_2(aq)$ enrichment on the structural and chemical characteristics of the tropical seagrass, *Thalassia testudinum*. $CO_2(aq)$ availability was manipulated for six months in clear, open-top chambers within a shallow seagrass meadow in the Florida Keys (USA), reproducing the forecasted CO_2 environment for the year 2100. Structural characteristics (leaf area, leaf growth, shoot mass, and shoot density) were unresponsive to $CO_2(aq)$ enrichment. However, leaf nitrogen and phosphorus content declined on average by 11 and 21%, respectively. Belowground, non-structural carbohydrates increased by 29%. These results indicate that increased $CO_2(aq)$ availability may primarily alter the chemical composition of seagrasses, influencing both the nutrient status and resilience of these systems.

Gattuso, J.P., W. Kirkwood, J.P. Barry, E. Cox, F. Gazeau, L. Hansson, I. Hendriks, D.I. Kline, P. Mahacek, S. Martin, P. McElhany, E.T. Peltzer, J. Reeve, D. Roberts, V. Saderne, K. Tait, S. Widdicombe, and P.G. Brewer. 2014. Free-ocean CO_2 enrichment (FOCE) systems: present status and future developments. Biogeosciences 11: 4057-4075.

Free-ocean CO_2 enrichment (FOCE) systems are designed to assess the impact of ocean acidification on biological communities in situ for extended periods of time (weeks to months). They overcome some of the drawbacks of laboratory experiments and field observations by enabling (1) precise control of CO_2 enrichment by monitoring pH as an offset of ambient pH, (2) consideration of indirect effects such as those mediated through interspecific relationships and food webs, and (3) relatively long experiments with intact communities. Bringing perturbation experiments from the laboratory to the field is, however, extremely challenging. The main goal of this paper is to provide guidelines on the general design, engineering, and sensor options required to conduct FOCE experiments. Another goal is to introduce xFOCE, a community-led initiative to promote awareness, provide resources for in situ perturbation experiments, and build a user community. Present and existing FOCE systems are briefly described and examples of data collected presented. Future developments are also addressed as it is anticipated that the next generation of FOCE systems will include, in addition to pH, options for oxygen and/or temperature control. FOCE systems should become an important experimental approach for projecting the future response of marine ecosystems to environmental change.

Kline, D.I., L. Teneva, K. Schneider, T. Miard, A. Chai, M. Marker, K. Headley, B. Opdyke, M. Nash, M. Valetich, J.K. Caves, B.D. Russell, S.D. Connell, B.J. Kirkwood, P. Brewer, E. Peltzer, J. Silverman, K. Caldeira, R.B. Dunbar, J.R Koseff, S.G. Monismith, B.G. Mitchell, S. Dove, and O. Hoegh-Guldberg. 2012. A short-term in situ CO_2 enrichment experiment on Heron Island (GBR). Scientific Reports 2, Article number: 413.

Ocean acidification poses multiple challenges for coral reefs on molecular to ecological scales, yet previous experimental studies of the impact of projected CO_2 concentrations have mostly been done in aquarium systems with corals removed from their natural ecosystem and placed under artificial light and seawater conditions. The Coral–Proto Free Ocean Carbon Enrichment System (CP-

FOCE) uses a network of sensors to monitor conditions within each flume and maintain experimental pH as an offset from environmental pH using feedback control on the injection of low pH seawater. Carbonate chemistry conditions maintained in the −0.06 and −0.22 pH offset treatments were significantly different than environmental conditions. The results from this short-term experiment suggest that the CP-FOCE is an important new experimental system to study in situ impacts of ocean acidification on coral reef ecosystems.

Da Gama, B.A.P., R.C. Pereira, A.G.V. Carvalho, R. Coutinho, and Y. Yoneshigue-Valentin. 2002. The effects of seaweed secondary metabolites on biofouling. Biofouling 18: 13- 20.

Antifouling activity is one poorly investigated property of seaweed natural products. To determine whether seaweeds contain chemicals able to influence the settlement of fouling organisms in the field, crude organic extracts from *Stypopodium zonale*, *Dictyota menstrualis* (Phaeophyceae) and *Laurencia obtusa* (Rhodophyceae) were incorporated at natural volumetric concentrations, into hard stable gels that served as substrata for fouling in the experiments. Fouling organisms settled at a significantly higher rate on plates treated with *S. zonale* extracts than on control gels, while settlement was strongly inhibited on gels containing *K. obtusa* extracts. Fouling on gels treated with the *D. menstrualis* extract was not significantly different from the fouling found on control gels. The findings suggest that the broad antifouling properties of the crude extract of *L. obtusa* inhibit the settlement of fouling as well as hinder the development of settled fouling species, thereby reducing the richness of species. The results imply that *L. obtusa* possibly harbors powerful agents that can be explored for the development of antifouling technology.

Qian, P., Y. Xu, Y. Li, and N. Fusetani. 2015. Mini-review: marine natural products and their synthetic analogs as antifouling compounds: 2009–2014. Biofouling 31: 101-122.

Since early 2008, an increasing number of countries have ratified an international treaty to ban the application of antifouling (AF) coatings based on organotin compounds (e.g. tributyltin (TBT) and triphenyltin). As a result, the demand for environmentally-friendly, non-toxic or low-toxic AF compounds and technologies (green AF agents) has become an urgent reality. Marine coatings based on Cu_2O and various other biocides have a negative impact on the environment, and they must eventually be replaced by new, effective, and environmentally-friendly AF compounds. This mini-review describes important AF compounds discovered from a variety of organisms from 2004 until mid-2009, and discusses recent and general trends in the discovery of AF compounds. Finally, a perspective on the future of AF compound development is presented. The discussion is aimed at updating scientists and engineers on the current challenges facing AF research.

Stark, J.S., E.T. Peltzer, D.I. Kline, A.M. Queiros, T.E. Cox, K. Headley, J. Barry, F. Gazeau, J.W. Runcie, S. Widdicombe, M. Milnes, N.P. Roden, J. Nlack, S. Whiteside, G. Johnstone, J. Ingels, E. Shaw, L. Bodrossy, J.D. Gaitan-Espitia, W. Kirkwood, and J. Gattuso. 2019. Free Ocean CO_2 Enrichment (FOCE) experiments: scientific and technical recommendations for future in situ ocean acidification projects. Progress in Oceanography 176: 89-107.

Free Ocean CO_2 Enrichment (FOCE) experiments are a relatively recent development in ocean acidification research, designed to address the need for in situ, long-term, community-level experiments. FOCE studies have been conducted across different marine benthic habitats and regions, from Antarctica to the tropics. Based on this previous research we have formed some core operating principles that will aid those embarking on future FOCE experiments. FOCE studies have potential to provide important insight into the effects of ocean acidification that can add to or refine conclusions drawn from laboratory or single species studies, because they are conducted in situ on intact assemblages. Scaling up from sub-organismal and individual effects to also include indirect impacts on the ecosystem and ecosystem services, make FOCE experiments essential to filling in current knowledge gaps in the understanding of ocean acidification. While FOCE systems are complex, relatively costly, and somewhat difficult to operate, the challenges they pose are tractable and they have proven to be a useful approach in ocean acidification research. The aim of this paper is to draw from the experiences of past FOCE experiments and provide practical advice for designing, building and operating a FOCE experiment. Some of the most important recommendations include: field testing the system design; having a backup power supply; using replicate treatment enclosures; monitoring and maintaining the chemistry appropriately; allowing sufficient time to achieve near CO_2 equilibrium conditions; and having a scientific focus with a core set of hypotheses. Future FOCE experiments could focus on longer durations, multiple factors, and testing more intact benthic marine communities and ecosystems. The authors hope this paper will enable further FOCE deployments and experiments, as well as provide some guidelines to improve future FOCE studies and advance ocean acidification research.

Effects of Ocean Acidification on the Early Developmental Stages of the Commercially Important Gastropods, Ezo Abalone and Horned Turban, in Japan

Ryo Kimura[1*], Hideki Takami[2], Toshihiro Onitsuka[3], Tsuneo Ono[4], and Yukihiro Nojiri[5]

[1] Japan Fisheries Research and Education Agency (FRA), 15F Queen's Tower B, 2-3-3 Minatomirai, Nishi-ku, Yokohama, Kanagawa, 220-6115, JAPAN [2] Tohoku National Fisheries Research Institute, FRA, Shiogama, 985-0001 JAPAN

[3] Hokkaido National Fisheries Research Institute, FRA, Kushiro, 085-0802 JAPAN

[4] National Research Institute of Far Seas Fisheries, FRA, Yokohama, 236-8648 JAPAN

[5] Hirosaki University, Hirosaki, 036-8560 JAPAN

Corresponding author: rkim@affrc.go.jp

Keywords: Atlantic salmon, eastern oyster, breeding program, disease resistance

ABSTRACT

Commercially valuable shellfish species will be affected more seriously by ocean acidification (OA) in conjunction with overfishing. We investigated the effects of OA on fertilization, larval development, metamorphosis, and post-larval growth of valuable gastropods; Ezo abalone *Haliotis discus hannai* and turban shell *Turbo cornutus*, using a CO_2 manipulation system (AICAL pCO_2 control system, Kimoto Electric Co., Ltd, Japan). There was no effect of exposure to <1000 µatm pCO_2 seawater observed in fertilization, malformation, or mortality rates in either gastropod. However, fertilization and hatching rates decreased with exposure to >1500 µatm pCO_2 seawater and the malformation rates at >1000 µatm increased significantly in Ezo abalone. The diameter of larval shells in >800 µatm seawater was significantly smaller compared to those in control seawater (400-450 µatm pCO_2) for both species. Scanning electron microscope images of larval shells of Ezo abalone from >1000 µatm showed abnormal development, suggesting problems with shell deposition and/or increased shell dissolution. The composition of developmental stages of embryos in horned turban differed between the experimental levels of pCO_2 seawater. These results indicate that pCO_2 >1000 µatm reduced developmental performance of these gastropods in early life stages.

The natural habitat of these gastropods is kelp beds where photosynthesis and respiration by macroalgae lead to marked pCO_2 diel fluctuations. These fluctuations are larger than the pCO_2 change projected to be caused by OA for open ocean waters by 2100. We also conducted laboratory investigations on the effects of ocean acidification, in relation to diel cycles of seawater pCO_2 levels projected for the near and distant future, on the early development of *Haliotis discus hannai*. Shell length, abnormality and mortality of larval abalone were examined under different pCO_2 levels with mimicked diel cycle using the AICAL pCO_2 control system. Adult abalone, with fully matured gonads, were induced to spawn and gametes were artificially fertilized in concentrations of ca. 400 (atmosphere-balanced, control treatment), 800 or 1200 pCO_2 µatm. Sixteen hours post-fertilization, hatched larvae were exposed to running seawater adjusted to a constant level [ca. 400, 800 or 1200 µatm pCO_2] or fluctuating on a diel cycle [800±400 (400-1200) or 1200±400 (800-1600) µatm pCO_2] pCO_2 in 4-day experimental periods. There were no significant differences in the mortality rate, abnormality rate and shell length of larvae among the 400, 800, and 400-1200 µatm pCO_2 treatments.

In contrast, the abnormality rate was significantly higher in the 1200 and 800-1600 µatm pCO_2 treatments than in the control treatment. Larval shell length in the 800-1600 µatm pCO_2 treatment was significantly smaller than in the other pCO_2 treatments. The exposed duration time exceeding the aragonite saturation state was a critical factor determining the negative impacts on the larval development of abalone.

INTRODUCTION

Anthropogenic elevation of the partial pressure of CO_2 (pCO_2) in seawater has resulted in a lowering of seawater pH (= ocean acidification) and most calcifying organisms investigated showed reduced calcification in response to increased pCO_2 (Fabry et al., 2008). Kurihara (2008) reviewed ocean acidification (OA) effects on the early developmental stages of marine invertebrates including bivalves (oysters and mussels); however, no reports on larval gastropods were included. In order to clarify impacts of OA on fishery-important marine gastropods, we investigated the OA effects on the early development of Ezo abalone and the horned turban by constant elevated pCO_2 levels. We also investigated the effects of diel fluctuating pCO_2 levels on the survival and larval shell formation of Ezo abalone.

METHODS

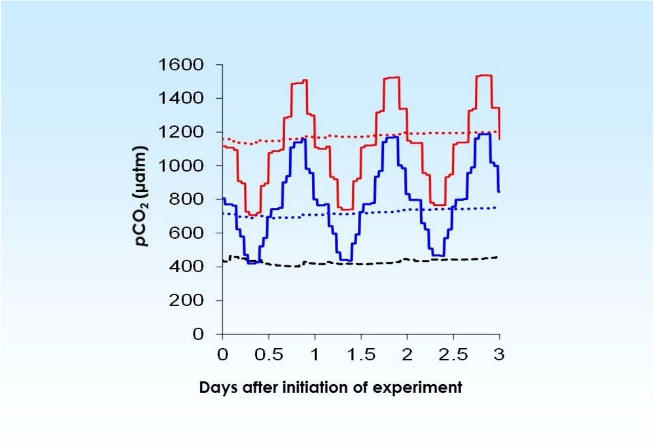

Figure 1. Changes in partial pressure of CO_2 in experimental seawater during the diel cycle treatment experiment conducted 21- 24 December 2010 in the Shiogama laboratory. Lines represent the values monitored by the AICAL system every hour. Dotted lines represent values in the constant treatment (1200, 800, 450 (atmosphere balanced) µatm) and line represents those in the diel cycle treatment (400-1200, 800-1600 µatm), respectively.

We used the AICAL pCO_2 control system, described in Kimura et al. (2011), for making controlled seawater of specific pCO_2 levels. The system showed stable and good separation of pCO_2 between tested groups through the gas monitoring and water sample (Kimura et al., 2011). We investigated fertilization rate, hatching rate, malformed rate, developmental stage retardation, and maximum larval shell length. These methods for rearing and testing were described in Kimura et al. (2011) for Ezo abalone and in Onitsuka et al. (2014) for horned turban. In the experiment testing diel-fluctuated pCO_2 level on Ezo abalone, we set the pCO_2 levels as follows: 450 (atmosphere balanced), 800, 800±400, 1200, 1200±400 µatm (Fig. 1).

RESULTS

There was no effect of exposure to <1000 µatm pCO_2 seawater observed in fertilization, malformation, or mortality rates in either species. The fertilization rate and hatching rates decreased with exposure to >1500 µatm pCO_2 seawater in Ezo abalone. The malformation rates at >1000 µatm increased significantly compared to those in control seawater in Ezo abalone. The maximum larval shell length of larvae from >800 µatm seawater treatments were significantly smaller compared to those in control seawater in both species. Scanning Electron Microscope (SEM) images of larval shells of Ezo abalone in >1000 µatm seawater showed abnormal development with shell deposition and/or increased shell dissolution. The proportions of embryos in horned turban that attained the 4-cell stage and 8-cell stage were significantly lower at the higher pCO_2 seawater treatments than those at the control levels 2 h after fertilization. Similar trends were observed in the ratio of veligers at 24-26 h after fertilization. These results are described in Kimura et al. (2011) for abalone, and Onitsuka et al. (2014) for horned turban, respectively.

In the diel fluctuation of pCO_2 level experiment, significantly higher malformation rates and smaller maximum shell lengths were detected in the 1200 and 1200+-400 µatm pCO_2 treatments than in the 450 µatm pCO_2 treatments. These negative impacts were greater in the 1200+-400 µatm than in the 1200 µatm. To test cumulative effects of elevated pCO_2, the index "Integral pCO_2 over 1100 µatm" was used because the aragonite saturation state of seawater around the omega value of 1.0 was equivalent to 1100 µatm pCO_2. The index was defined by the following formula:
Integral pCO_2 over 1100 µatm =Σ(P-1100)i P: pCO_2 over 1100 µatmi: exposed hours to pCO_2 over 1100 µatm

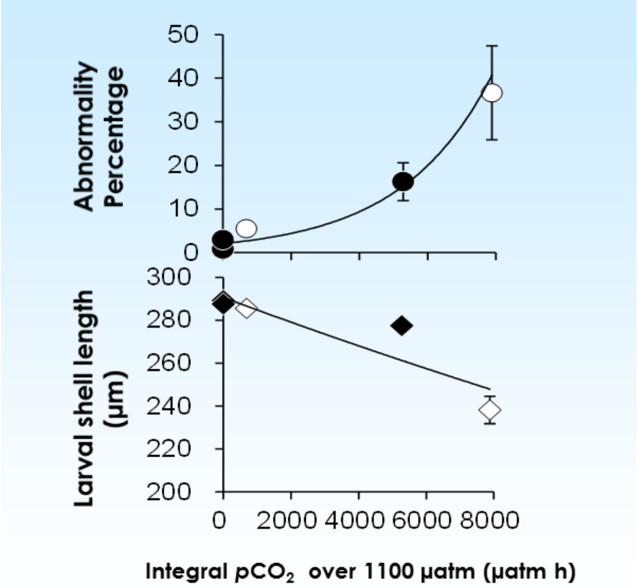

Figure 2. Relationship between larval malformation rates (upper), and shell length (lower) of Haliotis discus hannai 72 h after initiating the cycle treatment experiment and the integral pCO2 over 1100 µatm. White- and black-colored symbols represent the results of the treatments with a constant and cyclically fluctuating partial pressure of CO2, respectively. Each bar represents standard error.

The abnormality rate increased with the increment of "Integral pCO_2 over 1100 µatm". Furthermore, the larval shell length decreased as the index value increased (Fig. 2). (The results of the diel fluctuation of pCO_2 experiments were described in Onitsuka et al., 2018 and were included with another experiment's results).

DISCUSSION

In these experiments, our results show that pCO_2 >1000 µatm reduced developmental performance of these gastropods in early life stages (Kimura et al. 2011; Onitsuka et al. 2014). The mortality / abnormality rate, and larval shell length were significantly different between the 1200 and 1200±400 µatm pCO_2 treatments, indicating pCO_2 diel fluctuation can produce greater negative impacts on the larval life of abalone. The exposed duration time exceeding the aragonite saturation state was a critical factor determining the negative impacts on the larval development of abalone (Onitsuka et al. 2018: In the paper, the authors introduced a new index "ECPC", the effective cumulative value of seawater pCO_2 over a specific criterion value, which was calculated using the threshold pCO_2 level defined as the pCO_2 value corresponding to 1.1 of the aragonite saturation state in seawater).

Since mitigation measures against ocean acidification are limited to reducing CO_2 emissions, small scale pCO_2 monitoring in coastal areas is thought to be important to properly assess OA effects on marine invertebrates.

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Kimura R., H. Takami, T. Ono, T. Onitsuka, and Y. Nojiri. 2011. Effects of elevated pCO_2 on the early development of the commercially important gastropod, Ezo abalone, *Haliotis discus hannai*. Fish. Oceanogr. Vol 20: 357-366.

This is one of the first papers that investigated the effect of ocean acidification on commercially important gastropod species. This is also a very early paper using an AICAL system that precisely controls the pCO_2 concentration during the experiment.

Kurihara H. 2008. Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. Mar. Ecol. Prog. Ser. Vol 373: 275-284.

This review paper describes the effects of ocean acidification on the early development of many marine invertebrates including bivalves (oyster and mussel). Levels of carbon dioxide that affect the development of marine invertebrates were reviewed.

Onitsuka, T., H. Takami, D. Muraoka, Y. Matsumoto, A. Nakatsubo, R. Kimura, T. Ono, and Y. Nojiri. 2018. Effects of ocean acidification with pCO_2 diurnal fluctuations on survival and larval shell formation of Ezo abalone, *Haliotis discus hannai.* Mar. Env. Res. Vol 134: 28-36.

This paper assessed the effects of constant and diurnally fluctuating pCO_2 (mimicking the rhythm of the ocean) on development and shell formation of larval abalone using the AICAL system, which can precisely control pCO_2. It is suggested that the effects of ocean acidification on development and shell formation of larval abalone can be determined by intensity and time of exposure to pCO_2 over the threshold associated with aragonite saturation state (Ω-aragonite).

Chemical Changes in the Environment: What Does This Mean for Shellfish?

Shannon L. Meseck*, Mark Dixon, Yaqin Li, George Sennefelder, Dylan Redman, Renee Mercaldo-Allen, Paul Clark, Lisa Milke, and Gary H. Wikfors

National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Ave., Milford, CT 06460

Corresponding author: Shannon.Meseck@noaa.gov

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ABSTRACT

Coastal areas, estuaries, and river deltas are experiencing an increase in carbon dioxide resulting from human activities. Approximately 30% of atmospheric carbon dioxide (CO_2) dissolves in the oceans. As this CO_2 dissolves, it reacts with water and produces carbonic acid (H_2CO_3), which dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and hydrogen ions (H^+). With increased CO_2 in the water, there are multiple ways that shellfish may be affected: (1) food availability; (2) physiological responses, and (3) sediment acidification.

Food availability may be influenced by changes in phytoplankton community structure and nutritional value. For example, increased carbon dioxide may cause a shift in phytoplankton biomass and community composition depending upon species' efficiency of inorganic carbon acquisition. Shifts in phytoplankton communities may favor phytoplankton with more- or less- nutritional content for support of human food production.

In addition to potential changes in food supply, coastal acidification may be causing physiological changes in marine bivalves that affect how they feed. Bivalve gills are constantly exposed to ambient water, with the gill cilia moving water through the shell for respiration and feeding. Under environmental stress, physiological changes to the heart, gills, gonads, and general metabolism and regulation may occur. Physiological changes could result in slower growth, higher mortality, or inability to grow. The influence of ocean acidification on bivalve physiological functions is important to understand so that aquaculture may be modified to respond to a changing environment.

Finally, the same reaction that occurs in water during ocean acidification occurs at the sediment-water interface, where bivalves settle, following bacterial decomposition of settling organic matter. As particles settle, aerobic and anaerobic reactions, combined with redox processes, result in the production of CO_2 in sediment porewater. Corrosive sediment can impede successful settlement, recruitment, and abundance in bivalve populations. Nutrient-enriched coastal areas in the United States are already experiencing sediment acidification, and this might be limiting bivalve recruitment at the sediment-water interface. The synthesis presented here will focus on the myriad of ways that ocean acidification may affect marine bivalves.

INTRODUCTION

Increased atmospheric carbon dioxide (CO_2) is being absorbed by the world's oceans and is thought to be a concern for some groups of marine organisms, especially those that contain structures composed of calcite or aragonite (Andersson et al., 2008; Dickinson et al., 2013; Gazeau et al., 2013).

In the ocean, CO_2 reacts with water to form carbonic acid (H_2CO_3), which dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and hydrogen ions (H^+), resulting in acidification. Currently average ocean pH is 8.1 and by 2100 ocean pH is predicted to drop to 7.7. (Caldeira and Wickett, 2003; Feely et al., 2004; Raven et al., 2005; Turley and Findlay, 2016).

Environmental variations (e.g., tidal, daily, and season changes) can influence metabolism, growth, and survival of marine shellfish (Norkko et al., 2006). Most bivalves can be classified as euryhaline organisms (Flügel, 2013) and live in areas which experience changes in environmental parameters that are natural (e.g., temperature) and/or a result of anthropogenic pollution (e.g., excess nutrients). Marine bivalves tolerate a range of environmental conditions; however anthropogenic pollution may affect bivalve energy balance such that a change in physiological processes (growth, reproduction) could result. Increasing our understanding of how organisms physiologically respond to specific changes in the environment will help in assessing how a community functions (Menge et al., 2002; Niemi et al., 2004).

In this study, we synthesize data from our ocean acidification experiments with phytoplankton growth and nutrition to examine physiological effects on bivalves exposed to ocean acidification, and discuss how sediment acidification may influence settlement.

Phytoplankton

The correct nutritional components of phytoplankton are a key component regulating physiological performance of marine bivalves. The assimilation of key nutrients from phytoplankton, especially polyunsaturated fatty acids (PUFA), is required for growth and can only be acquired through trophic transfer (Ezgeta- Balić et al., 2012; Milke et al., 2004). Fatty acids are critical for growth, survival, neural development, stress resistance, membrane fluidity, and enzyme activity with enhanced growth and reproductive rates when fatty acids, especially PUFA and carbon (C), nitrogen (N), and phosphorus (P), are optimized (Elser et al., 2003; Elser et al., 2000).

Ocean acidification effects on phytoplankton may include changes in cellular carbon quotas (Burkhardt et al., 2001; Burkhardt et al., 1999), changes in phytoplankton species composition (Riebesell et al., 2013), and nutritional quality to other trophic levels (Rossoll et al., 2012). To better understand what happens to marine phytoplankton following increased carbon dioxide, we conducted a series of seven experiments with temperate phytoplankton species, grown under pre-glacial pCO_2 conditions to predicted 2100 levels of ρCO_2. Table 1 summarizes the growth rates, C:P, N:P, and PUFA, MUFA, and SFA analysis from the seven species studied (King et al., 2015). This study found no significant difference in nutritional composition for any of these species, however some species experienced a change in growth rate. This research highlighted that although there were CO_2 driven changes in growth rate, it did not reflect changes in elemental composition or fatty acid composition. This research highlights that the complexing of ocean acidification on marine phytoplankton, will clearly depend on how the phytoplankton community changes. Understanding what drives community succession will help determine how changes in carbon dioxide might influence nutritional quality in the future.

Bivalve Physiology

Future ocean acidification conditions could result in decreased fertilization success, changes in growth rate, reduced calcification rates, and increased mortality (Melzner et al., 2009). Our current understanding of how shellfish physiology is affected by ocean acidification is incomplete, but there is a growing amount of research suggesting that bivalves may be highly sensitive to ocean acidification (Dickinson et al., 2013; McCorkle et al., 2015; Sugano et al., 2015; Widman Jr et al., 2012). Understanding what physiological traits are responsible for sensitivity to ocean acidification may provide useful information for optimizing aquaculture practices with regard to bivalves.

Preliminary research suggests that under ocean acidification, growth rates and survival of surfclams and sea scallops may decrease (McCorkle et al., 2015; Sugano et al., 2015; Widman Jr et al., 2012). Furthermore, shell deformities increased as carbon dioxide levels increased. Change in filtration rates is one possible mechanism for reduced growth. Preliminary short term experiments (two weeks) in the laboratory tested how ocean acidification affects the filtration rate of blue mussels, *Mytilus edulis*, and the eastern oyster, *Crassostrea virginica*, under current conditions and increased carbon dioxide. Feeding rates changed with increased carbon dioxide in both blue mussels and eastern oysters. Measurements of cilia beat function for blue mussels found that as carbon dioxide levels increased cilia beats decreased, suggesting that changes in feeding rates might be related to changes in cilia beat function (Meseck at al. 2020).

Dynamic energy budget (DEB) models may be a useful tool to understand how changes in physiological rates would impact marine bivalves under ocean acidification. By understanding the metabolic costs of ocean acidification, we can model how biovolume, development, and reproduction might change. Further research on how bivalve physiology is affected under ocean acidification is necessary to understand how aquaculture practices may be adapted to the changing environment.

Sediment acidification

Acceptance or rejection of habitat by bivalves as they settle from the water column to the benthos is not well understood, but research suggests that environmental, chemical, and physical variables at the sediment-water interface promote settlement and abundance of bivalves.

Recently, sediment porewater carbonate chemistry has been linked to larval settlement and survival in life stages of many marine bivalves (Clements and Hunt, 2014, 2017; Clements et al., 2016; Green et al., 2004; Green et al., 2013). Sediment acidification can occur at the sediment surface, as particulate material undergoes aerobic and anaerobic reactions, combined with redox processes, which produces CO_2. Once CO_2 is produced, it reacts with water to form carbonic acid (H_2CO_3), and dissociates into bicarbonate ions (HCO_3-), carbonate ions (CO_3^{2-}), and hydrogen ions (H+) resulting in sediment acidification (Green and Aller, 2001; Tang and Kristensen, 2007; Wenzhöfer and Glud, 2004). These corrosive sediments (aragonite, $\Omega < 1$) result in low pH conditions in the upper millimeters, where bivalves settle, and may reduce settlement, recruitment, and abundance in bivalve populations (Aller, 1982; Green and Aller, 2001; Zwarts and Wanink, 1989).

Over the last three decades, bottom water in Long Island Sound (LIS) has been documented to have low pH (acidified sediments) and to be undersaturated with respect to carbonate minerals

Table 1. Relative difference in specific growth rate, carbon: phosphorus ratio (C:P), nitrogen: phosphate ratio (N:P), PUFA, MUFA, and SFA for each of the seven phytoplankton species using a one way ANOVA P<0.05. The = sign means no change between present day levels and Year 2100 pCO2 levels. Adapted from data presented in King et al. (2015).

Species	Growth rate	C:P	N:P	PUFA	MUFA	SFA
Thalassiosira pseudonana	=	=	=	=	=	=
Thalassiosira rotula	Increase	Decrease	=	=	=	=
Thalassiosira weissflogii (CCMP2599)	=	=	=	=	=	=
Thalassiosira weissflogii (CCMP1010)	=	=	=	=	=	=
Thalassiosira oceanica	Decrease	=	=	=	=	=
Chlorella autotrophica	Increase	=	=	=	=	=
Dunaliella salina	=	=	=	=	=	=

(Aller, 1982; Green and Aller, 2001; Green et al., 1993). In Connecticut, the seafloor is leased to shellfish growers and they rely on natural reseeding of northern quahog *Mercenaria mercenaria* to occur. Understanding the influence that sediment acidification may have on natural settlement, recruitment, and abundance of commercial bivalves is essential for sustainable harvesting of shellfish. During 2013, we studied the relationships between abundance of bivalve molluscs and physical (grain size) and chemical variables (carbonate parameters) at the sediment-water interface on a leased shellfish bed during the bivalve settlement period when Ω in LIS tends to be low (Meseck et al., 2018).

Briefly, Meseck et al. (2018) found that throughout the season, grain size in combination with carbonate chemistry was influencing bivalve settlement. When the settlement season was narrowed down to two weeks to remove temporal effects, grain size was no longer a significant contributor, but carbonate chemistry still was. The contribution of carbonate chemistry parameters in predicting bivalve settlement explained 44% of the total bivalve benthic community, but the contributions of carbonate chemistry to settlement of individual species varied. For the two dominant species, carbonate chemistry explained 33% *of M. arenaria* and 71% of *Nucula* spp. abundance (Table 3). These findings were consistent with previous research in LIS which reported increased mortality of bivalves (*N. annulata* and *Tellina agilis*) in sediments that were undersaturated ($\Omega < 1$) with respect to carbonate (Green et al., 1993, 1998). Our study did not focus on determining whether mortality or dissolution was occurring because of undersaturated sediments, however mean Ω values in LIS were undersaturated during most of the sampling season. Results from the Meseck et al (2018) study suggest that increased pH and alkalinity may enhance bivalve community recruitment. These findings were similar to those reported by Clements and Hunt (2016) suggesting that carbonate chemistry may influence bivalve settlement.

DISCUSSION

Environmental conditions of ocean acidification may influence bivalve survival, reproduction, growth, and overall health in both direct and indirect ways. Ocean acidification may influence multiple modes for marine bivalves. This summary illustrates how ocean acidification may influence phytoplankton production, shellfish physiology, and seafloor chemistry, and the importance of understanding these interactions for sustainable marine aquaculture.

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The authors conducted a field study assessing the relationship between juvenile soft-shell clam abundance and spatial, temporal, and environmental variables in the Bay of Fundy. Sediment pH, water temperature, location, and date was monitored in 2012 during the settlement season for *Mya arenaria* at four different study sites along the shore. Using Akaike's information criterion models that incorporated environmental variables alone, sediment pH, and minimum air temperature best predicted bivalve recruitment. By removing temporal effects, there was a significant positive relationship between sediment pH and grain size. These results suggest that carbonate geochemistry might influence bivalve settlement.

Meseck, S.L., R. Mercaldo-Allen, C. Kuropat, P. Clark, and R. Goldberg. 2018. Variability in sediment- water carbonate chemistry and bivalve abundance after bivalve settlement in Long Island Sound, Milford, Connecticut. Marine Pollution. 135. Pp. 165-175.

The authors conducted field experiments in 2013 to assess the relationship between porewater sediment carbonate chemistry (pH, Alkalinity, dissolved inorganic carbon), grain size, and bivalve abundance at two sites in Long Island Sound (LIS), Connecticut, USA. Akaike's linear model was used to predict total bivalve community abundance and specific species abundance when appropriate. Over the entire season, 29% of bivalve abundance at the sites could be explained by grain size, salinity, and pH. When temporal effects were removed, up to 71% of *Nucula* spp. abundance could be accounted for by pH, phosphate, and silica concentrations in the porewater. These results suggest that bivalve abundance in LIS may be influenced by carbonate geochemistry and grain size.

Bermúdez, R., M. Winder, A. Stuhr, A. Almén, J. Engström-Öst, and U. Riebesell. 2016. Effect of ocean acidification on the structure and fatty acid composition of a natural plankton community in the Baltic Sea. 2016. Biogeosciences. 13. Pp. 6625-6635.

The authors conducted mesocosm perturbation experiments in coastal waters to investigate the response of phytoplankton to increased carbon dioxide with respect to community composition and fatty acid composition (nutritional statues). Their results found little change in plankton community composition from the natural condition (~347 μatm) to predicted levels for the year 2100 (~1333 μatm). The fatty acid composition was influenced by community composition, which was driven by phosphate and silica availability. The results presented here suggest that phytoplankton community composition is influenced more by nutrient availability than by CO_2, with little change in nutritional composition.

King, A. L., B.D. Jenkins, J.R. Wallace, Y. Liu, G.H. Wikfors, L.M. Milke, and S.L. Meseck. 2015. Effects of CO2 on growth rate, C: N: P, and fatty acid composition of seven marine phytoplankton species. Marine Ecology Progress Series. 537. Pp. 59-69.

The authors conducted laboratory-based experiments on seven species of phytoplankton common in coastal estuaries of the North Atlantic, where carbon dioxide was manipulated to four levels ranging from the glacial minimum (<290 ppm) to geological maximum levels (>2900 ppm).

Results indicated that carbonate system-driven changes in growth rate did not result in changes to elemental composition of the species studied. Fatty acid composition was not affected by elevated CO_2. The results showed little sensitivity of common coastal marine phytoplankton species to elevated CO_2 that will result in changes in nutritional status.

Current Trends in pH and Ocean Acidification in Aquaculture Sea Areas in Gokasho Bay, Japan

Masayuki Minakawa[1*], Satoshi Watanabe[2], Yuka Ishihi[2], Natsuki Hasegawa[2], and Toshie Matsumoto[2]

[1] National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, 224-1 Hiruda, Tamaki, Mie, 519-0423, Japan
[2] National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, 422-1 Nakatsuhamaura, Minamiise, Mie, 516-0193, Japan
* Present address: Fisheries Stock Assessment Center, Fisheries Resources Institute, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa 236-8648, Japan.

Corresponding author: minmas@affrc.go.jp

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ABSTRACT

Monthly variation in pH and carbonate saturation state (Ω) of aragonite and calcite were investigated in Gokasho Bay, Mie, Japan, from November 2016 to October 2019. During the winter season, pH was almost constant (8.02 - 8.13) in the whole bay area, which was approximately the same level as those reported in surface waters off the south coast of Japan. However, pH varied greatly during summer seasons (7.75 - 8.60). The lower value was thought to be caused by the remineralization of organic matter in the seabed, and the higher value was likely derived from high phytoplankton photosynthetic productivity. Excluding variation of pH caused by apparent oxygen utilization (AOU), acidification in the bay water was calculated to be 8.13 pH at present. The pH values currently measured are generally lower than those recorded in the 1980s, suggesting the advance of ocean acidification (OA) in the bay water with a reduction rate of 0.08 pH/decade. The calculated average values of Ω (> 3) were relatively high in the whole bay area, indicating calcium carbonate is supersaturated in the current environment of the bay. If OA advances in surface water around Japan in the near future, the degree of Ω in the bay water would drop with the reduction in pH, and some negative impacts on calcareous organisms may be brought about by this change.

INTRODUCTION

Coastal environments are influenced by various human-induced stressors. Recently, the global reduction of pH in surface seawater, which is referred to as ocean acidification (OA), has arisen as a consequence of oceanic uptake of anthropogenic CO_2. It is thought that OA affects the biogeochemical dynamics of calcium carbonate in the oceans (Doney et al., 2009; Kawano, 2010; Garrard et al., 2013). OA is suggested to damage not only marine phytoplankton production but fisheries and seafood industries (Fujii, 2018). Shellfish aquaculture occurs in many parts of coastal and bay areas in Japan and there is a possibility that shellfish aquaculture will also be damaged by OA in the near future since the shells are composed of calcium carbonate whose deposition is controlled by carbonate species and the pH of seawater (Poach et al., 2019). While there are many research studies on pH in open ocean and coastal areas (Fujii et al., 2001; Ishii et al., 2011: Zhai et al., 2014; Lipuma, 2016; Kubota et al., 2017), the effects of OA are not well understood in sea aquaculture areas due to large variations in pH associated with many biogeochemical processes. To obtain basic information for water quality of the coastal sea in Japan, monthly hydrographical observations were conducted in a sea aquaculture area in Gokasho Bay, Mie, Japan from November 2016 to October 2019. This paper provides recent, high-quality pH values and carbonate saturation states (Ω) of aragonite and calcite in the bay water. Spatial and temporal variations in pH values will be discussed in relation to ocean acidification in Japanese aquaculture sea areas.

METHODS

Study area

Gokasho Bay is located on the Pacific coast of Japan, and consists of 3 coves (Gokasho, Funakoshi and Hazamaura) and the main bay which opens to the western North Pacific (Fig. 1). The Bay has a relatively small area of 22.2 km^2 and a water volume of 0.11 km^3. Mean depth of the Bay is 12.7 m, and maximum depth is 30 m at the central part of the bay (Toda et al., 1990). Hydrographical features of the bay are reported in detail by Toda et al. (1990) and Abo (2000).

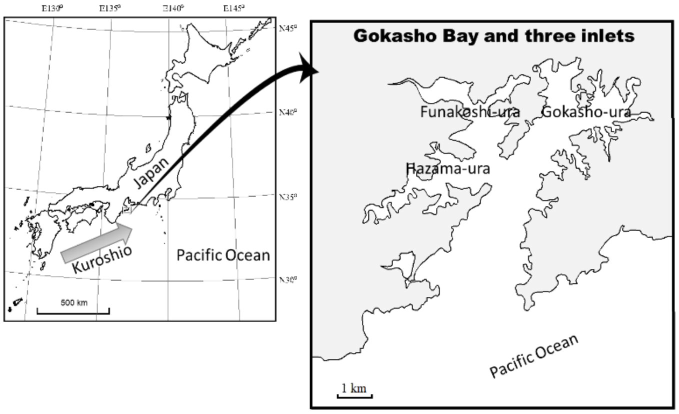

Figure 1. Map of Gokasho Bay.

Aquaculture is flourishing in Gokasho bay. Pearl aquaculture used to be the main industry in this area but has decreased significantly (Yokoyama et al., 1996). Now, the aquaculture of red seabream (*Pagrus major*) in Hazamaura-Cove, Pacific bluefin tuna (*Thunnus orientalis*) in the main bay, and oysters (*Crassostrea gigas* and *C. nippona*) and green alga (*Monostroma nitidum*) in areas close to the shore are operating in the bay.

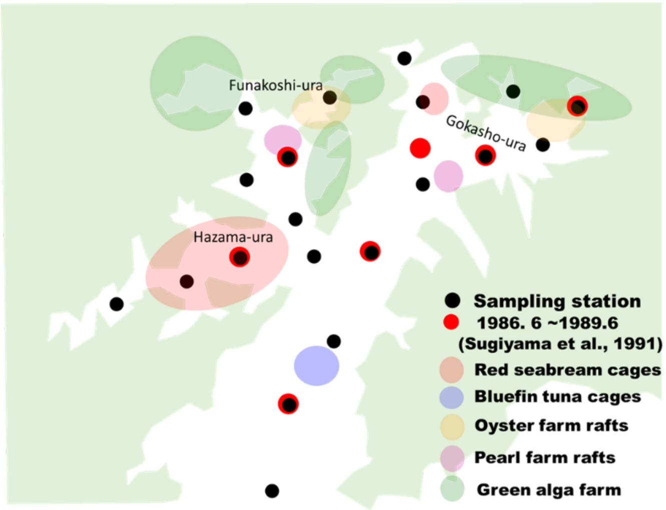

Figure 2. Sampling stations in Gokasho Bay. Black dots show the sampling stations from November 2016 to October 2018 (this study), and Red dots from June 1986 to June 1989 (Sugiyama et al., 1991). Major aquaculture area in the bay is also shown.

Sampling and analysis

Monthly hydrographic surveys in Gokasho Bay were conducted from November 2016 to October 2018 from the research vessel *Hamabou*. Seawater samples were collected in the bay at three depth layers using a 1.3-L Kitahara's water sampler at 19 stations from November 2016 to June 2017 and 20 stations from July 2017 to October 2018 (Fig. 2). Samples were collected in 25-ml co-stoppered glass test tubes for measurement of pH to avoid the contamination of CO_2 from the atmosphere and in 250 ml polyethylene bottles for measurements of total alkalinity. A CTD system (RINKO profiler, JFE Advantech) was also used to obtain hydrographic data (Watanabe et al., 2019). Dissolved oxygen (DO) concentration obtained from the CTD system was calibrated in the laboratory with seawater analysis data from a DO meter (MM-60R, TOA-DKK) with dissolved oxygen electrodes (OE-470AA, TOA- DKK).

The pH of seawater was measured more precisely by potentiometric method using a pH meter

(MM-60R, DKK-TOA Co.) with a glass electrode (ELP-0320, DKK-TOA Co.). The glass electrode was standardized against the JIS buffer scale (4.008, 6.865 and 9.180 pH at 25°C) which adapted the NBS buffer scale. Any drift in pH was corrected against the seawater buffer scale (Ono et al., 1998; Dickson et al, 2007) and the precision of pH was ± 0.003. For measurement of pH, different pH scales were used. It is reported that the pH of JIS (NBS) buffer scale are about 0.15 pH higher than that of the total scale (Gattuso and Hansson, 2011). In this study, because the large variation of pH value observed was much higher than the systematic error of pH between NBS and total scales, the systematic error between these scales was ignored.

To calculate carbonate systems, total alkalinity was measured from May 2016 to October 2018 using a one-point titration method (Culberson et al., 1970; Tsunogai and Noriki, 1983; Ono et al., 1998) modified by using a small sample method (Suga et al., 2013). An aliquot (15 ml) of seawater sample was used to measure total alkalinity. In this research, systematic error of total alkalinity values was not corrected. The precision of total alkalinity was ±0.006 meq/L.

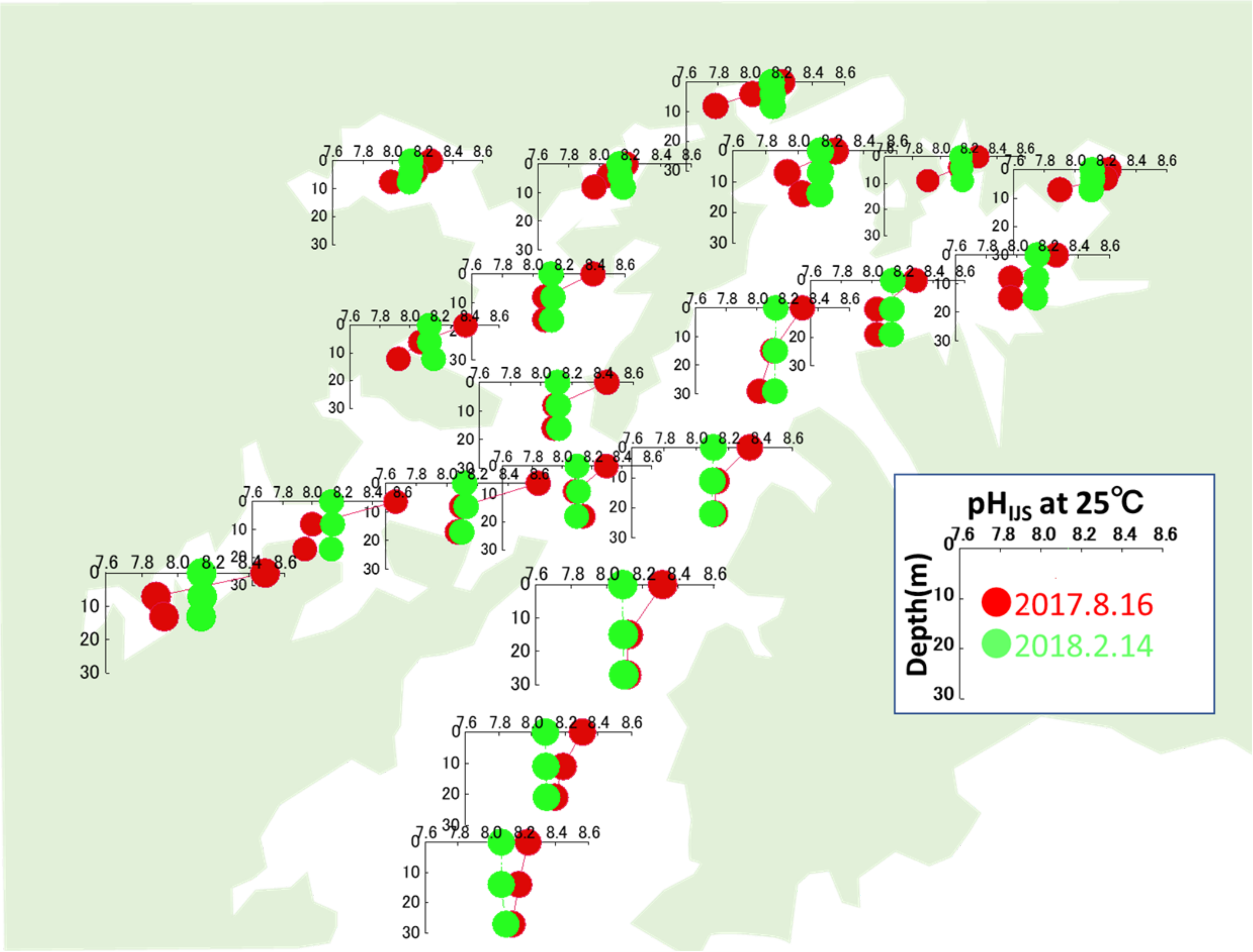

Figure 3. Vertical profile of pH at each station on August 2017 and February 2018 in Gokasho Bay, Mie, Japan.

Calcium carbonate saturation state (Ω)

Calcium carbonate saturation state of seawater (Ω) is one of the indexes for health of the ocean calcareous organisms. Ω was calculated using the equation as follows;

$$\Omega_{cal,\,arag} = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}^{cal,\,arag}} \quad \cdot\ \cdot\ \cdot\ (1)$$

where $[Ca^{2+}]$ is the calcium concentration calculated from salinity (Millero, 2013), $[CO_3^{2-}]$ is the carbonate concentration determined from observed pH and TA (Dickson at al., 2007), and $K_{sp}^{cal,arago}$ is the solubility product for calcite and aragonite, calculated from salinity, temperature and water pressure (Mucci, 1983; Millero, 1983), respectively.

RESULTS AND DISCUSSION

An example of vertical profile of pH was shown at each station in August 2017 and February 2018 (Fig. 3). High pH of more than 8.3 was observed for surface water in August 2017 while pH decreased toward the sea bottom to less than 8.0.

Vertical variation of pH tended to be greater at stations closer to the shore. However, pH in winter (February 2018) stayed constant around 8.1 because the bay water was homogenized due to winter vertical and horizontal mixing.

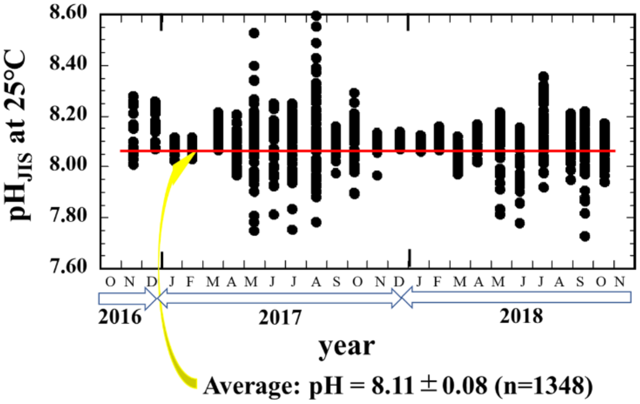

Figure 4. Monthly trend of pH at all data from November 2016 to October 2018 in Gokasho Bay, Mie, Japan. The average of pH value is also shown.

Monthly trends of pH are shown in Fig. 4 from November 2016 to October 2018. pH values with a large variation ranging from 7.7 to 8.6 were found from spring to summer seasons, whereas the variation was relatively small, centered around 8.1, from autumn to winter. During phytoplankton blooms in surface water, particularly high pH of around 8.6 was observed in August 2017. Low pH values were seen in the suboxic bottom water during these periods. Seasonal average pH was calculated to be 8.11 ± 0.08 (1σ, n = 1384). This level was similar to those reported for Japan's coastal waters and the western North Pacific surface water (Kawano, 2010; Ishii et al., 2011; Japan Meteorological Agency).

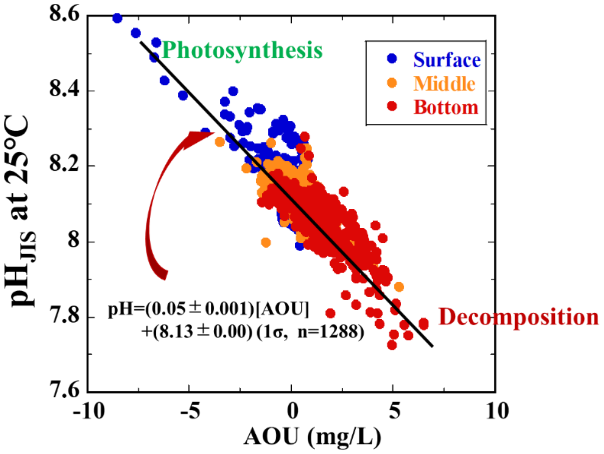

Figure 5. Relationship between AOU and pH from November 2016 to October 2018, except the data on January 2018, in Gokasho Bay, Mie, Japan. The regression line is also shown.

Large seasonal variations of pH and dissolved oxygen levels come from biogeochemical and physical processes in the bay water. The relationship between pH and apparent oxygen consumption (AOU) at all stations are shown in Fig. 5. In general, pH is affected by photosynthesis and decomposition of organic matter. When photosynthesis is active, CO_2 is taken up from the seawater to synthesize organic matter and to release oxygen. As a result, AOU decreases, and pH increases in the water column. On the contrary, decomposition of organic matter consumes oxygen and releases CO_2 to seawater. As a result, AOU rises, and pH decreases. The observed correlation between AOU and pH suggests that the large seasonal variation of pH was at least partially caused by photosynthesis in the surface layer and decomposition of organic matter near the bottom.

Marine phytoplankton have a stoichiometric composition of carbon to oxygen (Anderson 1995). The slope of the regression line between pH and AOU was calculated to be -0.05 ± 0.001 pH/(mg/L) (or -0.0016 ± 0.00003 pH/(μmol/L)) (1σ, n = 1288). The pH of surface water is dominated by photosynthesis, decomposition of organic matter and uptake of atmospheric CO_2. When AOU is in condition of 0 mg/L in sea water, the pH is apparently affected by only uptake of atmospheric CO_2. Therefore, the vertical intercept of the regression line can be defined as the acidification degree in the bay water under the conditions of absence of biochemical processes and AOU being 0 mg/L. In Fig. 5, the pH of acidification degree was calculated to be 8.13 ± 0.00. These results showed that the pH in the bay water changed with the AOU at the ratio of - 0.05 ± 0.001 pH/(mg/L) around pH of 8.13.

A similar study was conducted in the bay from June 1986 to May 1989 by Sugiyama et al. (1991).

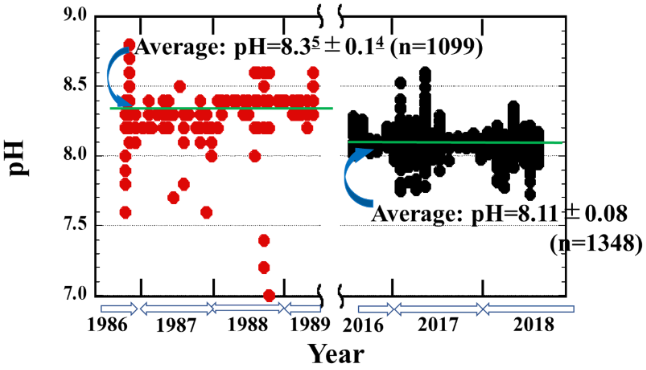

Figure 6 Comparison with pH values between the trend of pH from June 1986 to November 1989 (Sugiyama et al., 1991) and November 2016 to October 2018 (this work) in Gokasho Bay, Mie, Japan. Since the pH data from 1986 to 1989 was measured to be up to 2 digits after decimal point, the overall uncertainty of the pH could be regarded to be 0.01 pH units. Each average value of pH is also shown.

Compared with the seasonal variation of pH values at present, the seasonal variation reported from 1986 to 1989 was larger, ranging from 7.0 to 8.8 (Fig. 6). If the ratio of pH/AOU was constant in the bay water, the seasonal variation of pH could have also been larger than that of AOU at present. This suggests that the production and decomposition of organic matter were more active in the 1980s than now. The average value of pH from 1986 to 1989 was calculated to be 8.35±0.14, which was 0.24±0.16 higher than the average value of 8.11 ± 0.08 from 2016 to 2018. Assuming the pH decreased by 0.24 in the 30 years from 1988 to 2018, the pH reduction rate is calculated to be -0.08±0.005 pH/decade. This reduction value is greater than -0.02 ± 0.007 pH/decade of the south west coast Japan (Ishii et al., 2011) or 0.0082 ± 0.0057 pH/decade of the shelf break of the East China Sea (Lui et al., 2015). This suggests that OA may be progressing more rapidly in Gokasho Bay, though a longer-term data set is required to be more conclusive.

The monthly trends of omega calcite (Ωcal) and aragonite (Ωarago) were calculated for all data from May 2017 to October 2018 (Fig. 7). In summer, high Ω value was observed in surface water and Ω decreased with depth. The vertical variation of Ω tended to be greater at the stations closer to the shore.

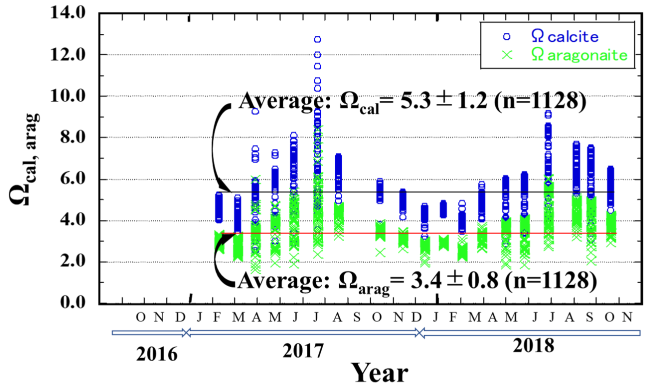

Figure 7. Monthly trends of calcium carbonate saturation states of seawater for calcite (Ωcal) and aragonite (Ωarago) from May 2017 to October 2018, except the data on October 2017, in Gokasaho Bay, Mie, Japan. Each average value of calcium carbonate saturations is also shown.

On the other hand, Ω hardly changed vertically nor horizontally in winter because the water was well mixed. These characteristics of Ω were similar to those of pH. Under thermodynamic conditions of carbonate chemistry, when CO_2 dissolves in seawater, pH decreases and the following chemical reaction is taking place:

$$H_2O + CO_2 + CO_3^{2-} \rightleftarrows 2HCO_3^{-} \quad \cdot \cdot \cdot \cdot (2)$$

CO_2 reacts with CO_3^{2-} to form HCO_3^- in seawater and as a result, CO_3^{2-} concentration decreases. On the other hand, when CO_2 is taken up by photosynthesis, pH increases and CO_3^{2-} is supported by dissociation of HCO_3^{2-}. In that case, CO_3^{2-} concentration increases. Since Ω is a function of CO_3^{2-} concentration as shown in equation (1), Ω changes with a change of CO_2 concentration and pH in seawater. That was a reason why the characteristics of Ω were similar to those of pH. These results suggest that the seasonal variations of Ω_{cal} and Ω_{arago} were affected by photosynthesis in surface water and decomposition of organic matter in bottom water. The lowest values of Ω_{cal} and Ω_{arago} were found to be 2.4 and 1.5, respectively. The average Ω_{cal} and Ω_{arago} was calculated to be 5.3 ± 1.2 and 3.4 ± 0.8 (1σ, n = 1128), respectively. These results show that the bay water is supersaturated for calcite and aragonite at present, although ocean acidification may be advancing slowly in the bay. If the reduction rate of pH remains at 0.08±0.005 pH/decade, Ω will be less than 1 in 50 years. In the near future, the degree of Ω in the bay water would drop with reduction of pH and could bring negative impacts to the calcareous organisms of the bay.

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The authors determined that a significant trend in ocean acidification is superposed on the large seasonal and interannual variabilities of acidity in surface waters off the south coast of Honshu Island, Japan, based on their observation of the carbonate system (partial pressure of CO_2, total inorganic carbon and pH). Multiple regression analysis of these parameters showed that pH and the aragonite saturation state (Ω_{arago}) decrease at a rate of -0.020 ± 0.007 pH/decade, and -0.12 ± 0.05 Ω_{arago}/decade, respectively. If future atmospheric CO_2 levels keep increasing as predicted by IPCC scenario A1FI, which postulates tentative fossil fuel use associated with very rapid economic growth, a further reduction of -0.8 pH and -0.1 Ω_{arago} is likely in the next 50 years. The authors suggest that such a rapid reduction of Ω_{arago} could have negative impacts on a variety of calcareous organisms.

Kubota, K., Y. Yokoyama, T. Ishiakwa, A. Suzuki, and M. Ishii. 2017. Rapid decline in pH of coral calcification fluid due to incorporation of anthropogenic CO_2. Nature, 7, 7694, DOI:10.1038/s41598-017-07680-0.

Based on Boron isotopic ratio ($\sigma^{11}B$) measurements of corals, the authors show clear evidence that ocean acidification is affecting the pH of the calcification fluid (pH_{CF}) in Porites corals within the western North Pacific Subtropical Gyre at two separate locations, Chichijima Island (Ogasawara Archipelago) and Kikaijima Island. A comparison with the pH of the ambient seawater (pH_{SW}) near these islands, estimated from a large number of shipboard measurements of seawater CO_2 and atmospheric CO_2, indicated that pH_{CF} is sensitive to change in pH_{SW}. The authors suggested that the calcification fluid of corals will become less supersaturated with respect to aragonite by the middle of this century (pH_{CF}=~8.3 when pH_{SW}=~8.0 in 2050), earlier than previously expected, despite the pH_{CF}-unregulating mechanism of corals.

Suga, H.P, S. Sakai, T. Toyofuku, and N. Ohkouchi. 2013. A simplified method for determination of total alkalinity in seawater based on the small sample one-point titration method. JAMSTEC Rep. Res. Dev., 17, 23-33.

Measurement of pH and total alkalinity is the most convenient way to describe the carbon system in a seawater sample. In this paper, the authors introduced a modified method for determination of total alkalinity focused on reduced volume seawater samples (1 mL) based on the one-point titration method. The precision of this method is 0.1 - 0.2% (relative standard deviation). On the other hand, with no correction, the precision was 0.1 - 1.0%. Even though values showed a systematic error, precision was maintained through the correction based on concurrent measurement of commercially available standards. This method allows us to describe the carbonate system in a small amount of water with sufficient precision and accuracy to investigate the mechanism of calcification using only a pH meter.

Fujii, T., Y. Komai, and T. Fujiwara. 2001. Carbon dioxide dynamics in coastal regions of Osaka Bay. Japan Sci. Civil Eng. Papers B2, 67, 911-915.

In coastal regions, the biological production is far greater than that of the open ocean and the short-term change of hydrographical conditions is significant. The authors conducted continuous measurements of salinity, pH, and dissolved oxygen

(DO) at three stations in Osaka Bay. Also, they calculated the values of carbonate species in the bay water by using the values of pH and total alkalinity. The results showed that dissolved inorganic carbon and DO fluctuated synchronously with high correlation (R^2=0.97), and the records of DO and partial pressure of CO_2 (pCO_2) indicated the prominent diurnal variations which correspond to diurnal irradiation variations due to high productivity in the bay.

Ecological Interactions Among Wildlife and Shellfish Farms: A Case Study from New Jersey, USA, on Horseshoe Crabs and Oyster Farms

Daphne Munroe

Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Ave., Port Norris, NJ, USA 08349

Corresponding author: dmunroe@hsrl.rutgers.edu

Keywords: Oyster aquaculture, *Crassostrea virginica*, horseshoe crab, *Limulus polyphemus*, wildlife interactions

ABSTRACT

Globally, shellfish aquaculture is growing rapidly. For the first time in history, global seafood supply from aquaculture exceeded that of wild fisheries. Although shellfish culture is viewed as an ecologically sound industry, as farm production expands it faces key challenges in assuring ecological and social sustainability. Understanding the ways shellfish farms interact with coastal wildlife, particularly species of special concern, is among those challenges.

Farms for Eastern oyster (*Crassostrea virginica)* are commonly located in shallow coastal areas along the U.S. east coast, use a range of farm equipment, and involve regular access to care for and harvest livestock. In some cases, these farms are located in areas used by American horseshoe crabs (*Limulus polyphemus*) as they come ashore during spring to spawn along Atlantic Coast beaches. The sandy shores of the Delaware Bay, bordered by the states of New Jersey to the north and Delaware to the south, host the largest spawning aggregations of the species in the world. Horseshoe crabs are economically and ecologically important; their blood is highly valuable in the medical industry and migratory shorebirds including the red knot (*Calidris canutus rufa)*, a threatened migratory shorebird, feed on crab eggs when stopping in Delaware Bay.

Limited studies have been done to examine the interactions among horseshoe crabs and intertidal oyster farms, and recently concern has been raised about the horseshoe crab's ability to traverse oyster farms to reach spawning habitat. A case study examining potential farm interactions with horseshoe crabs was done in Delaware Bay, New Jersey, during the 2018 crab spawning season. The investigations included a range of controlled experiments and surveys during high and low tide using sonar and walking respectively, to observe crab behavior at farm and non-farm sites. In all cases, results indicate that crabs can successfully traverse farms and reach spawning beaches, and crabs do not differentially use farm versus non-farm areas of the Delaware Bay mudflats. These results provide important context for developing frameworks for managing ecological interactions among farms and wildlife species of concern.

INTRODUCTION

For the first time in history, global seafood supply from aquaculture exceeded that of wild fisheries (FAO, 2018). As aquaculture continues to develop globally (FAO, 2018), as well as domestically in the United States (National Marine Fisheries Service, 2018), the industry faces key challenges assuring ecological and social sustainability (Billing 2018). Understanding the ways shellfish farms interact with coastal wildlife, particularly species of special concern, is among those challenges (Barrett et al., 2018; Collier et al., 2018). The ways in which molluscan aquaculture interacts with fundamental ecosystem processes such as particle depletion, nutrient cycling, and benthic-pelagic coupling has been relatively well studied (Newell 2004, Dumbauld et al. 2009, Rose et al. 2015). Likewise, attractiveness of farms in marine habitats, such as fish net pens and shellfish farm structures, to mobile fish and crustaceans is well documented (Callier et al, 2017). However, central to sustainability is appreciating the nature of the interaction among farms and wildlife that may use habitat near to or occupied by farms (Price et al., 2017; Barrett et al., 2018), interactions that are as of yet poorly studied.

Shellfish aquaculture in the Delaware Bay, Cape Shore region of New Jersey has a long history as a low impact, sustainable food production system (Hilborn et al., 2018; van der Schatte Olivier et al. 2018). Oyster farms currently occupy approximately 10 acres in this area of New Jersey and produce over 1.8 million market-sized oysters annually (Calvo, 2016); however, during the first half of the 20th century, oyster farming was much more expansive, using large wooden intertidal racks to cultivate oysters over wide stretches of this region (Ford and Haskin 1982). The vast intertidal mudflats of the lower bay, with rich oyster food resources, make this region a desirable location to grow high quality oysters; it is also a region used by important wildlife species.

Horseshoe crabs (*Limulus polyphemus*) are an economically, medically (Novitski, 2009) and ecologically important species. Known to be the largest spawning aggregations in the world (Shuster and Botton, 1985), hundreds of thousands come ashore during the spring to mate and lay eggs along sandy beaches of the Delaware Bay (Smith et al., 2002). The rufa subspecies of the red knot (*Calidris canutus rufa*), a migratory shorebird that uses stopover areas along the Atlantic coast of the U.S., including the Delaware Bay, was designated 'threatened' in 2015 under the Endangered Species Act in the U.S. by the U.S. Fish and Wildlife Service (50 C.F.R. § 17 2014). An important food source for red knots are lipid-rich horseshoe crab eggs, deposited on beaches by mating crabs during the spring northward bird migration (Castro & Myers, 1993). The eggs become available to the transitory bird flocks as they are exhumed from nests by sediment disturbance such as crab burrowing and wave action (Kraeuter & Fegley, 1994; Smith, 2007) and become concentrated in the upper intertidal zone as the tide rises (Nordstrom et al. 2006).

A small portion of the total Delaware Bay shoreline is collectively used by crabs for spawning. This is a nexus of shorebird migratory stopover habitat, and is home to oyster farming. The overlap in both time and space of these two iconic and ecologically important species, and oyster farm activities, presents a unique opportunity to examine potential ecological interaction among wild species/stocks

Figure 1: Photo of a typical oyster rack with bag on top at a Cape Shore farm. Horseshoe crabs can be seen among the racks and in the flooding tide waters. Crab spawning habitat (sandy beach slope) can be seen in the background.

and shellfish farms. Little data has been collected specifically addressing the ability of horseshoe crabs to traverse intertidal rack-and-bag oyster farms to reach their spawning habitat (Figure 1). If crab spawning migrations are impeded by farms, it is possible that crab populations themselves may suffer, and in turn shorebird foraging patterns and opportunities may be altered. In this case study, our primary goal is to characterize the ways that horseshoe crabs interact with farm structures while passing through farms *en route* to inshore spawning habitat, and determine if the farms themselves present a barrier to crabs reaching beaches to lay eggs. Ultimately, this information is important for both wildlife species conservation and sustainable farm management.

METHODS

All experiments and surveys were carried out during the horseshoe crab spawning season (Smith and Michels 2006; Shuster and Botton 1985), from May through July of 2018, at the Rutgers University Cape Shore Laboratory and nearby oyster farms located along the lower Delaware Bay. First, an experiment was conducted in which crabs were placed in a tank, along with oyster farm equipment (rack-and-bag) to observe the ways that crabs are, or are not, able to move past this equipment. Second, surveys were conducted on the flats during low and high tide conditions to assess crab presence/absence relative to farm footprints, and to evaluate whether crabs use intertidal habitats differently when farm gear is present.

For the observation of crab movement around racks, a large fiberglass tank (3.7 m long x 1.5 m wide x 0.51 m deep) was filled with filtered baywater to a depth of 30 cm. Mature horseshoe crabs were collected by hand from the adjacent Cape Shore mudflats. Experiments began May 9, 2018, and continued through June 21, 2018, during the spawning period for horseshoe crabs; thus, all animals used in the experiment were actively coming ashore to spawn and assumed mature. Twenty crabs, collected at random, were used for each experimental trial. Before being placed into the tank, each crab was measured (widest distance across the prosoma) and sexed, then assigned an identification number that was written on both sides of the shell in yellow waterproof crayon (this mark was removable and was wiped off before returning these crabs to the flats after the experiment). The size, sex, and id number of each crab was recorded and marked animals were placed into the tank and allowed to acclimate for 15 minutes before the experimental treatment was placed in the tank with them. During this acclimation period, many of the females in the tank were attached to by males, forming amplexus pairs.

A total of eleven treatments were tested. Treatments included a control, in which the footprint of an oyster rack was drawn on the tank bottom but no physical structure was put in the tank, and 10 farm gear treatments of varying heights and configurations. The suite of treatment types included three rack heights (3”, 5” and 8” above the bottom) with and without oyster bags attached, an oyster bag on the bottom of the tank (no rack), a floating oyster bag tethered to the bottom with ¼” braided sinking line, oyster bags leaning on the side of a rack and a rack on its side (no bag). All racks were rebar racks that are used by farmers, had elastic cords with metal hooks attached (farmers use these to hold bags onto the racks), and bags contained oyster shells to mimic adult oysters. The designated treatment was placed in the tank with 20 crabs and a timer started. The crabs were observed continuously for 15 minutes as they moved about the tank and interacted with the treatment structure. A note was recorded for every crab each time that individual crab passed to the side, beneath, or over a structure. This 15 minute observation period was considered one replicate for the given treatment. Each treatment was replicated a minimum of nine, and maximum of 14 times, for a total of 139 trials.

Low tide surveys were conducted using paired transects, laid out and marked on four intertidal farms in the Cape Shore region. At each farm, paired transects (1m wide) were oriented perpendicular to the shore with one transect intersecting a farm, and a parallel control transect passing through adjacent un- farmed intertidal habitat following methods described in Munroe et al. (2017). During daytime low tides, starting on May 8, 2018, and continuing through May 25, 2018, transects on all four farms were walked and all crabs encountered along the transect were documented, and their location (inshore of or within farm) was noted. Walks were repeated a minimum of weekly, for a total of nine repeat surveys during the 2018 spawning season. A Kruskal-Wallis test was used to test if differences were observed in the number of crabs observed inshore, and within farm gear among paired farm:control transects.

Waters in the Delaware Bay are sufficiently turbid, that even in shallow water and over short distances, visual observation of crabs on the bottom during high tide is not possible. Therefore, specialized sonar (DIDSON sonar) mounted to the front of a small aluminum boat was used to observe crabs on the bottom during high tide. Sonar video was recorded as the boat motored slowly along parallel transects set in oyster farm and non-farm habitats, laid out as described for the low tide surveys. In addition to the transect videos, sonar video was taken at two paired mooring locations for durations of 10-15 minutes. One mooring point allowed observation within a farm; the other in a comparable control location with no farm gear. A total of nine paired mooring videos were taken.

RESULTS

In total, 330 male and 129 female crabs were used in the experimental trials, ranging in size from 16 to 28 cm prosoma width for males (mean=20.1 cm), and from 21 to 30.5 cm for females (mean = 25.8 cm). Across all eleven rack treatments tested, all categories of crabs (single male, single female and amplexus pairs) were observed moving around and under/over/through the farm gear treatment. Interestingly, this includes single female crabs and crabs engaged in amplexus successfully passing both under and over the two shortest rack heights. In zero of the 128 trials involving oyster racks and/or bags (i.e. all non-control treatments) was a crab observed to be stuck or impeded from moving past or through the oyster gear.

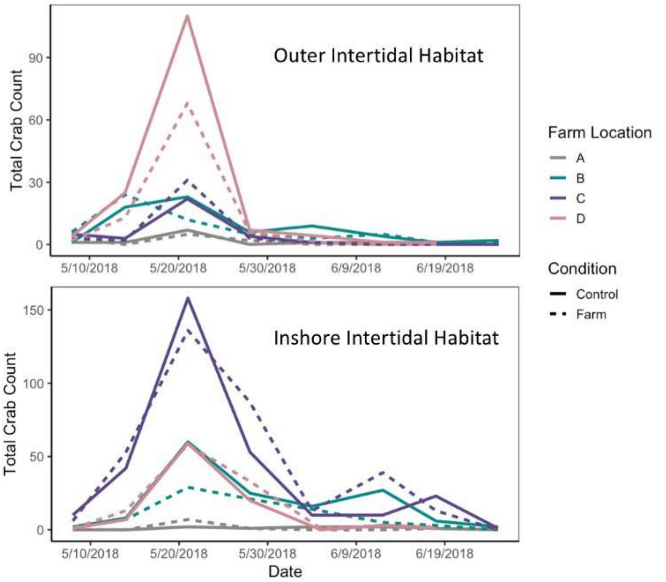

Figure 2: Crab counts along transects made during low tide. Upper panel shows crabs observed within the outer intertidal region, among the farm gear. Lower panel shows crabs observed inshore of the farm gear. Note that the length of transects varies among farms, and differs between outer and inshore habitats. Line colors depict each of the four farms, and dashed lines are counts along the transect bisecting the farm, solid lines show counts made along the control transect.

Numbers of crabs observed during the low tide surveys varied through the season, and among farms (Figure 2). Counts ranged from a low of zero at the beginning and end of the observation period, to a high of 135 per transect during the observation made on May 21st, 2018; this period fell between the New and Full moon in late May. In total, over all transects and across the entire observation period, 1,176 crabs were observed on the four farms, zero of which were impinged in farm gear. No difference exists among the number of crabs counted at habitat inshore of farm gear compared to controls (Kruskall-Wallace test, p=0.73), nor at habitat within the farm footprint compared to controls (Kruskall-Wallace test, p=0.45). The numbers of crabs counted during low tide differed significantly among farm sites (Kruskall- Wallace test, p<<0.001 inshore habitat, p=0.004 within farm habitat).

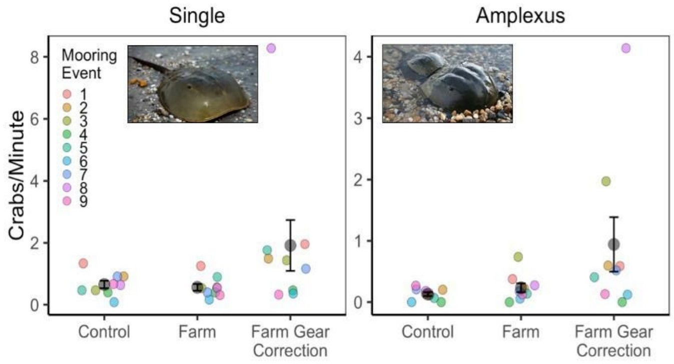

Figure 3: Single and amplexus crab counts made using sonar from all paired mooring events. Gray points represent the mean ± standard deviation. Colored points show data per observation event, with each event colored the same.

Sonar video surveys of crabs during high tide showed large aggregations of crabs in sloughs (muddy depressions), and sparsely distributed crabs moving independent of other crabs in seemingly random directions outside of sloughs. On many occasions, crabs were observed to move under and out from farm gear unimpeded. The number of crabs observed during high tide along transects varied depending on the day of observation, the farm, and whether counts were inshore of the farm gear versus in the area of the farm gear. For the region inshore of the farm gear, no significant difference was detected among control and farm counts for single crabs (p=0.54) nor amplexus pairs (p=0.53). In the intertidal area within the footprint of the farm gear (outer intertidal), no significant difference was detected among control and farm counts made for single or amplexus crabs, (all p values>0.13). Counts of crabs made during mooring events, standardized by effort to crabs/minute, ranged from zero to nearly two crabs per minute (Figure 3). No significant difference was detected among paired control and farm counts for single crabs (paired T-test, p=0.37), nor amplexus pairs (Wilcoxon signed rank, p=0.33). When counts were corrected for view obstruction by farm gear, no significant difference among single crab counts at control vs. farm was detected (Wilcoxon signed rank, p=0.16); however, significantly more amplexus pairs were observed at the farm mooring (Wilcoxon signed rank, p<0.05).

DISCUSSION

Experiments and surveys conducted during the horseshoe crab spawning season in 2018 tested the ability of horseshoe crabs to move among and through oyster farm gear and evaluated the ways that crabs use habitat around oyster farms in the Delaware Bay region. Across all of the surveys and experiments, our results indicate that crabs can successfully traverse farms and reach spawning beaches, and that crabs do not avoid farm gear when accessing spawning beaches. These results provide important context for developing frameworks for ecological interactions among farms and wildlife species of concern, specifically with respect to concerns about the potential for changes in abundance of horseshoe crabs or their behavior due to the presence of oyster farm gear.

Mature crabs ranging in size and including single male and female crabs, as well as amplexus pairs, were observed to move beneath, over and around oyster racks ranging in height from three to eight inches off the bottom when underwater. Likewise, sonar data collected during high tide at farm and control locations on the Cape Shore intertidal flats documented crabs (both single crabs and amplexus pairs) moving unobstructed among farm gear in daylight and moonlight conditions. Crabs were able to access habitat inshore of farms at the same rate as those inshore of comparable control sites, suggesting that the presence of farms does not impede crabs from accessing upper intertidal spawning sites; consequently, the reproductive ability of the crabs is not impacted by oyster farms. When sonar counts of crabs at farm sites were corrected for obstruction of the view of the bottom, there in fact appears to be more crabs moving within farms at high tide compared to control sites without gear. Attractiveness of farm structures in marine habitats, such as fish net pens and shellfish gear, to mobile fish is well documented (Callier et al. 2017). It is possible that horseshoe crabs also find oyster farm gear attractive due to increased foraging opportunities, shelter or other cues; future research may address this possibility.

Concepts of conservation aquaculture highlight the important contributions shellfish farms and other forms of aquaculture can make in achieving local and global conservation goals (Froehlich et al 2017). When evaluating aquaculture impacts and interactions with wildlife, it is important to (1) establish appropriate reference sites at which to compare wildlife abundance to farms, and (2) collect data on potential differences in behavior and reproductive capacity for wildlife at farm sites (Barrett et al, 2018). In the studies documented in this report, appropriate controls were used in an effort to best illuminate changes, if any exist, in abundance of horseshoe crabs or their behavior due to the presence of oyster farm gear, with the intention to serve the needs of an adaptive management process aimed at balancing wildlife species conservation and sustainable farm management. Adaptive approaches that allow for inclusion of new data are critical to coastal management, and particularly so when considering appropriate ecosystem-based decisions that ensure protection of wildlife and viability of important coastal industries.

ACKNOWLEDGEMENTS

I am indebted to the fearless field crew that helped count crabs at all hours of day and night, and in particular to hard-working interns Josh Daw and Niki Cleary who were instrumental in data collection and analysis. I extend further gratitude for the continued cooperation of the oyster farmers of Delaware Bay who allow us access to their farms. Support for part of this project was provided by Cape May County. This white paper is the result of research sponsored by the New Jersey Sea Grant Consortium (NJSGC) with funds from the National Oceanic and Atmospheric Administration (NOAA) Office of Sea Grant, U.S. Department of Commerce, under NOAA grant Award # NA10OAR4170085 and the NJSGC. The statements, findings, conclusions, and recommendations are those of the author and do not necessarily reflect the views of the NJSGC or the U.S. Department of Commerce. NJSG-19-964.

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ANNOTATED BIBLIOGRAPHY OF KEY WORKS

In this annotated bibliography, I have selected recent works that focus on interactions among wildlife - in particular horseshoe crabs or *kabutogani* - and shellfish farms. Limited research has focused on this issue; therefore, I have included two experimental studies, and two broader reviews. Horseshoe crabs are an important and iconic species along the eastern United States, as well as in Japan.

Barrett, Luke T., Stephen E. Swearer, and Tim Dempster. 2018. Impacts of marine and freshwater aquaculture on wildlife: a global meta-analysis. Reviews in Aquaculture. 1-23. doi: 10.1111/raq.12277

In this review, authors from the University of Melbourne in Australia demonstrate evidence that supports higher wildlife biomass and diversity found around aquaculture farms. They argue that to properly understand the possible impacts of farms on wildlife, studies have thus far failed to address issues of impacts on behavior, reproduction or fitness. If animals that tend to aggregate around farms suffer poor fitness relative to those occupying non-farm habitats, the farm becomes an 'ecological trap'.

Callier, M.D., C.J. Byron, D.A. Bengtson, P.J. Cranford, S.F. Cross, U. Focken, H.M. Jansen, P. Kamermans, A. Kiessling, T. Landry, and F. O'beirn. 2017. Attraction and repulsion of mobile wild organisms to finfish and shellfish aquaculture: a review. Reviews in Aquaculture.

The ways in which finfish and shellfish farms act as attractant or repulsive areas for mobile marine species is reviewed. At finfish farms, food inputs and physical structure of the farm tend to drive aggregating effects. Likewise, at shellfish farms, the farm structure (both farm gear and shellfish themselves) and increased feeding opportunities via biodeposition, the shellfish crop or fouling organisms tend to attract wildlife to farms. In general, the authors note that a great deal of variability in attractiveness or repulsiveness exists when considering the suites of habitats, farms, and mobile fauna that have been studied thus far.

Kwan, Billy K.Y., Chan Hoi Kin, and Siu Gin Cheung. 2018. Habitat use of globally threatened juvenile Chinese horseshoe crab, *Tachypleus tridentatus* under the influence of simulated intertidal oyster culture structures in Hong Kong. Aquatic Conservation: Marine and Freshwater Ecosystems. 28: 124-132.

The authors conducted this study to test if juvenile Chinese horseshoe crabs differ in abundance and if their feeding trails differ at oyster farm spat collection locations. They performed their test at a shallow mudflat in Hong Kong which is home to a large population of juvenile horseshoe crabs. Addition of oyster spat collectors (bricks on the mudflat) lead to a significant reduction in the number of juvenile horseshoe crabs, and a reduction in the length of feeding trails.

Munroe, D., D. Bushek, P. Woodruff, and L. Calvo. 2017. Intertidal rack-and-bag oyster farms have limited interaction with Horseshoe Crab activity in New Jersey, USA. Aquaculture Environment Interactions. 9: 205-211. https://doi.org/10.3354/aei00227

In this study, the authors sought to assess whether intertidal oyster farms altered the distribution of mature horseshoe crabs as they came ashore to spawn in the lower Delaware Bay. In total, they used a combination of field surveys of crabs on mudflats and stranded along the upper shore, and controlled tests to determine if crabs can move past farm gear. Results show no evidence that horseshoe crab distribution within this area is altered by the presence of oyster farms.

Use of Point-of-View Video Cameras to Assess Fish Interactions with Oyster Aquaculture Cages

Gillian Phillips[1, 2*], Renee Mercaldo-Allen[1], Peter J. Auster[3], Paul Clark[1], Erick Estela[1], Yuan Liu[1, 2], Lisa Milke[1], Dylan Redman[1], and Julie Rose[1]

[1] NOAA Fisheries, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Avenue, Milford, CT 06460 USA
[2] Integrated Statistics, 16 Sumner Street, Woods Hole, Massachusetts 02543 USA
[3] Department of Marine Sciences & Mystic Aquarium, University of Connecticut, 1080 Shennecossett Road, Groton, CT 06340 USA

Corresponding author: gillian.phillips@noaa.gov

Keywords: Aquaculture, Shellfish, Habitat, Ecosystem Services, eDNA

ABSTRACT

Oyster bottom cages are an increasingly popular style of aquaculture gear in the Northeastern United States. Cages are used to contain and protect shellfish during grow-out but also likely provide three- dimensional structure that may benefit the local wild fish community. Understanding the ecosystem services provided by aquaculture gear may help to inform regulatory decisions and inform a broader public discussion of shellfish farming and how farms interact with the local environment.

A low-cost camera mounting system was developed and tested to allow visual assessment of fish activity near an oyster cage farm and in a surrounding low seafloor structure environment. Minimal-structure t-platform stands were created to mount cameras adjacent to boulders, enabling comparison of fish interactions with cages to fish activity observed on natural structured rock reef habitat. Seawater samples were collected monthly during pilot camera deployments for environmental DNA (eDNA) analysis to detect fish species that may be present but not observed in video.

Video collected by cameras attached to oyster cages and near boulders facilitated visualization and quantification of the wild fish communities along with documentation of behavioral interactions between animals and these habitats. Analysis of eDNA detected significant differences in fish community composition across the three months sampled, and significant differences between sites in two of the three months sampled.

*The Federal Government does not endorse the use of GoPro™ cameras

INTRODUCTION

Bottom cages are an increasingly common type of aquaculture gear for cultivating oysters in the Northeastern United States (Archer *et al,* 2014; Flimlin *et al*, 2010). Cages are used to contain and protect shellfish during grow-out but also provide three-dimensional structure that may attract the local wild fish community and provide habitat services (Dumbauld *et. al,* 2009). A trapping and mark- recapture study in Narragansett Bay, Rhode Island, found the seafloor with oyster grow-out cages provided valuable habitat for juvenile and adult temperate reef fish (Tallman and Forrester, 2011). Habitat offered by oyster aquaculture gear may provide food resources, shelter, refuge from currents, and protection from predation for commercially and recreationally important species of fish. Documenting and quantifying the ecosystem services provided by aquaculture gear may help inform regulatory decisions and inform a broader public discussion about shellfish farming and how farms interact with the local environment.

Several structure-oriented species common to Long Island Sound are known to support important recreational and commercial fisheries in the Northeast. Demersal species including Black Sea Bass and Scup are federally managed under the "Summer Flounder, Scup, and Black Sea Bass Management Plan" for New England stocks (Atlantic States Marine Fisheries Commission, 2018). Tautog is a temperate reef fish also highly valued by fishermen, with 90% of landings contributed from recreational harvest (Atlantic States Marine Fisheries Commission, 2015). An understanding of habitat preferences and use, particularly among species of commercial and recreational interest, may aid in regional management decisions related to aquaculture leasing.

Small self-contained point-of-view video cameras can enable fine spatial-scale studies to quantify the interactions between fish and oyster aquaculture gear. Here we report on a pilot study using these cameras and other commercially-available accessories to create an optimized and transferable system for the deployment of underwater video cameras across multiple sampling periods and geographic study locations. The information gained from this pilot program is presented in this report. The ultimate goal of our research program is to utilize this system to gain information on species composition and abundance of fish interacting with oyster cages, to quantify and document details of how fish species interact with oyster cages, to compare how the habitat services provided by oyster cages compare with those provided by natural structured habitats, and to pilot the use of eDNA metabarcoding to characterize finfish communities associated with our study habitats over time and space.

METHODS

We studied a style of commercial oyster bottom cage commonly used in the Northeast (Figure 1). Cages measure 4 x 3 x 2 ft (1.2 x 0.9 x 0.6 m) and consisted of three shelves, with two bags of oysters per shelf. We stocked each bag with 150 seed oysters (~1 in; 2.5 cm) based on information from industry partners. Bricks were added to the base of the cage for ballast. Cages were deployed from May-September, 2017, at two nearshore sites off Milford, Connecticut; one adjacent to a high-density cage farm, and one in a low-structure environment. Water depths at the study sites ranged from 15-20 feet at high tide.

Figure 1. Oyster cage used in this study. Full view showing three shelves, each with two bags of oysters. Feet of cages show added bricks for ballast.

Figure 2. On bottom cage with camera views. (A) Close-up of cage fitted with cameras, showing side view in the front of the photo and top camera at the rear. (B) Close-up of top camera looking across the horizontal surface of the cage. (C) The resulting view of the top camera. (D) View of the side camera looking at two sides of the cage and where the cage meets the sea floor. (E) Resulting view of the side camera.

We experimented with several methods for attaching point-of-view (GoPro®) cameras to oyster cages. Initially a spring attachment was placed at a corner of the cage to provide flexibility to protect cameras during deployment and retrieval, but unfortunately resulted in too much camera motion during deployment and retrieval of cages. Camera mounts were instead constructed with flexible marine hose and ¼ inch PVC, which provided stability during video recording but allowed some flexibility for camera protection. We found a combination of two time-synced cameras to provide adequate visual coverage of most of the cage and immediate surroundings. The first camera was affixed to a cage corner by ~2 feet (0.6 m) of 7/8' flex marine wet exhaust and water hose, allowing the camera to hang off the side of the cage, enabling a view of two cage sides as well as the area where the cage feet met the seafloor. We initially experimented with a second camera positioned to look down at the top of the cage, but the poor water clarity prevented viewing the entire top of the cage. The final configuration had the second camera positioned in a periscope like mount, which was able to capture the top horizontal surface of the cage, including viewing the top of the oyster bags proximal to the camera. (Figure 2).

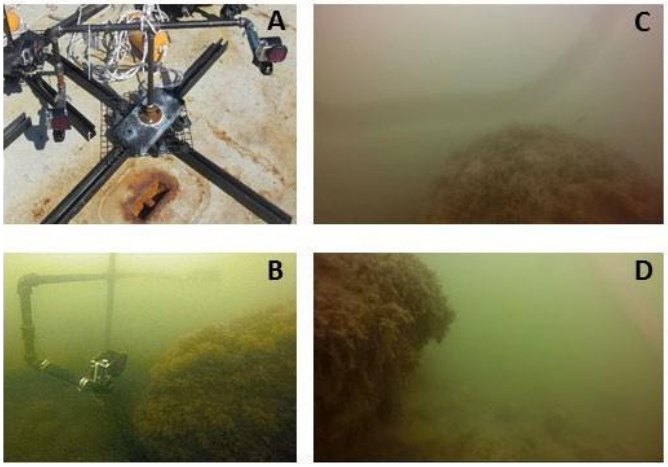

Figure 3. T-platform construction to mimic camera views on natural structured habitat. (A) Full view of t-platform with cameras attached. (B) Image of t-platform deployed on a natural rock reef adjacent to boulder. (C) Resulting top view of the boulder. (D) Resulting side view of the boulder which also captures where the boulder and sea floor meet.

To compare fish interactions with cages to interactions with natural structured rock reef habitat, "t-platform" stands were devised to mount cameras adjacent to boulders while adding minimal additional structure (Figure 3). T-platforms were built using threaded metal pipe and were designed to provide a field of view similar to that on the cage, capturing the top of the boulder and one side where the boulder meets the seafloor. The platforms are intended to be deployed at the beginning of the field season and left in place, using divers to attach and remove cameras. This should minimize the disturbance associated with camera deployment/retrieval, and ensure consistent fields of view across multiple deployments.

Intervalometer Blink® timers (CamDo, Vancouver, Canada) were used with GoPro cameras to delay the start of video recording by 24-hours, to minimize any deployment-related disturbance prior to recording. Video was recorded for eight minutes every hour from 7AM to 7PM to cover a complete tidal cycle and most daylight hours. A Polar Pro® magenta filter, with a 0.5 stop reduction in exposure, was affixed to each camera lens to reduce green coloration of video footage caused by the natural attenuation of light in water, as well as phytoplankton growth in Long Island Sound. The addition of any filter reduces total light capture by the cameras, which limited the depth of our deployments, but the importance of this deployment restriction was outweighed by the large reduction in time required for video post-processing. One TCM-1 tilt current meter (Lowell Instruments LLC, North Falmouth, MA, USA) was deployed at each site to measure current speed and direction during trials. HOBO pendant temperature and light meters (Onset Computer Corporation, Bourne, MA, USA) were also deployed to document light penetration and seawater temperature.

Environmental DNA (eDNA) analysis was used to detect fish species that may have been present but not observed on video. Following camera retrieval, seawater was collected near the seafloor using a Niskin Bottle during one deployment each in the months of June, July, and August 2017. Samples were kept on ice until filtration (0.45 µm) and then frozen (-20 °C) until analysis. DNA was extracted and PCR was performed on the 12S mitochondrial rDNA region. Next generation sequencing was then conducted on PCR amplicons, followed by bioinformatics analysis.

Pilot-scale deployments over the May-September 2017 field season yielded 20+ hours of video on weeks with the cage density comparisons. During weeks where t-platforms were deployed, 18+ hours of video was recorded on a continuous recording scheme. Video was downloaded immediately following trips and duplicate copies stored in two separate locations for data management. Each file was renamed with a unique identification to ensure proper organization. Observer XT software (v14.0; Noldus Information Technology, Wageningen, Netherlands) was used to analyze videos. Advantages of this software platform included multi-video playback, inter-coder analysis and flexible coding scheme, making it an ideal tool for our analysis.

Video analysis is currently underway. Fish abundance will be reported using the MaxN metric (Watson *et al.* 2005). The likelihood of double-counting the same fish within each interval of video recording was increased by the typical behavior of fish associated with a structured environment. The MaxN metric avoids double-counting by limiting abundance to the maximum number of fish observed at any one time, and is thus likely a conservative estimate of total fish abundance associated with a structure. For our study, MaxN was defined as the maximum number of individuals of a given species present in a single frame within each 1-minute segment of video (Watson *et al.* 2005). MaxN was calculated separately for each species observed. A behavior matrix under development will quantify and describe specific behaviors observed in video records. Precise definitions allow distinct behaviors to be identified and reduce the subjectivity in behavior analysis and identification among coders. For example, the current working definition for “station keeping above the cage” is “small fin movements to maintain a position both in a period of foraging and while in the water column or above cage.”

RESULTS AND DISCUSSION

Video analysis is ongoing, so only initial observations are reported here. The four most frequently observed fish species thus far were: cunner (*Tautogolabrus adspersus*), black sea bass (*Centropristis striata)*, tautog (*Tautoga onitis*) and scup (*Stenotomus chrysops)*, which are all commonly found in and around structurally complex habitat in Long Island Sound. These four species have been observed both on boulders and on oyster cages. Other species of fish captured on video include: butterfish (*Peprilus triacanthus*), banded rudderfish (*Seriola zonata*), hake (*Urophycis chuss*), sea robin (*Prionotus carolinus*), striped bass (*Morone saxatilis*), summer flounder (*Paralichthys dentatus*), windowpane flounder (*Scophthalmus aquosus*), smallmouth flounder (*Etropus* microstomus*)* and yellow jack (*Carangoides bartholomaei*). Animals observed inside cages during camera deployment or retrieval, but not in video, include: naked goby (*Gobiosoma bosci*), oyster toadfish (*Opsanus tau*), conger eel (*Conger* sp.), and rock gunnel (*Pholis gunnellus*). Some of these species are more active at night, which may account for their absence from video to date. Invertebrates associated with cages (either in video or observed during deployment/retrievals) include oyster predators such as oyster drill and sea star, as well as prey species for fish such as black-fingered mud crab. Other invertebrate species noted adjacent to a cage in video include: spider crab, blue mussel, slipper shell, horseshoe crab, blue crab, and channel whelk.

The results of eDNA metabarcoding efforts yielded 17 major and 20 rare finfish species from samples collected monthly during June, July, and August deployments in 2017. Fish communities obtained by eDNA metabarcoding were significantly different across the three months, and communities between sites were different during the months of July and August (Liu et al. 2019). eDNA was detected for all of the fish species observed thus far in the videos. Using ordination analysis, the axis associated with sampling month represented the highest variation (73.4%) among samples while the axis associated with habitat type represented minor variation (15.6%) among samples.

Outreach is an important component of this project. Public perception and understanding of aquaculture practices can be enhanced by news articles and other types of outreach (Froehlich *et al.* 2017). Heightening awareness of aquaculture practices and positive outcomes associated with aquaculture gear can increase support for aquaculture in the scope of the growing demand for shellfish. Our methodology was based on low-cost, readily-available cameras and mounts. An advantage of this design is that it can easily be replicated by aquaculture industry members, extension specialists, and citizen scientists. We have provided our methods in detail, with photos, within a .pdf document that is publicly available online (https://www.fisheries.noaa.gov/webdam/download/89562100). Our intention is to broaden video collection to other geographic locations, shellfish aquaculture industry practices, and seasons, to ultimately enable a more comprehensive understanding of the habitat services provided by shellfish aquaculture. Selected video clips collected from oyster cages are posted on our project website (https://www.fisheries.noaa.gov/aquaculture-habitat-NE). There has also been traditional news and social media coverage of this project via local news outlets as well as Twitter and Facebook coverage from both local and national NOAA Fisheries accounts.

This project has yielded a low-cost, easily replicated system for observing fish activity associated with oyster aquaculture gear and natural structured habitat. Video analysis generated during pilot-scale deployments will provide data on fish abundance and community composition in these two habitats, as well as insights into fish behavior. The continued investigation of fish interactions with aquaculture gear may help inform regulators, policy makers and fishery managers who make decisions about aquaculture practices. Video generated by this study could additionally contribute to a broader public understanding of interactions between shellfish aquaculture and the local environment.

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Watson D.L., E.S. Harvey, M.J. Anderson, and G.A. Kendrick. 2005. A comparison of temperate reef fish assemblages recorded by three underwater stereo-video techniques. Marine Biology 144: 415-425.

The authors deployed underwater stereo-video cameras to sample the relative density and species richness of temperate reef fishes in Southwest Australia. This study compared diver deploy, un-baited remote and baited remote cameras. They defined MaxN as “species presence and the maximum number of individuals belonging to each species in the field of view at one time (MaxN).”

This metric avoids repeated counting of fish within a given time frame and gives a conservative estimate of relative density relating to the area of survey.

DeAlteris J.T., B.D. Kilpatrick, and R.B. Rheault. 2004. A comparative evaluation of the habitat value of shellfish aquaculture gear, submerged aquatic vegetation and a non-vegetated seabed. Journal of Shellfish Research 23(3): 867-874.

The authors set out to evaluate habitat value of shellfish aquaculture gear (SAG), submerged aquatic vegetation (SAV), and shallow non-vegetated seabed (NVSB) over one year in Port Judith, RI, USA. Oyster cages were sampled using lift nets that were diver deployed under oyster cages and left to soak for two weeks prior to retrieval. Submerged aquatic vegetation and non-vegetated seabed sites were sampled with quadrats, drop nets, and a venturi- driven suction dredge deployed from a skiff. Sessile invertebrate growth was documented on eelgrasses and on oysters and oyster cages. All fish >5mm were collected. The authors documented that the physical habitat of shellfish aquaculture gear had greater surface area compared with the other two habitat types as well as a significantly higher abundance and species richness of organisms per meter squared throughout the year. The authors concluded that shellfish aquaculture gear had greater habitat value compared to their control sites.

Tallman J.C. and G.E. Forrester. 2007. Oyster grow-out cages function as artificial reefs for temperate fishes. Transactions of the American Fisheries Society 136 (3): 790-799.

The authors compared fish habitats within Narragansett Bay, RI, USA using trap surveys. They compared three oyster grow-out sites, six natural rock reefs, and one artificial reef built for fish habitat, looking to identify patterns in fish density, growth and disappearance rates. Their traps were designed to sample both juvenile and adult fish and were deployed in the summer and fall. The study showed that oyster cages provide habitat for fish associated with hard bottom habitats including scup, tautog, and black sea bass. Dumbauld, B.R., J.L. Ruesink, and S.S. Rumill. 2009. The ecological role of bivalve shellfish aquaculture in the estuarine environment: A review with application to oyster and clam culture in West Coast (USA) estuaries. Aquaculture 290:196-223.

This review article focuses on bivalve shellfish aquaculture on the US West Coast. Much like natural rock reefs, cages become colonized by epibenthic, emergent, and encrusting organisms and may provide sheltering fish with camouflage, refuge from predation, respite from high current flow, and a source of food. Multi-tiered off-bottom cages, an increasingly popular method for growing oysters on a smaller footprint, offer vertical three-dimensional structure and surface area similar to that afforded by natural reefs. In traditional on-bottom shellfish culture, where live oysters and shell cultch are distributed on sediments for grow-out and spat collection, oysters act as ecosystem engineers creating hard bottom irregular substrate on otherwise featureless seafloor. Evaluation of habitat services provided by oyster aquaculture can be challenging. Shellfish farms vary in spatial size and bottom area covered, variety and quantity of aquaculture gear, and have a seasonally variable footprint as gear is relocated or harvested. Aquaculture of oysters, whether in cages or grown on the seafloor, may represent valuable estuarine habitat.

Vibrio coralliilyticus Induction of Virulence Toward Larval Oysters and Corals at Elevated Seawater Temperatures and Potential Mitigation Strategies

Gary P. Richards[1*] and Blake Ushijima[2]

[1] United States Department of Agriculture, Agricultural Research Service, Dover, Delaware USA
[2] Current Address: University of North Carolina Wilmington, Dept. of Biology and Marine Biology, Wilmington, North Carolina USA

Corresponding author: gary.richards@usda.gov

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ABSTRACT

Recent research has advanced our understanding of the role pathogenic vibrios play in disease of various aquaculture species. *Vibrio coralliilyticus*, formerly thought to infect just corals, is now known to infect larval oysters, causing major losses in hatchery settings. The negative effect of *V. coralliilyticus* on wild-type oyster production remains uncertain, but circumstantial evidence suggests it may be substantial. We identified eight strains of *V. coralliilyticus* that infect both Eastern oyster (*Crassostrea virginica*) larvae and Pacific oyster (*Crassostrea gigas*) larvae in U.S. East and Pacific coast hatcheries. West Coast hatcheries have experienced major production losses over the years due to the presence of *V. coralliilyticus*. Some of the largest hatcheries have reported losses of up to 80%, resulting in shortages in seed oysters needed for commercial oyster production. Losses can exceed 10 billion larvae per year in the larger hatcheries. Most U.S. East Coast hatcheries are considerably smaller, but also experience losses in Eastern oysters, possibly from *V. coralliilyticus*, but also from the known shellfish pathogen *V. tubiashii*. Interestingly, *V. tubiashii* only infected Eastern oyster larvae, unlike *V. coralliilyticus* which causes acute mortalities with Eastern and Pacific species.

The infection and death of corals by *V. coralliilyticus* is known to be enhanced at seawater temperatures ≥ 27°C. In our studies, multiple strains of *V. coralliilyticus* produced high larval oyster mortalities with LD_{50}'s ranging from 3.8×10^3 to 4×10^4 CFU/ml of seawater, depending on the *V. coralliilyticus* strain and the oyster species (Eastern vs. Pacific oysters). Studies with knockout mutations demonstrated that the transcriptional regulator ToxR and the outer membrane protein OmpU were important in larval oyster (and coral) infections caused by *V. coralliilyticus*. Gross pathological changes occur in *V. coralliilyticus*-infected larvae, initially in the velum and cilia and then in the internal organs, which become liquefied. Infection of larval oysters is likely induced by stress and a lowering of resistance to these pathogens. Stressors, particularly in hatcheries, likely include incorrectly adjusted seawater temperatures, salinities, pHs, and dissolved oxygen levels or inadequate nutrition, over stocking, high overall bacterial loads, etc.

To begin to mitigate vibrios in hatcheries, we identified a variety of predatory bacteria which in nature help to modulate *Vibrio* levels in seawater and/or shellfish. They include several predatory bacteria such as *Pseudoalteromonas piscicida*, which secrete digestive enzymes that inhibit and kill many kinds of bacteria including vibrios; and *Halobacteriovorax*, a *Bdellovibrio* and Like Organism (BALO), which infect Gram-negative bacteria, like vibrios, and replicate within them, killing the vibrios in the process. Together these

bacteria may serve as probiotics in reducing hatchery mortalities and in killing vibrios and other bacterial pathogens in a variety of other aquaculture settings. Bacteriophages (phages) are another means to inactivate many undesirable pathogens in the environment, and are increasingly being used in aquaculture. To date, we isolated multiple phages against *V. coralliilyticus* and *V. tubiashii* and showed the practical application of phage therapy to reduce or eliminate mortalities from *V. coralliilyticus* and *V. tubiashii* in larval oysters.

INTRODUCTION

Naturally-occurring *Vibrio coralliilyticus* is a well-recognized pathogen of some corals. More recently, it has been found to be highly pathogenic toward larval Pacific oysters (*Crassostrea gigas*) and Eastern oysters (*Crassostrea virginica*) (Richards et al., 2015; Ushijima et al., 2018). These two oyster species are the primary commercially-harvested oysters in the United States. Another pathogen of larval Eastern oysters is *Vibrio tubiashii*, which is better known for disease outbreaks in East Coast hatcheries. Although literature has commonly referred to *V. tubiashii* as a pathogen in West Coast shellfish hatcheries, some of the more prevalent strains associated with the mortalities were misidentified and were confirmed by genomic sequencing to be *V. coralliilyticus* (Richards et al., 2014, 2018). Together, these two vibrios have contributed to episodic losses in hatchery production and shortages in seed oysters needed for commercial shellfish aquaculture. Interestingly, *V. coralliilyticus* and *V. tubiashii* are only known to infect and kill oysters while in the larval stage, which is only two to–three weeks in most cases. Once the larvae undergo metamorphosis, they appear resistant to infection by these vibrios.

Methods are needed to mitigate larval oyster diseases in shellfish hatcheries. Several potential probiotic approaches include the use of predatory bacteria, like *Halobacteriovorax* (Richards et al., 2012, 2013, 2016) and *Pseudoalteromonas piscicida* (Richards et al., 2017a, 2017b). In addition, bacteriophages (phages) against both *V. coralliilyticus* and *V. tubiashii* offer some promise as a therapeutic treatment for reducing hatchery-associated larval mortalities. Phage therapy is increasingly being used to combat bacterial disease in aquaculture (reviewed by Richards 2014).

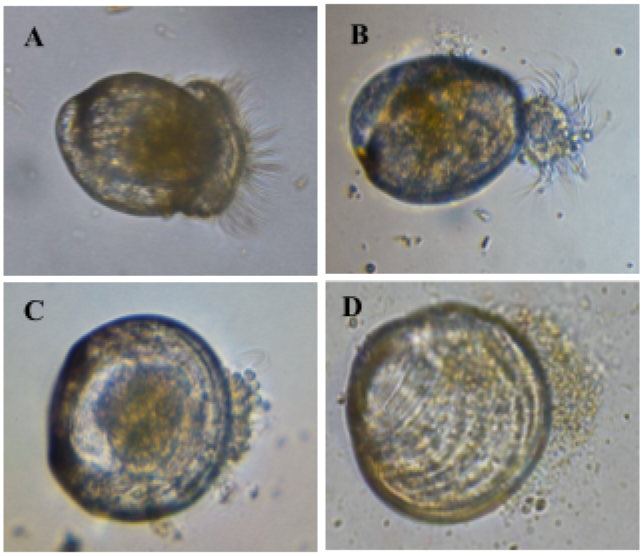

Figure 1. Normal and diseased Pacific oyster larvae. A) Normal, healthy larva and (B-D), larvae infected with V. coralliilyticus. B) Early-stage infected larva with tangled cilia and lumpy velum. C) Later stage infection with loss of cilia and velar cells. D) Final stage showing dead larva and release of digested/liquified tissues from within the valves.

We conducted studies of larval oysters to compare disease pathologies associated with both *V. coralliilyticus* and *V. tubiashii* (Ushijima et al., 2018). We identified the infectious doses of these pathogens in larval oysters. It is well known that elevated seawater temperatures enhance coral disease from *V. coralliilyticus*, so we also determined if similar high temperatures enhanced larval oyster mortalities. We also explored genes potentially responsible for the virulence of *V. coralliilyticus* in knock-out experiments (Ushijima et al., 2018) and are currently investigating mitigation strategies to reduce larval oyster mortalities using bacteriophages and predatory bacteria.

RESULTS AND DISCUSSION

Disease Pathology

Normal, uninfected, Pacific oyster larvae had a smooth velum and ordered cilia (Fig. 1A). They remained actively motile. Larvae infected with *V. coralliilyticus* showed tangled cilia and a lumpy-looking velum within a day or two post infection (Fig. 2B), which greatly restricted their motility. Shortly thereafter, the cilia were either digested or released into the surrounding milieu while individual velar cells were also released into the milieu. Usually within a day, the tissues within the valves appeared to liquefy by microbial digestion and were released from the valves (Fig. 1C). Eastern oysters exhibited the same pathology from *V. coralliilyticus*. Pathological signs of *V. tubiashii* infection were the same as for *V. coralliilyticus* infection in Eastern oysters, but *V. tubiashii* did not infect larval Pacific oysters in our studies.

Table 1. *Increased larval oyster mortality from four V. coralliilyticus strains due to elevated seawater temperatures*

***Vibrio coralliilyticus* strain**	**Percent mortality at 23°C**	**Percent mortality at 27°C**	**Increased mortality due to higher temperature**
ATCC BAA-450	23.4	38.8	15.4%
OCN008	54.9	78.8	23.9%
OCN014	50.9	93.7	42.8%
RE98	53.3	79.2	25.9%

Infectious Dose of *V. coralliilyticus* and *V. tubiashii* in Eastern and Pacific Oysters

Our studies showed that the dose of pathogens causing 50% mortality (the LD_{50}) for both Eastern and Pacific oysters varied somewhat based on the strain of *V. coralliilyticus* used. In general, LD_{50}'s ranged from approx. 1×10^3 to 1×10^4 CFU/ml of seawater. In the case of *V. tubiashii*, LD_{50}'s for strains ATCC 19106 and ATCC 19109 were 3.8×10^3 and 1.2×10^4 CFU/ml, respectively, in Eastern oysters; however, these strains were not infectious to Pacific oysters. These ATCC strains are obtained from the American Type Culture Collection, Manassas, Virginia (USA).

Effects of Elevated Seawater Temperature on Larval Mortalities

We also evaluated and determined that elevated seawater temperatures (27°C) increased larval oyster mortalities by 15.4% to 42.8%, depending on the *V. coralliilyticus* strain, compared to larval oysters maintained at 23°C (Table 1).

Virulence Genes in *V. coralliilyticus*

We also evaluated the ToxR transcriptional regulator and the outer membrane protein OmpU to determine if they were virulence factors in *V. coralliilyticus* infection of larval Pacific oysters. Studies with knockout mutations demonstrated that ToxR and OmpU in *V. coralliilyticus* were important in larval oyster (and coral) infections (Table 2). Mutants lacking ToxR had significantly reduced virulence compared to the wild type strain, while the mutant lacking OmpU had completely attenuated virulence at doses $<10^6$ CFU/ml. Larvae exposed to the OmpU mutant did not experience significantly more mortalities compared to replicates exposed to filtered sea water or a non-pathogenic bacterium, suggesting OmpU is essential for *V. coralliilyticus* infection of Pacific oyster larvae. Furthermore, both the ToxR and OmpU mutant are avirulent towards coral, suggesting these proteins play similar roles for infections of multiple hosts (Ushijima et al. 2016, 2018). In contrast, the mannose-sensitive hemagglutinin (MSHA) type IV pili that are required for coral infection (Ushijima et al. 2016) were not required for larval oyster infection (Ushijima et al. 2018).

The protein ToxR is believed to positively respond to various environmental signals, including elevated water temperatures, which is then involved with upregulating the expression of various genes, including the gene encoding OmpU. Therefore, ToxR is not believed to be directly involved with shellfish mortalities, however, represents a "molecular messenger" that translates environmental signals, most of which have yet to be identified, to physiological responses. Hence, understanding the conditions conducive to increased *V. coralliilyticus* virulence (i.e. the regulators of virulence) would complement, and potentially enhance, the various mitigation efforts described here.

Table 2. *Larval mortalities after deletion (Δ) mutation of various genes believed to be important for V. coralliilyticus virulence.*

Vibrio coralliilyticus **strain**	**Percent mortality at 23°C**	**Percent mortality at 27°C**	**Statistically different from wild type?**	**Statistically different from the negative control?**
Wild type	54.9	78.8	--	Yes
Δ*toxR* mutant	15.8	29.4	Yes	Yes
Δ*ompU* mutant	4.5	6.1	Yes	No
ΔMSHA mutant	44.1	72.4	No	Yes

Mitigation Hatcheries Strategies for *V. coralliilyticus* in Hatcheries

We have conducted considerable research over the past 10 years to isolate, characterize and test the efficacy of phages against *V. coralliilyticus* and *V. tubiashii* strains in Eastern and Pacific oyster larvae. From over 20 phages isolated and characterized, several have been shown to be lytic phages (based on genomic sequencing). Such lytic phages are desirable for use in mitigation efforts because their replication cycle consistently results in host death. Currently, we have phages against eight strains of *V. coralliilyticus,* including the principal strains associated with hatchery outbreaks on the West Coast (Table 3), and against *V. tubiashii* strain ATCC 19106. Proof-of-principle testing has shown that these phages can be combined in a cocktail to provide a safe treatment for *V. coralliilyticus* in hatcheries. Testing of a preliminary phage cocktail at a West coast hatchery at Oregon State University showed nearly a 100% reduction in mortalities after challenging larval oysters with a lethal dose of *V. coralliilyticus*. East coast hatchery trials are planned in the spring. The goal is to market a phage cocktail for use in hatcheries world-wide. Probiotic means to eliminate *V. coralliilyticus*, *V. tubiashii* and other pathogens in hatcheries are also being explored using the predatory bacteria *Halobacteriovorax* (a genus of *Bdellovibrio* and Like Organisms [BALO's]) and *Pseudoalteromonas piscicida*. Both predators have broad host specificity, with the *Halobacteriovorax* targeting Gram-negative bacteria and *P. piscicida* targeting both Gram-positive and Gram-negative bacteria.

Table 3. Eight strains of V. coralliilyticus killed by phage treatment.

Vibrio coralliilyticus **strain**	***Vibrio coralliilyticus*** **strain**
atccATCC BAA-450*	RE98
ATCC 19105*	RE09-105-8
RE22	OCN008
RE90	OCN014

*Indicates strains known to infect both corals and oyster larvae

CONCLUSION

There are many obstacles to successful hatchery operations. Since *V. coralliilyticus* and *V. tubiashii* appear to be opportunistic pathogens, anything that stresses larval shellfish makes them more susceptible to infection. High seawater temperature is one factor that enhances *V. coralliilyticus* virulence and may be managed by maintaining temperatures under 27°C in the hatcheries.

Likewise, close monitoring of other hatchery parameters (like salinity, dissolved oxygen, pH, food supply, larval densities, etc.) and adequate hatchery sanitation will reduce larval stresses to enhance productivity. With new treatments on the horizon to combat vibrios, the future is looking brighter for hatcheries and the commercial oyster industry as a whole.

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Richards, G.P., J.P. Fay, K.A. Dickens, M.A. Parent, D.S. Soroka, and E.F. Boyd. 2012. Predatory bacteria as natural modulators of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seawater and shellfish. Appl. Environ. Microbiol. 78:7455−7466.

Levels of the human pathogenic *Vibrio parahaemolyticus* and *Vibrio vulnificus* are increasing in shellfish harvesting areas in many parts of the world due, in part, to elevated seawater temperatures. A means to reduce these vibrios in shellfish is needed. When seawater and oysters were spiked with *V. parahaemolyticus* or *V. vulnificus*, the *Vibrio* levels rapidly diminished over a 3-day period when natural seawater was used, but counts quickly climbed when sterile seawater was used. In an effort to determine what was inhibiting the vibrios in natural seawater, tests showed the presence of *Vibrio* predatory bacteria, later classified as *Halobacteriovorax* species. The *Halobacteriovorax* in natural seawater readily eliminated vibrios that were added to the seawater. In contrast, oysters spiked with vibrios showed some initial increase in *Vibrio* counts within the first 24 h, followed by a rapid decline to baseline levels. Thus, it appears that *Halobacteriovorax* are one of nature's tools to modulate bacterial levels in shellfish. Harnessing this tool may provide a means to reduce or eliminate pathogens in aquaculture operations and in the seafood industry.

Richards, G.P., M.A. Watson, D.S. Needleman, K.M. Church, and C.C. Häse. 2015. Mortalities of Eastern and Pacific oyster larvae by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. Appl. Environ. Microbiol. 81:292−297.

Vibrio tubiashii has been a well-known pathogen in Eastern oyster (*Crassostrea virginica*) larvae on the United States (U.S.) East Coast for many years and has also been associated with Pacific oyster (*Crassostrea gigas*) larvae on the U.S. West Coast. *Vibrio coralliilyticus*, a well-known coral pathogen and a cause of coral bleaching, has also been associated with disease of Pacific oyster larvae, particularly in oyster hatcheries. It became evident that some of the outbreaks on the West Coast that were thought to be from *V. tubiashii* were actually caused by *V. coralliilyticus*. In this study, we evaluated two strains of *V. tubiashii* and four strains of *V. coralliilyticus* in both Eastern and Pacific oyster larvae to determine their ability to cause larval mortalities. We showed that *V. tubiashi*i strains infected primarily the Eastern oyster larvae while all four of the *V. coralliilyticus* strains infected the Eastern and Pacific oyster larvae. The LD_{50} values for the *V. coralliilyticus* strains ranged from 1.1×10^4 to 3×10^4 CFU/ml of seawater in Eastern oyster larvae. In Pacific oyster larvae, the LD_{50} range was 1.2×10^4 to 4×10^4 CFU/ml of seawater. Together, these studies defined the host specificity of these *Vibrio* strains with regard to Eastern and Pacific oyster larvae and demonstrated the ability of coral- associated pathogens to also infect and kill larval oysters. Subsequent research (Ushijima et al., 2018, see abstract below) showed similar results with other strains of *V. coralliilyticus* and an up-regulation of mortalities at elevated seawater temperatures.

Richards, G.P., J.P. Fay, J. Uknalis, M. Olanya and M.A. Watson. 2016. Purification and host specificity of predatory *Halobacteriovorax* isolates from seawater. Appl. Environ. Microbiol. 82: 922−927.

Halobacteriovorax are small predatory bacteria found in the marine environment and may serve as biocontrol agents against human, fish and shellfish pathogens. They are within a group of predatory bacteria known as the *Bdellovibrio* and Like Organisms (BALO). *Halobacteriovorax* were isolated from seawater and had broad specificity toward five strains of *Vibrio parahaemolyticus,* while two additional *Halobacteriovorax* strains isolated from low salinity seawater readily infected and killed the human pathogens *E. coli* O157:H7 and *Salmonella* Typhimurium DT104. The replication rate of *Halobacteriovorax* against *E. coli* and *Salmonella* increased as salinities decreased. It is likely that *Halobacteriovorax* could readily be isolated against a broad range of Gram-negative seafood pathogens. Improved methods were also developed to purify infectious *Halobacteriovorax* from their host cells. Overall, the use of *Halobacteriovorax* in various aquaculture applications may serve as an environmentally friendly, non-antibiotic treatment to reduce pathogens in fish and shellfish.

Richards, G.P., M.A. Watson, D.S. Needleman, J. Uknalis, E.F. Boyd, and J.P. Fay. 2017. Mechanisms for *Pseudoalteromonas piscicida*- induced killing of vibrios and other bacterial pathogens. Appl. Environ. Microbiol. 83:e00175-17, https://doi:10.1128/AEM.00175-17

Pseudoalteromonas are marine bacteria that are known to secrete antimicrobial compounds which inhibit competing bacteria in the marine environment. We identified a second method by which some *Pseudoalteromonas* (*P. piscicida*) kill competing bacteria. It involves the direct transfer of digestive vesicles from the surface of the *Pseudoalteromonas* to the surface of competitors, digestion of holes in the competitor's cell wall by proteolytic enzymes associated with the vesicles, and apparent feeding of the *Pseudoalteromonas* off the nutrients released by the digested bacterium in a predatory fashion. Among the *P. piscicida* enzymes identified were aminopeptidase B, a trypsin-like serine protease, a chymotrypsin-like serine protease, and a cysteine protease. *Pseudoalteromonas piscicida* inhibited and killed the pathogens *Vibrio parahaemolyticus, V. vulnificus, V. cholerae, Photobacterium damselae, Shewanella algae*, and *Staphylococcus aureus.* Together, this data indicates that *Pseudoalteromonas piscicida* produce important antibacterial compounds that have a potential role in the probiotic treatment of aquaculture products and in reducing biofilm formation.

Ushijima, B., G.P. Richards, M.A. Watson, C.B. Schubiger, and C.C. Häse. 2018. Factors affecting infection of corals and larval oysters by *Vibrio coralliilyticus*. PLoS One. https://doi:10.1371/journal.pone.0199475

Vibrio coralliilyticus is a naturally occurring marine bacterium which infects and kills corals and larval shellfish. It is best known as the cause of coral bleaching, which has contributed to the loss or damage to coral reefs worldwide. We determined that *V. coralliilyticus* strains also infect and kill larval oysters and other shellfish, particularly in shellfish hatcheries, causing shortages of seed oysters needed for commercial shellfish operations. We determined that larval oyster mortalities were significantly higher at an elevated seawater temperature (27°C) compared to a lower temperature (23°C). Thus, multiple coral pathogens were found to infect larval oysters in a temperature- and dose-dependent manner. Also identified were virulence factors that promote the infection of both coral and oyster larvae. This work demonstrates for the first time that elevated seawater temperatures enable *V. coralliilyticus* to more readily infect oyster larvae. It also serves to warn hatchery operators to maintain seawater temperatures below 27°C.

Cyst Distribution Patterns of the Paralytic Shellfish Poisoning Plankton Species *Alexandrium catenella* and *A. pacificum* off the Pacific Coast of Eastern Japan

Tomoko Sakami[1*], Tetsuroh Ishikawa[2], and Toru Udagawa[3]

[1] National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, 422-1 Nakatsuhamaura, Minami-ise, Mie, Japan 516-0193
[2] Miyagi Prefectural Government, 3-6-16, Honcho, Aoba-ku, Sendai, Miyagi, Japan 980-0014
[3] National Research Institute of Fisheries Engineering, Japan Fisheries Research and Education Agency, 7620-7, Hasaki, Kamisu, Ibaraki, Japan 314-0408

Corresponding author: sakamisiro3@gmail.com

Keywords: paralytic shellfish poisoning*, cyst, Alexandrium catenella, Alexandrium pacificum*

ABSTRACT

Mainly two dinoflagellate plankton species, *Alexandrium catenella* and *A. pacificum,* cause paralytic shellfish poisoning in Japan. To examine how the two *Alexandrium* species' cysts distribute off the Pacific coast of eastern Japan, we measured cyst abundance of the two species by quantifying their specific gene abundance in DNA extracted from marine sediments. Both species were detected in some ria-type inner bays in the Sanriku region. However, only *A. catenella* was detected from the Joban area, which is located to the south of Sanriku region, opening widely to the Pacific Ocean. In addition, only *A. pacificum* was detected in the Sotobo area, which is located to the south of the Joban area and is under the strong influence of the warm Kuroshio current. These results suggest that the distribution patterns of the two toxic phytoplankters differ depending on topography and the ocean current system.

INTRODUCTION

Paralytic shellfish poisoning (PSP) is caused by eating bivalves contaminated with a marine biotoxin that is produced by microalgae. In Japan, it is a serious problem for fishermen because many bivalves cause PSP, and a voluntary shipment regulation is carried out when a PSP is detected from their products. PSP occurs in the north-east Pacific coast and western part of Japan, Seto inland sea, and Kyushu area. However, PSP does not occur in the middle parts of the Pacific coastal area and Japan sea coastal area except in a few inner bays. Moreover, PSP occurrence has been shown to increase suddenly, for example, after the Great East Japan Earthquake in 2011 (Ishikawa *et al*., 2015). It is, therefore, essential to study the risks of PSP outbreaks in shellfish aquaculture areas without previous reports. One of the important precautions is monitoring of the toxin producing organisms. There are four PSP producing phytoplankton species in Japan. *Alexandrium catenella* and *A. pacificum* are the major causative organisms. These phytoplankton species used to be called *A. tamarense* and *A. catenella,* respectively; however, recent molecular work has indicated a new nomenclature for species within the *Alexandrium tamarense* species complex (Litaker *et al*., 2018). *A. catenella* prefers cold water and often causes PSP in the northern areas of Japan. On the other hand, *A. pacificum* prefers warm water. It is found mainly in the western part of Japan, and also in north-eastern Japan during summer. The toxicity level of *A. pacificum* is relatively low compared to *A. catenella* and is not generally considered to cause PSP in the north-eastern Japan areas.

These phytoplankton produce a cyst after blooming stimulated by nutrient depletion. The germinating plankton become a seed population in the year of PSP outbreaks. To assess risks of PSP outbreaks, cyst abundances are often investigated in the area. The fluorescent dye method is commonly used to determine *Alexandrium spp.* cyst abundance (Yamaguchi *et al*., 1995). However, it is difficult to determine whether the cysts belong to *A. catenella* or *A. pacificum* species (Nagai *et al*., 2012). There are some reports where molecular techniques have been used for identification of *A. catenella* and *A. pacificum* cysts (Kamikawa *et al*., 2007; Erdner *et al*., 2010). In this study, we measured cyst abundances of *A. catenella* and *A. pacificum* by quantifying their specific gene abundance in the DNA extracted from marine sediments to examine how the cysts of the two *Alexandrium* species are distributed off the Pacific coast of eastern Japan.

METHODS

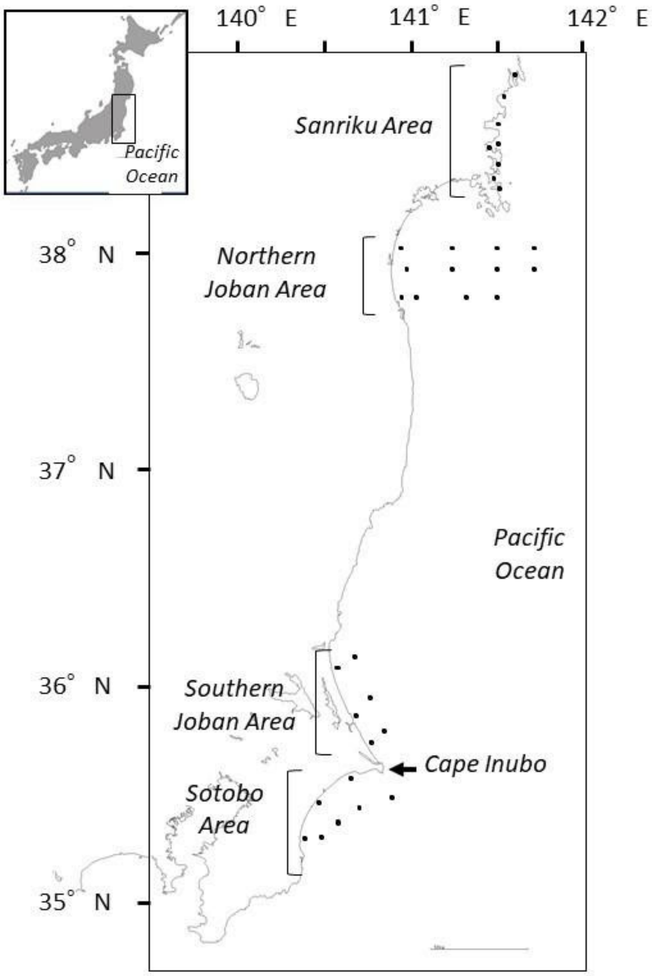

Figure 1. Sample collection points along the Pacific Coast of eastern Japan in 2015, located in the Sanriku rias coastal area, northern part of the Joban area, southern part of the Joban area, and the Sotobo area.

Sediment samples were collected from the Sanriku area, northern and southern parts of the Joban area, and the Sotobo area (Fig. 1). The Sanriku area is a rias coast where mountains approach the sea and the coast line is very complex. The Joban and Sotobo areas are geographically flat and are widely open to the Pacific. The Sanriku and Joban areas are regions where the cold Oyashio current mixes with the warm Kuroshio current. The Sotobo area is mainly under the influence of the Kuroshio current. PSP generally occurs in the Sanriku and Joban areas but not in the Sotobo area.

Sediment samples were collected using a grab or core type bottom sampler during the summer of 2015 when *Alexandrium* vegetative cells were not observed. In the Sanriku area, samples were collected at 3-12 points for each examined bay. A surface layer sediment of approximately 2 cm depth was taken and kept in cold and dark conditions immediately after collection. Samples were preserved at -30 °C until DNA extraction.

DNA was extracted from marine sediments using a commercial kit, FAST DNA-SPIN kit for soil (MP Biomedicals). DNAs of *A. catenella* and *A. pacificum* were amplified by qPCR from the sediment DNA using specific primer sets reported by Nagai (2011). PCR reactions were performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) with ABI StepOne™ System (Applied Biosystems). Plasmid DNA (pTaq2, DynaExpress co. ltd) into which the PCR target fragment was inserted was used as a standard DNA. To estimate the density of cysts, one cyst was picked under the microscope, its DNA extracted, and the copy number determined using qPCR.

RESULTS

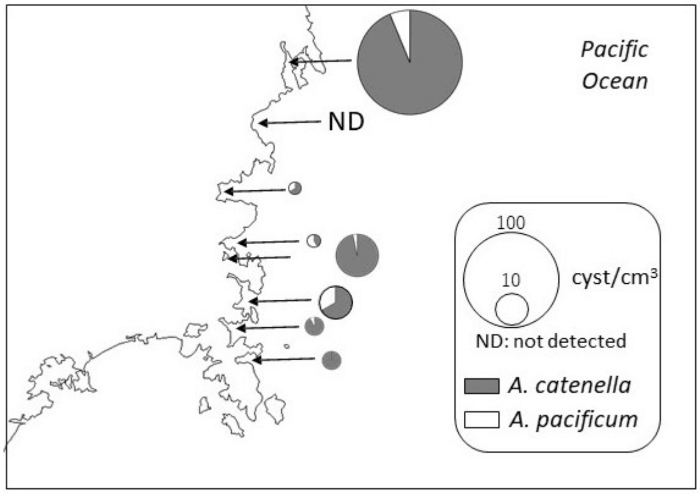

Figure 2. Cyst abundances of Alexandrium catenella and A. pacificum determined by qPCR in sediments collected in the Sanriku rias coastal area

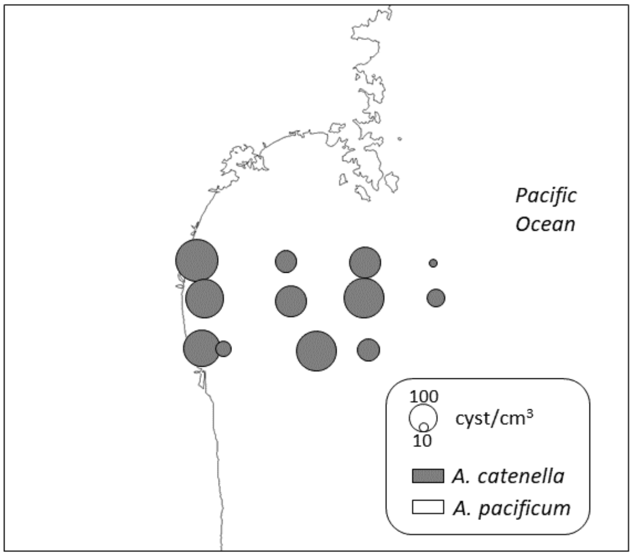

Figure 3. Cyst abundances of Alexandrium catenella and A. pacificum determined by qPCR in sediments collected in the northern part of Joban area.

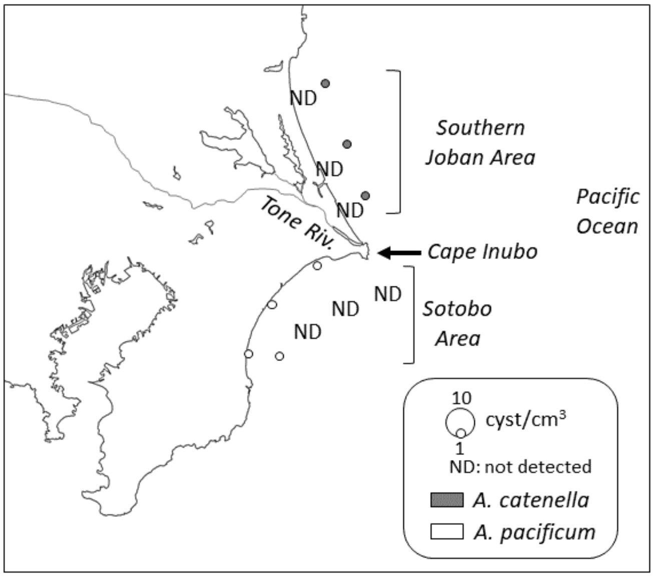

Figure 4: Cyst abundances of Alexandrium catenella and A. pacificum determined by qPCR in sediments collected in the southern part of Joban area and the Sotobo area.

In the Sanriku rias coastal area, both *A. catenella* and *A. pacificum* cysts were detected in the sediment samples, except in one small bay located at the southernmost part of the area (Fig. 2). The proportion of *A. catenella* cysts was found to increase with the total cyst abundance. In the northern Joban area, only *A. catenella* cysts were detected (Fig. 3). Cyst abundance was high in this area compared to the rias coastal area. Also in the southern part of the Joban area, only *A. catenella* cysts were detected, although the density observed was very low (Fig. 4). In the Sotobo area, only *A. pacificum* cysts were detected and the density was as small as that observed for *A. catenella* cysts in the southern Joban area.

DISCUSSION

Both *Alexandrium* species cysts were detected in the Sanriku rias coastal area, whereas only *A. catenella* was detected in the Joban area. It is presumed that *A. pacificum* can survive in the Sanriku area because of high water temperature, which allows it to grow in summer in the small shallow bays. Although *A. pacificum* is not considered to cause PSP, recently PSP is found to occur during high temperature periods in the Sanriku area. The relationship between PSP occurrence and population dynamics of the two *Alexandrium* plankton should be studied, as these species concomitantly occur in some areas (Sakami unpublished data) and their vegetative cells have very similar forms. Therefore, it is important to monitor both species of PSP-causing phytoplankton to assess PSP outbreak risks in the Sanriku Rias areas, where cysts of both species are present.

In the Joban area, only cysts of *A. catenella* were detected. A high cyst density of more than one hundred per square centimeter was observed at the offshore area, more than 50 km away from the coastline with a water depth greater than 100 m. This result suggests that not only coastal areas, but offshore areas should be investigated for the presence of PSP-causing plankton. The cyst density was found to be much higher in the northern part than in the southern part of the Joban area. One of the reasons for the lower cyst density in the southern part is that the sediment in the southern part is sand, in which cyst densities are generally low (Yamaguchi *et al*., 2002; Shimada and Miyazono, 2005; Yamamoto *et al*., 2009). In the past fifteen years, PSP occurred in the northern and southern part in thirteen years and seven years, respectively. Therefore, the difference in cyst abundance seems to indicate the difference in PSP outbreak risk in these areas.

In the Sotobo area, cysts of only *A. pacificum* were detected. The Sotobo area, located south to Cape Inubo, is under the strong influence of the strong and warm Kuroshio current which runs along Japan's south coast. The lowest water temperature is 12-13°C to the north of Cape Inubo, and 14-15°C in the Sotobo area. The water temperature may be too high for *A. catenella* in the Sotobo area. There is a report that *A. catenella* cysts seldomly germinate at temperatures higher than 15°C (Yamamoto *et al*., 2009). In fact, the lowest water temperatures are around 10-13°C in Hiroshima and Osaka Bays in the western part of Japan, where *A. catenella* causes PSP. In addition, the river mouth of the Tone River, the largest river in Japan, opens to the north of Cape Inubo. The large freshwater discharge can be a barrier for the distribution of the phytoplankton. Considering that PSP occurrence has not been reported in the Sotobo area, it seems that distribution of *A. catenella* is limited to the south of Cape Inubo.

In conclusion, we determined cyst abundances of two PSP-causing *Alexandrium* species by using a qPCR method from samples collected off the Pacific coast of eastern Japan. We observed that: 1) both *A. catenella* and *A. pacificum* cysts are found in the Sanriku rias coastal area, 2) only *A. catenella* cysts are found in the Joban flat coastal area, extending to the southern tip area where the warm Kuroshio current mixes with the cold Oyashio current and 3) only *A. pacificum* cysts are found in the Sotobo area under the strong influence of the Kuroshio current. These results suggest that the cyst distributions of the two species are influenced by the coastal geographic features, the water temperature and/or the current systems in different ways. Although more data are required to clarify relationships between cyst distribution and environmental factors, our study seems to indicate that cyst abundances and/or fluctuation patterns are useful parameters for forecasting PSP outbreak risks in shellfish aquaculture areas.

ACKNOWLEDGEMENTS

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Yamaguchi, M., S. Itakura, K. Nagasaki, and Y. Kotani. 2002. Distribution and abundance of resting cysts of the toxic *Alexandrium* spp. (Dinophyceae) in sediments of the western Seto Inland Sea, Japan. Fish Sci. 68: 1012–1019.

Yamamoto, K., Y. Nabeshima, M. Yamaguchi, and S. Itakura. 2009. Distribution and abundance of resting cysts of the toxic dinoflagellates *Alexandrium tamarense* and *A. catenella* in 2006 and 2007 in Osaka Bay. Bull Jpn Soc Fish Oceanogr. 73(2): 57–66. (in Japanese with English abstract)

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Baerwald, M. R., M. H. Meek, M. R. Stephens, R. P. Nagarajan, et al. 2015. Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. Molecular Ecology 25: 1785-1800.

This study investigates the role of epigenetics (DNA methylation) in migration-related life history traits in *Oncorhynchus mykiss*. The authors used reduced representation bisulfite sequencing to perform comparative DNA methylation analysis between juvenile resident and smolt F2 siblings generated from a cross between steelhead (migratory) and rainbow trout (nonmigratory). Fifty-seven differentially methylated regions, many of which were in gene regulatory regions, were identified between residents and smolts, suggesting a relationship between epigenetic variation and variation in migration-related phenotypes.

Gavery, M.R. and S.B. Roberts. 2013. Predominant intragenic methylation is associated with gene expression characteristics in a bivalve mollusc. PeerJ 1: e215.

This dataset, generated using methylation-enriched high-throughput bisulfite sequencing, represents the first high-resolution methylome in any mollusc. DNA methylation data were compared to gene expression datasets and a positive relationship between intragenic methylation and gene expression levels was identified. These data suggest that DNA methylation patterns may play a role in regulating gene expression in molluscs.

Navarro-Martín, L., J. Viñas, L. Ribas, N. Díaz, A. Gutiérrez, et al. 2011. DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. PLoS Genetics 7: e1002447.

The authors report that in European sea bass (*Dicentrarchus labrax*), which exhibits temperature-dependent sex determination, exposure to high temperature in early development was associated with increased DNA methylation in the promoter of the aromatase gene (*cyp19a1a*) and a higher proportion of phenotypic males. Furthermore, *in vitro* methylation of the aromatase promoter was sufficient to suppress transcription of the gene, supporting a role for DNA methylation as a mechanistic link between temperature and sex ratios in species exhibiting temperature-dependent sex determination.

Potok, M. E., D. A. Nix, T. J. Parnell, and B. R. Cairns. 2013. Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. Cell 153: 759-772.

Genome-wide DNA methylation patterns in zebrafish gametes, various stages of embryos and a somatic tissue (muscle) were analyzed using whole genome bisulfite sequencing. This high-resolution approach identified dynamic and unique patterns of DNA methylation during development in zebrafish. Results suggest that the functional significance of sperm DNA methylation patterns in fish is to provide transcriptional competency to the early embryo, which 'inherits' the DNA methylation pattern in the sperm.

Shao, C., Q. Li, S. Chen, P. Zhang, J. Lian, et al. 2014. Epigenetic modification and inheritance in sexual reversal of fish. Genome Research 24: 604-615.

The half-smooth tongue sole (*Cynoglossus semilaevis*) was used as a model to investigate the role of epigenetic regulation in species with environmental sex determination. Using genome-wide bisulfite sequencing of normal male, female and pseudomale fish (generated by exposing genetic females to high temperature during a sensitive developmental window), the authors showed that sex-reversed genetic females exhibit methylation patterns consistent with genetic males, both of which differ from the methylome of normal females. Furthermore, it was reported that global methylation patterns are inherited by F1 pseudomale offspring generated by crosses between temperature-induced sex-reversed pseudomales and normal females, suggesting transgenerational epigenetic inheritance of sex reversal in this species.

Gavery, M.R. and S.B. Roberts. 2014. A context specific role for DNA methylation in bivalves. Briefings in Functional Genomics doi:10.1093/bfgp/elt054.

A review of current knowledge of DNA methylation in bivalves. A primary conclusion is that the functional role of the gene could influence the role of DNA methylation in influencing expression.

Olson, C.E. and S.B. Roberts. 2014. Indication of family-specific DNA methylation patterns in developing oysters. bioRxiv doi: http://dx.doi.org/10.1101/012831

This study provides the first single-base pair resolution DNA methylomes for both oyster sperm and larval samples from multiple crosses. While sample sizes are very low, this work suggests DNA methylation patterns could be inherited.

Roberts, S. 2015. Compilation of DNA Methylation Genome Feature Tracks (*Crassostrea gigas*). figshare https://dx.doi.org/10.6084/m9.figshare.145626.v2

Genome feature tracks and accompanying IGV session file to visualize DNA methylation data for the Pacific oyster (*Crassostrea gigas*).

Trends in Aquaculture Production in Japan

Satoshi Watanabe* and Tomoko Sakami

Fisheries Technology Institute, Japan Fisheries Research and Education Agency, 2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa, 236-8648, Japan

Corresponding author: swat@affrc.go.jp

Keywords: demographic problems, earthquake, tsunami, declining industry

ABSTRACT

A wide variety of aquatic organisms are commercially cultured in Japan. For marine species, national statistical data is published by the Ministry of Agriculture, Forestry and Fisheries on the aquaculture production of seven species of teleost fish (*Oncorhynchus kisutch, Seriola* spp., *Trachurus japonicus*, *Pseudocaranx dentex*, *Pagrus major*, *Paralichthys olivaceus*, *Tetraodontidae* spp., *Thunnus orientalis*), two species of bivalves (*Mizuhopecten yessoensis* and *Crassostrea* spp.), one species of prawn (*Marsupenaeus japonicus*), one species of tunicate (*Halocynthia roretzi*), and four species of aquatic plants (*Saccharina* spp., *Undaria* spp., *Pyropia* spp. and *Nemacystus* spp.). Four freshwater species of teleost fish (*Oncorhynchus mykiss*, *Plecoglossus altivelis*, *Cyprinus carpio* and *Anguilla japonica*) are also included in the statistics. Despite the historically strong affinity of Japanese consumers to seafood, production of aquaculture and capture fisheries are on a long-term moderate decreasing trend largely due to socio-economic factors in Japan. The total marine aquaculture production decreased from 1.28 × 10^6 t in 1996 to 1.03 × 10^6 t in 2016. Bivalve aquaculture production was relatively constant around 4.5 × 10^5 t. The production of oysters (*Crassostrea* spp., mainly Pacific oyster) and Japanese scallop (*M. yessoensis*) was heavily damaged by the Great East Japan Earthquake and subsequent tsunamis in 2011, and it has not recovered to the level prior to the earthquake. Teleost fish and seaweed production are on a continuous decreasing trend. Teleost fish production was not severely affected by the earthquake, except for Coho Salmon (*Oncorhynchus kisutch*), whose production level quickly recovered. Although the production of some seaweeds such as kelps (*Saccharina* spp.), *wakame* (*Undaria* spp.) and laver or *nori* (*Pyropia* spp.) was devastated by the earthquake, not only in the Tohoku region but in many parts of Japan, the long-term decreasing trend is not attributed to the earthquake. One of the causes for the production decline is considered to be the reduced number of management bodies due to the aging of the operators and shortage of successors.

The number of management bodies of *nori* culture, for instance, decreased from 51,354 in 1963 to 3,819 in 2013. Mergers of the management bodies is one of the reasons for the decrease, but the number of farmers is certainly decreasing. On the other hand, aquaculture production of teleost fish and bivalves has increased in value in recent years. The increase in teleost fish production is attributable to the recent inclusion of Pacific bluefin tuna (*T. orientalis*), which was added to the MAFF statistics list in 2012. For bivalves, production of the common scallop leaped from JPY 25.7×10^9 in 2012 to JPY 62.4 ×10^9 in 2016. In freshwater aquaculture, the Japanese eel (*A. japonica*) has by far the largest production in volume and value. The seeds for eel aquaculture are all wild caught, and the low availability of glass eel has been problematic in recent years. Prompt establishment of mass production technologies for eel seed is desired.

INTRODUCTION

Being surrounded by the sea, fish and seafood have been staple foods for the Japanese since ancient times. Japan has a long history of aquaculture, which mythically dates back to the era of Emperor Keiko's (BC13 – AD103, archeologically estimated to be in the 4th century) reign (Higurashi, 1912). The Emperor had a recreational fishing pond to which cultured fish were released for his amusement. Goldfish were introduced to Osaka from China in 1502, and they have been cultured in Japan ever since. Aquaculture of laver or *nori* (*Pyropia tenera*) is thought to have started in Shinagawa Bay, Tokyo, in the Bunki era (1684-1688) (Higurashi, 1912).

The history of aquaculture in Japan was summarized in a textbook (Tanigawa et al., 1966). For example, Gorozaemon Konishiya started pole (bouchot) aquaculture of oysters (*Crassostrea* spp) in Hiroshima Bay during the Enpo era (1673 - 1681), and it had been commonly operated in coastal intertidal areas with appropriate environmental conditions in Hiroshima Bay, Matsushima Bay, and Ariake Sound until the suspended culture method was established by Seno and Hori in 1923. The suspended culture grew significantly because of its high productivity and applicability in various environmental conditions. Japanese eel (*Anguilla japonica*) aquaculture was established by Kurajiro Hattori in Tokyo in 1879, and commercial scale culture was started in an 80,000 m^2 pond in Shizuoka Prefecture in 1897.

Japanese eel has by far the largest production by volume and value in inland aquaculture in Japan today. Yellowtail (*Seriola quinqueradiata*) aquaculture was started by Sakichi Amino in Kagawa Prefecture in 1927. It drastically expanded in western Japan after the end of World War II, owing to the introduction of synthetic fibers for net cages and the establishment of wild seed collection methods. Kuruma prawn (*Marsupenaeus japonicus*) aquaculture techniques are the fruit of 30-years of research and development by Motosaku Fujinaga (1903-1973). Some salmonids cultured or that used to be cultured in Japan were introduced from the U.S.: rainbow trout (*Oncorhynchus mykiss*, from Shasta, CA, in 1877), brook trout (*Salvelinus fontinalis*, in 1901-1902) and brown trout (*Salmo trutta*, accidentally introduced with rainbow trout). In addition to these, various other aquatic organisms are commercially cultured in Japan. Despite the historically strong affinity of the Japanese for seafood and fish, aquaculture production is on a long-term moderate decreasing trend largely due to socio-economic factors in Japan. This report describes the present status of aquaculture in Japan, and some of the problems confronting Japan's aquaculture industry.

MATERIALS

The data analyzed and presented in this report are based on the national statistical data for aquaculture production published by the Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF). The statistical data on the aquaculture production by volume and value of major species are available online in the Japanese language (http://maff.go.jp/j/tokei/kouhyou/kensaku/bunya6.html).
For marine aquaculture, the MAFF statistical data contain seven species of teleost fishes (*Oncorhynchus kisutch, Seriola* spp., *Trachurus japonicus*, *Pseudocaranx dentex*, *Pagrus major*, *Paralichthys olivaceus*, *Tetraodontidae* spp., *Thunnus orientalis*), two species of bivalves (*Mizuhopecten yessoensis* and *Crassostrea* spp.), one species of prawn (*Marsupenaeus japonicus*), one species of tunicate (*Halocynthia roretzi*) and four species of seaweeds (*Saccharina* spp., *Undaria* spp., *Pyropia* spp. and *Nemacystus* spp.). Four freshwater species of teleost fishes (*Oncorhynchus mykiss*, *Plecoglossus altivelis*, *Cyprinus carpio* and *Anguilla japonica*) are also included in the MAFF statistics.

There are also miscellaneous groups, such as other teleost fish, other molluscan shellfish and other seaweeds; production of each species is relatively small and pooled together for convenience. Some freshwater species were dropped from the statistical list after 2000 because of the relatively minor importance to the industry and the budgetary constraints of the related agencies. These species include the Japanese silver crucian carp (*Carassius auratus langsdorfii*), tilapia, Chinese soft-shelled turtle (*Pelodiscus sinensis*), and other miscellaneous groups.

Statistical data of the Fisheries Census is available online at the e-Stat website in Japanese and English languages (https://www.e-stat.go.jp/en). The statistical data are made available by the Minister of Internal Affairs and Communications to reveal the situation surrounding the fisheries industry in Japan, such as the production and employment structures of fishing communities, and the distribution and processing of fishery products. The data in the census include the number of fishery cooperatives, fisheries management entities, fisheries workers and fishing vessels.

Marine aquaculture

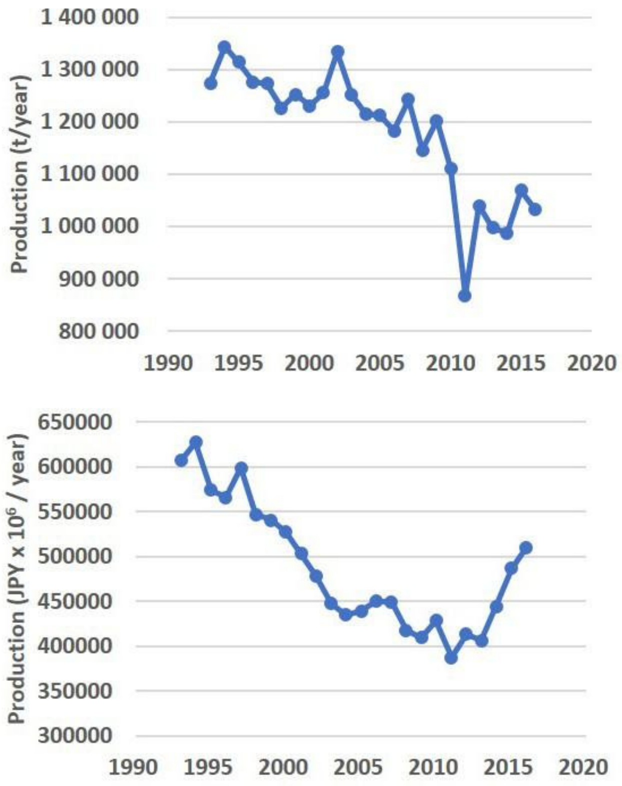

Figure 1. Total marine aquaculture production in Japan by volume (a) and value (b).

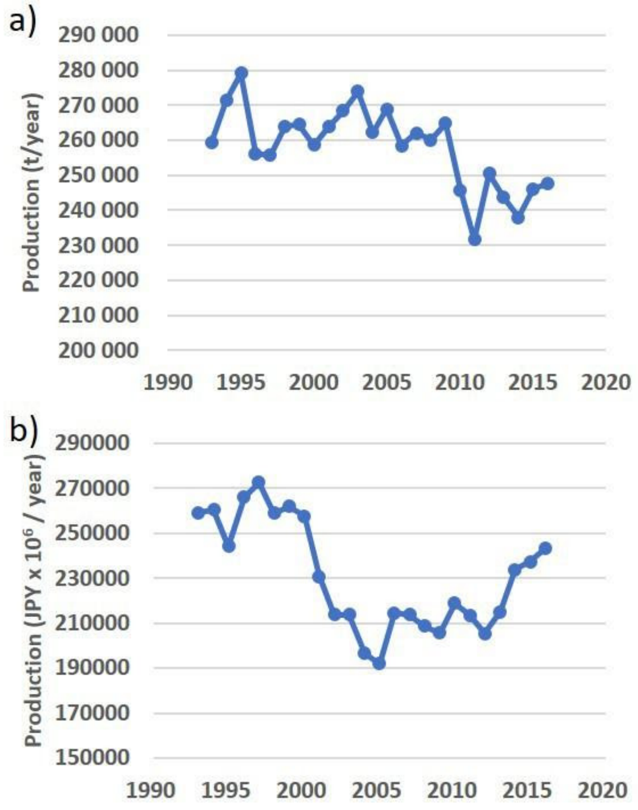

Fig. 2. Aquaculture production of marine teleost fish in Japan by volume (a) and value (b).

Total marine aquaculture production gradually and continuously decreased from 1996 (1.28×10^6 t, metric tons) to 2016 (1.03×10^6 t), i.e. a 19.6% decrease over the 20 years in Japan (Fig. 1a). It showed a sharp drop in 2011 (8.69×10^5 t) due to the damage caused by the Great East Japan Earthquake and consequent tsunamis, and it went back to the long-term decreasing track in 2012. The total marine aquaculture production by value, on the other hand, continuously decreased until hitting the bottom in 2011. It has been on an increasing trend since 2012 (Fig. 1b). The production value in 2016 was JPY 5.10×10^{11} (approx. US$ 4.72×10^9). A similar trend is seen in the production of marine teleost fish. The total aquaculture production volume of teleost fish is on a long-term moderate decreasing trend, with a drop in 2011 due to the earthquake and tsunamis (2.56×10^5 t and 2.48×10^5 t in 1996 and 2016, respectively; Fig. 2a).

Teleost fish aquaculture was not severely affected by the earthquake, except for Coho Salmon (*O. kisutch*), whose production level drastically decreased from 1.48×10^4 t in 2010 to 116 t in 2011 because it was conducted mostly along the north-east pacific coast close to the epicenter. However, it quickly recovered to 9.73×10^3 t in 2012.

Yellowtail (*Seriola quinqueradiata*, *S. dumerili* and *S. lalandi*) and red seabream (*P. major*) are the two major marine teleost species cultured in Japan (1.41×10^5 t and 6.70×10^4 t, respectively in 2016), and they had relatively minor damage to production. Yellowtail production was greater in 2011 (1.46×10^5 t) than in 2010 (1.39×10^5 t). Damage to yellowtail aquaculture was relatively minor since the main production area is in western Japan distant from the epicenter. About 12% of the yellowtail produced in Japan is exported, and the US is the largest importer (85% by value). The production of red seabream was on a long-term decreasing trend before the earthquake, the extent of which made the damage by the earthquake appear insignificant. The production, however, began an increasing trend after 2013.

Contrary to production volume, production value of teleost fish decreased from 1997 to 2005 (JPY 1.92×10^{11}) and thereafter turned into an increasing trend regardless of the earthquake (Fig. 2b). Production value was JPY 2.43×10^{11} in 2016. The recent increase in teleost fish production is in part attributable to the new inclusion of Pacific bluefin tuna (*T. orientalis*) to the MAFF statistics list in 2012. Kindai University established the complete aquaculture technique of Pacific bluefin tuna in 2002 and started providing hatchery produced juveniles to the private sector in 2007.

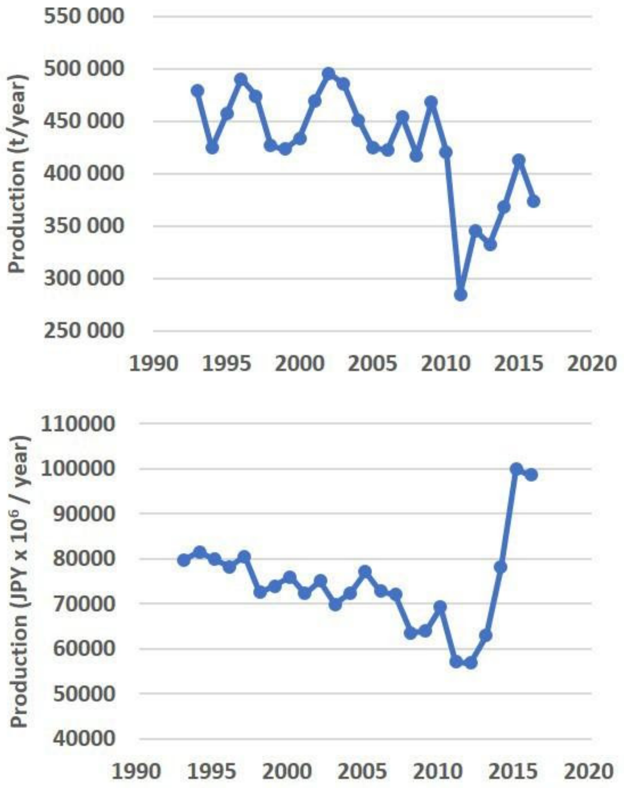

Fig. 3. Aquaculture production of marine molluscan shellfish in Japan by volume (a) and value (b).

The production of marine molluscan shellfish aquaculture was relatively constant around 4.5 × 10^5 t from 1996 to 2010 (Fig. 3a). However, production was severely impacted by the earthquake in 2011 (2.85 × 10^5 t) and has not recovered to the level prior to the earthquake as of 2016 (3.74 × 10^5 t).

The production value of marine molluscan shellfish, however, showed a big leap after the earthquake (Fig. 3b) increasing by 1.73 times from 2012 (JPY 5.69 × 10^{10}) to 2016 (JPY 9.86 × 10^{10}). The production of oysters (mainly Pacific oyster, *C. gigas*) and Japanese scallop (*M. yessoensis*) were both heavily affected by the earthquake. Japanese scallop production was around 2.3 × 10^5 t before it declined to 1.18 × 10^5 t in 2011. It recovered to 2.48 × 10^5 t in 2015, exceeding the value in 2010 (2.20 × 10^5 t). The production value of the scallop showed a big leap after 2012 (JPY 2.57 × 10^{10}), reaching JPY 6.24 × 10^{10} in 2016.

Oyster production, on the other hand, was on a long-term decreasing trend before the earthquake (2.2 × 10^5 t and 2.0 × 10^5 t in 1996 and 2010, respectively) and the trend persisted after the earthquake (1.66 × 10^5 t and 1.59 × 10^5 t in 2011 and 2016, respectively). The production value of oysters also showed an increase after the earthquake but to a lesser extent compared to that of the scallop (JPY 3.04 × 10^{10} t and 3.54 × 10^{10} in 2012 and 2016, respectively).

Seaweeds are important commodities in Japan as human food. Laver or *nori* (*Pyropia* spp, recategorized from *Porphyra*), for instance, has the second largest aquaculture production value (JPY 1.00 × 10^{11} in 2016) following yellowtail (JPY 1.18 × 10^{11}) in Japan. The aquaculture production of seaweeds is on a long-term decreasing trend both in volume and value (Fig. 4). The production volume

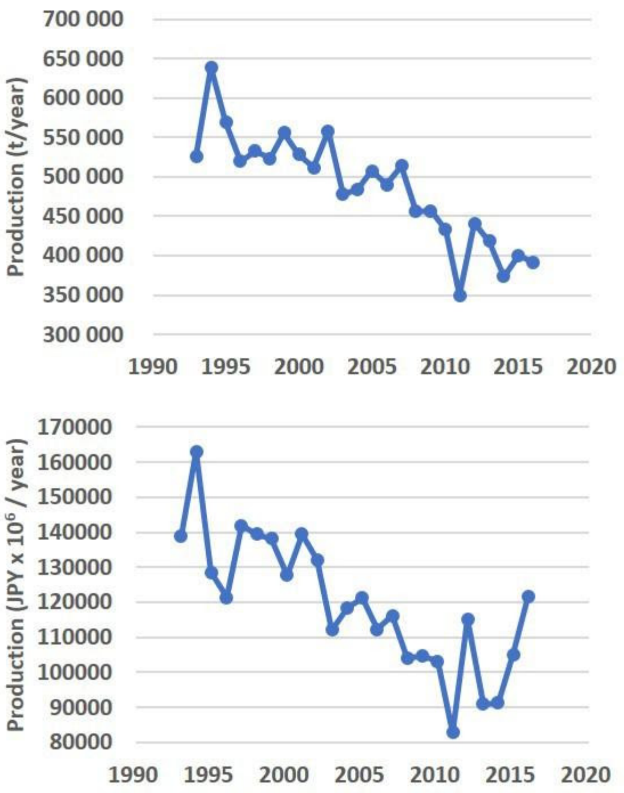

Fig. 4. Aquaculture production of seaweed in Japan by volume (a) and value (b).

and value of seaweed were 3.91 × 10^5 t and JPY 1.22 × 10^{11}, respectively in 2016. These were 25.6% and 12.3% less than those in 1993.

Aquaculture of kelps (*Saccharina* spp,) and *wakame* (*Undaria* spp.) are operated mainly in the Tohoku and Hokkaido regions and were damaged by the earthquake in 2011. Production of *wakame* decreased from 5.24 × 10^4 t in 2010 to 1.88 × 10^4 t in 2011 (i.e. 64% decrease), and it quickly increased to 4.83 × 10^4 t in 2012. Production of kelp was reduced by 42% from 2010 (4.33 × 10^4 t) to 2011 (2.51 × 10^4 t). Damage to *nori* aquaculture was relatively minor since the main production area is in western Japan.

Inland aquaculture

Japan's inland (freshwater) aquaculture production is small compared to marine aquaculture, only about 3% and 20% by volume and value, respectively in 2016. Japanese consumers generally choose seafood in preference to freshwater fish. Japanese eel (*A. japonica*) may be the only exception, although it is catadromous and not a freshwater species in a strict sense. It is not common to culture freshwater molluscs, crustaceans and algae in Japan. Major freshwater aquaculture species in the global market, such as carp, catfish, and tilapia are not consumed in large quantities in Japan.

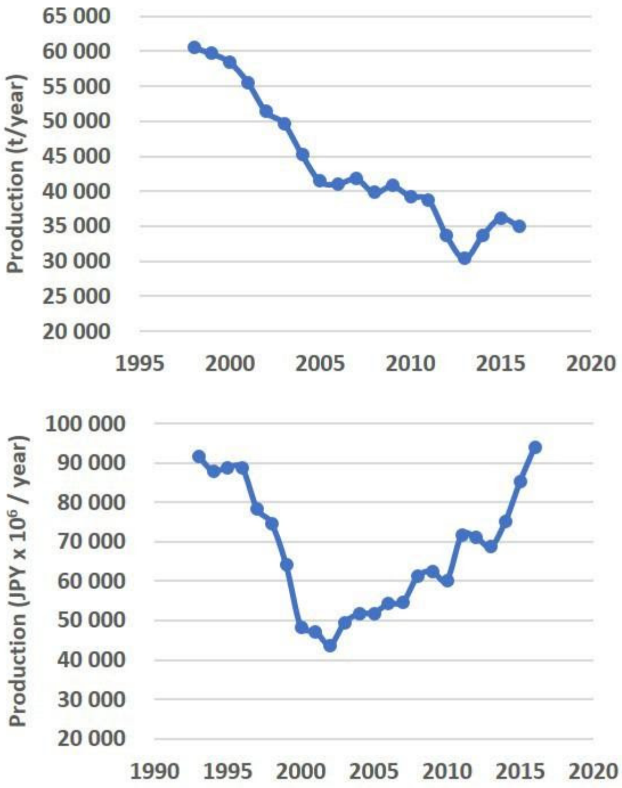

Fig. 5. Total inland aquaculture production in Japan by volume (a) and value (b)

The production volume of freshwater teleost fish has been on a continuous decline (Fig. 5a). The production decreased by 42% from 1998 (6.05 × 10^4 t) to 2018 (3.50 × 10^4 t). The production value of freshwater teleost fish decreased from 1993 (JPY 9.20 × 10^{10}) to 2002 (JPY 4.38 × 10^{10}) and has thereafter been on an increasing trend (Fig. 5b). The production value in 2016 (JPY 9.40 × 10^{10}) exceeded that in 1993.

Japanese eel has by far the largest aquaculture production, comprising about 70% of the total freshwater aquaculture production value. The seeds for eel aquaculture are currently all wild caught, and the low availability of the glass eel has been problematic in recent years. Japanese eel is listed as an endangered species in the IUCN Red List of Threatened Species. The unit price for the glass eel is soaring, marking the highest value (JPY 3.0 × 10^6/ kg) in 2018 (Fisheries Agency, 2019), while it was JPY 1.6 × 10^5 / kg in 2003. Studies on artificial spawning of Japanese eel were started in the 1960s, and the National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency finally succeeded in the complete aquaculture of Japanese eel at laboratory scale in 2010. However, technical difficulties remain to be overcome in scaling up the seed production in laboratory tanks to full industrial scale. Prompt establishment of mass production technologies for eel seeds is desired.

Declining aquaculture activities in Japan

As above, aquaculture production volume of many species has been on a decreasing trend in Japan for the past two decades. The earthquake and tsunamis had a big impact on the production of some of the species in 2011, but the long-term decreasing trend is not attributed to natural disasters. Although production values of some species are increasing due to increasing unit price, it can be said that aquaculture activities are generally dwindling in Japan. This is also true for fisheries activities as a whole in Japan.

One of the causes for the production declines is considered to be the reduced number of aquaculture management bodies due to the aging of the operators and a shortage of successors. The number of management bodies of yellowtail aquaculture, for instance, decreased 81.8% from 3,473 in 1978 to 632 in 2013. Soaring feed prices are putting pressure on the profits of fed aquaculture of teleost fish. Similarly, the number of management bodies of *nori* culture decreased by 92.6% from 1963 (51,354) to 2013 (3,819). The number of aquaculture farmers is decreasing in Japan. As of 2016, the fishing industry employed 166,000 people, which was about half the number in 1993 (325,000 people). The average age of fishing industry employees is 56.7 years old as of 2016. Merger is one of the reasons for the decrease in the number of the management bodies, but the majority of them are still small-scale, self-owned businesses, which are less competitive than enterprises within a large capitalist economy.

This is not simply a chicken-and egg-situation. Japan is facing a labor shortage due to demographic problems; Japan's population is shrinking and the average age is rising because of a low fertility rate. The rising education level of workers makes them reluctant to be engaged in menial jobs. It seems that the socio-economic reconstruction of the fisheries society is needed for future development of aquaculture and fisheries in Japan.

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Despite the increasing global demand for aquaculture products, aquaculture business management is not necessarily sustainable in Japan.

Some aquaculture companies producing pufferfish and red seabream went bankrupt in 2010 due to low fish prices and increasing feed prices. The author analyzed the supply and demand and price change trends of fishmeal and discussed their effects on aquaculture business in Japan. The author urged the importance of counter measures for insufficient fishmeal supply, such as use of unutilized fish and development of low fishmeal feed.

Preliminary Experiments on Using the Seaweed Extract, AMPEP, to Enhance Thermal Tolerance and Growth of the Sugar Kelp, *Saccharina latissima*, from New England

Schery Umanzor[1,3*], Sookkyung Shin[2], Simona Augyte[1], Charles Yarish[1], and Jang K. Kim[2]

[1] Department of Ecology & Evolutionary Biology, University of Connecticut, Stamford CT 06901- 2315, USA
[2] Department of Marine Science, Incheon National University, Incheon 22012, Korea
[3] Department of Marine Biology, University of Alaska Fairbanks, Juneau AK 99801

Corresponding author: Sumanzor@alaska.edu

Keywords: *Ascophyllum nodosum*, biostimulants, thermal stress, suboptimal temperature

ABSTRACT

Seaweed extracts are widely used as biostimulants to enhance the performance of land crops. Acadian Marine Plant Extract Powder (AMPEP), an extract derived from *Ascophyllum nodosum,* that has also been reported to enhance growth of *Kappaphycus alvarezii*, a red seaweed crop. Here we assessed if AMPEP can enhance the thermal tolerance of the kelp *Saccharina latissima*. To assess the effects of AMPEP, we first exposed early sporophytes to different concentrations (0.001, 0.005, 0.05, 1 and 5 mg L^{-1}) of the product and allowed individuals to grow for 20 days at an optimal temperature of 12 ±1°C. After determining that there were no significant differences in sporophyte growth related to the concentration of AMPEP, we transferred the sporophytes to the suboptimal temperature of 18°C where they remained for 14 days. Throughout the experiment, the photoperiod was maintained at 12:12 L:D with a photosynthetically active radiation (PAR) of 90 ±10 µmol m^{-2} s^{1}. We found that sporophytes previously exposed to AMPEP showed a higher percentage of survival and growth than control sporophytes never exposed to AMPEP. Sporophytes also showed thicker blades and a darker brown color than control sporophytes. These preliminary results indicate that AMPEP may enhance growth of the sugar kelp, *Saccharina latissima*, when exposed to suboptimal temperatures.

INTRODUCTION

The kelp farming industry is undergoing a fast global expansion, becoming one of the fastest growing industries in western countries (Cottier-Cook et al. 2016, Kim et al. 2017). In the United States for example, kelp farming, mainly of the sugar kelp – *Saccharina latissima* – is emerging as a sustainable aquaculture industry that is revitalizing the working waterfronts of New England by offering new economic opportunities for seafood production while at the same time removing excess nutrients from the coastal marine environment (Yarish et al. 2017). To keep its momentum, kelp aquaculture in the region needs to overcome challenges that may result in slowing its growth. For instance, it is necessary to invest in the development of strains that can resist diseases, biofouling, and increasing seawater temperature, while maintaining rapid growth rates and higher yields (Kim et al. 2017).

Rising seawater temperatures are already impacting most temperate marine ecosystems, affecting wild biomass production as a whole (Wernberg et al. 2016, 2018). Because kelp farming is a key component of aquaculture production (De Silva and

Soto 2009, Kim et al. 2017), rapid adaptive measures should be considered to support expanding operations. In the United States, there are several ongoing selective breeding programs as part of a national effort to domesticate and improve (*e.g.,* enhance thermal tolerance) native kelp cultivars, including *Saccharina spp.* (MARINER, 2017).

Experiments conducted principally on the economically important red alga, *Kappaphycus alverezii*, highlight what could be a faster alternative to enhance seaweed strains (Loureiro et al. 2014b, Marroig et al. 2016, Souza et al. 2018). These studies have shown that commercial extracts obtained from certain seaweeds can be used to improve the resistance and vigor of other non-related seaweeds and terrestrial plants. For example, similar to the results obtained in agriculture (Khan et al. 2009), applying AMPEP (Acadian Seaplants Ltd., Canada), a seaweed extract from *Ascophyllum nodosum*, to cultivars of *Kappaphycus alvarezii* resulted in growth enhancement (Hurtado et al. 2009; Tibubos et al. 2017; Souza et al. 2018), reduction in epiphyte loads, and higher thermal tolerance (Loureiro et al. 2014b; Hurtado and Critchley 2018).
Saccharina latissima, as other kelp species, is vulnerable to changes in water temperature. It shows an optimal growth between 10 and 15°C (Lee and Brinkhuis 1988, Egan et al. 1989, Yarish et al. 1990, Augyte et al. 2017), which decreases with temperatures exceeding 16°C, while 100% mortality is observed for temperatures exceeding 23°C (Lee and Brinkhuis 1986). Here we report on the effects of applying AMPEP to *S. latissima* as a potential method to increase thermal tolerance and enhance growth under optimal and suboptimal temperature conditions.

METHODS

The experiment consisted of testing the effect of AMPEP on the thermal tolerance and growth of juvenile sporophytes (500 µm in length) of *Saccharina latissima*. Sporophytes were produced by fertilizing gametophytes (SL18-UCONN-CC) isolated and cultivated in laboratory conditions from parental sporophytes collected from Cape Cod Canal (lat. 41.773818N; long. 70.499448W).

A stock of AMPEP solution was prepared by dissolving 10 g of AMPEP in 1L of filtered and sterilized seawater. We then prepared five dilutions (0.001, 0.005, 0.1, 0.5, and 1 g L $^{-1}$) and a control solution consisting of 100 ml filtered and sterilized natural seawater (30 ppt). Each of the AMPEP solutions and the control was then used to dip 36 kelp juvenile sporophytes (500 µm in length, 15 days old); 6 kelp sporophytes per treatment plus 6 controls, where they remained for 45 minutes. Immediately after the 45-minute dipping time, kelp sporophytes were rinsed with seawater and two sporophytes (pseudoreplicates) per treatment and control were transferred to 50 ml Erlenmeyer flasks containing nutrient-enriched seawater (half strength Provasoli Enriched Seawater (Provasoli 1968) plus germanium dioxide (GeO_2) following Lewis (1966) to inhibit the development of diatoms. Each pair of blades (N= 3 per treatment) was allowed to grow for 20 days at 12±1 °C with a PAR of 90 ±10 µM photons $m^{-2} s^{-1}$. Blades were photographed every five days using a PixeLINK® camera mounted onto an Olympus (SZH) dissecting scope. Photographs were processed as binary images using Fiji by Image J (Schindelin et al. 2012) to calculate the area occupied by each blade as a proxy to measure growth.
After the 20 days, the sporophytes were transferred to 125 ml Erlemeyers flasks containing media as described above. They were allowed to grow for 15 additional days inside an incubator set at the suboptimal temperature of 18° ±0.5°C. This allowed us to measure if the exposure to AMPEP promoted an increase in thermal tolerance by facilitating a higher percentage of survival and growth. Sporophytes were photographed at the end of the 15- day period and final blade area was calculated. After photographing, all of the survivors were cross-sectioned using a razor blade and photographed again to examine what appeared to be potential differences in the thickness of treated and non- treated blades.

Data analysis

The specific growth rate (SGR, expressed as percent increase day^{-1}) of juvenile kelp sporophytes was calculated as:

$$SGR = (Ln(A1) - Ln(A2)) \div (\ T1 - T2)\ x\ 100$$

where A1 and A2 are the area (mm^{-2}) at time T1 and T2, respectively (Kim et al. 2009).

Differences in SGR were analyzed using repeated measures ANOVA as a function of concentration of AMPEP (0, 0.001, 0.005, 0.1, 0.5, and 1 g L^{-1}) and time. For all of the analyses, significance was P > 0.05 unless otherwise noted.

RESULTS

Early juvenile sporophytes of *Saccharina latissima* showed the highest specific growth rates (SGR) at day ten post-treatment with AMPEP (Fig.1). Results show no significant difference in the SGR as a function of the concentration of AMPEP when growing at 12°C ($P > 0.05$). Nonetheless, when sporophytes were exposed to 18°C, treated samples did show a higher percentage of survival (Fig. 2), darker brown color, and increased overall growth (Fig. 3) compared with control sporophytes, which looked pale.

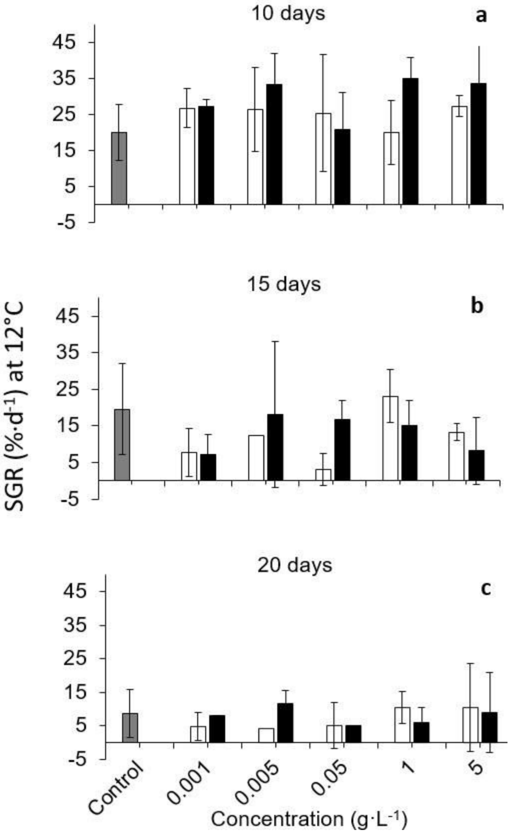

Figure 1. *Specific growth rates (mean values ±SE, N=3) of Saccharina latissima as a function of AMPEP concentration and time. The absence of error bars indicates only one survivor per treatment.*

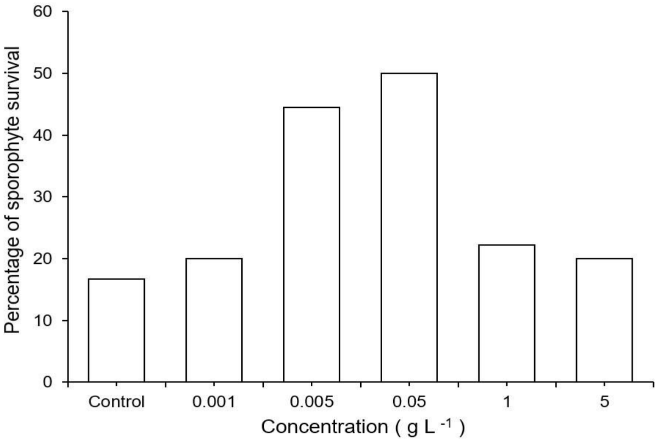

Figure 2. *Percentage of survival of juvenile sporophytes of Saccharina latissima growing at 18°C after exposure to different AMPEP solutions.*

Cross-section measurements also revealed that treated sporophytes showed thicker blades than non-treated individuals. Blade thickness of treated sporophytes (avg. 0.8 cm in length) was on average 1.5 ±0.2 mm, while thickness of control sporophytes was 0.4 ±0.03 mm. We could not confirm significance for either survival, growth, or thickness due to a reduced number of survivors across treatments.

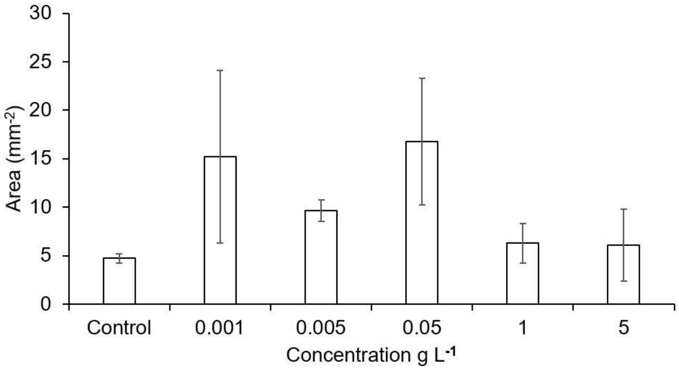

Figure 3. *Final area of treated and control blades of Saccharina latissima exposed to 18°C for 15 days.*

DISCUSSION

The commercial extract AMPEP derived from *Ascophyllum nodosum* has been used widely in agriculture and horticulture for the beneficial effects of enhancing yields and reducing damage caused by external biotic and abiotic stressors in a variety of land crops (refer to Craigie 2011 for a review). Similarly, studies conducted on red seaweeds have revealed a variety of benefits with an overall increase in seaweed vigor (Hurtado et al. 2012, Loureiro et al. 2014a, Marroig et al. 2016, Souza et al. 2018). To date, we are not aware of any study evaluating the effect of a brown seaweed extract (intertidal, Fucales) on kelp (Laminariales).

Hurtado and colleagues (2012) found that the use of AMPEP on *Kappaphycus alvarezii* growing in optimal conditions triggered higher growth rates than control thalli never exposed to AMPEP, and that low concentrations of the product (< 0.1 g L^{-1}) resulted in higher growth rates than higher concentrations (i.e. 1.0 g L^{-1}). In our study, we found that applying AMPEP to *Saccharina latissima* juvenile sporophytes growing at ideal 12°C did not result in increased growth rates.

However, similar to our observations related to the darker color exhibited by treated sporophytes, other experiments conducted on *K. alvarezzi* also report that at suboptimal temperatures samples without any AMPEP treatment showed bleaching of the thallus (Loureiro et al. 2014b). The dark brown coloration exhibited by the surviving kelp sporophytes exposed to AMPEP could be related to varying concentrations of fucoxanthin, however further analyses are required to establish this association.

Furthermore, Loureiro et al. (2014) also found that applying AMPEP to *Kappaphycus alvarezii* increased its tolerance to survive when exposed to suboptimal colder temperatures. Here we detected that by applying AMPEP to sugar kelp, individuals seem to increase their probability of thriving at the sublethal higher temperature of 18°C. Additionally, as in land plants, seaweed extracts applied to other seaweeds stimulate the increase of phytohormones, which in turn promote cell division and elongation (Khan et al. 2009, 2017, Hurd et al. 2014, Li and Mattson 2015, Hurtado and Critchley 2018), possibly related to the increased thickness observed on our treated blades. Nevertheless, although a higher number of treated sporophytes survived and these exhibited overall thicker and bigger blades than non-treated samples, a larger number of replicates is required to confirm significance. Also, field experiments are required to test how treated samples will perform over time in open ocean conditions and if there are any inheritable responses. However, our data suggests that applying AMPEP to juvenile kelp sporophytes while in the nursery could enhance thermal tolerance of *Saccharina latissima*, allowing treated blades to grow larger than non-treated counterparts over the same period.

ACKNOWLEDGEMENTS

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This contribution derives from the author's presentation at the "XX International Seaweed Symposium". Craigie provides an overview of the utilization of seaweeds by humans, and more relevant, a summary of seaweeds used in agriculture. Furthermore, this paper details with examples how the application of seaweed as bio-stimulants enhances land crops in different ways. The author includes brand names, countries of origin, and nutritional content of various commercially available products.

Tibubos, K.R., A.Q. Hurtado, and A.T. Critchley. 2017. Direct formation of axes in new plantlets of *Kappaphycus alvarezii* (Doty) Doty, as influenced by the use of AMPEP K^+, spindle inhibitors, and plant growth hormones. J Appl Phycol. 29: 2345-2349.

This is a recent contribution describing the effect of AMPEP K^+ on the formation of vegetative shoots of *Kappaphycus alvarezii*. AMPEP K^+ is a Canadian commercial extract of the brown seaweed *Ascophyllum nodosum* with enhanced levels of potassium. The authors tested this extract in combination with colchicine or oryzalin, in addition to synthetic plant growth regulators. They found that 5 mg L^{-1} of AMPEP K^+ with plant growth regulators produced the longest direct axis shoots (9.6 ± 0.33 mm), followed by 0.1 mg L^{-1} of AMPEP K^+ combined with 1.0 mg L^{-1} oryzalin and plant growth regulators (8.7 ±0.00 mm). Their results suggest that using AMPEP K^+ could improve shoot formation of *K. alvarezii* plantlets in land-sea-based nursery cultivation.

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The majority of publications related to using seaweed extracts on the aquaculture of other seaweeds have assessed the effect of *Ascophyllum nodosum* extracts on *Kappaphycus alvarezii.* This review mainly summarizes studies made on *K. alvarezii* exposed to different treatments of *A. nodosum* extracts. *A. nodosum* extracts are utilized to mitigate biotic and abiotic stressors of *K. alvarezii* subject to farming conditions. The authors provide examples related to micropropagation, field cultivation, endophyte mitigation, and impacts on the resulting carrageenan qualities of different *K. alvarezii* cultivars. In addition, the authors also mention alternative seaweed extracts that have been applied to boost the growth of other cultivated seaweeds. Altogether, this review paper provides evidence of the favorable results of using *Ascophyllum* extracts as biostimulants for *Kappaphycus* aquaculture.

Challenges to Establishment of Tetraploid Breeding Stocks for Shellfish Aquaculture

Huiping Yang

Fisheries and Aquatic Sciences Program, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32653

Corresponding author: huipingyang@ufl.edu

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ABSTRACT

In the Gulf of Mexico region, oyster farming has been rapidly increasing in recent years, in part, to help meet the seafood market demand that has resulted from a decline in fishery landings since 2012. For the oyster farming industry, triploids are recognized as the preferred products because of their fast-growth, better meat quality, and year-round harvest. With the rapidly growing oyster farming industry, there has been a significant increase in demand for oyster triploid seed. Seed shortages have occurred in the past several years and are becoming a major constraint to the industry. To address the demand for triploid oyster seed, establishment of tetraploid breeding stocks are needed because tetraploids are key for commercial all-triploid seed production. With support from the Gulf States Marine Fisheries Commission in cooperation with the National Oceanic and Atmospheric Administration's National Marine Fisheries Service, tetraploid induction research was initiated. It is expected that establishment of tetraploid breeding stocks will be useful for triploid seed production with diversified genetic resources to support the oyster farming industry. In this publication, challenges for tetraploid production were discussed to provide insights and better understanding of triploid-tetraploid technology for shellfish aquaculture.

INTRODUCTION

Molluscan shellfish aquaculture has traditionally been a major component of aquaculture production worldwide and occurs mostly in marine water environments (98.3% of the total production). In 2016, the total molluscan aquaculture production worldwide was 17.1 million metric tonnes ($29.2 billion) and the cultured molluscan species included oysters, clams, scallops, and mussels (FAO, 2018).

Sustainability of this large-scale aquaculture industry requires considerable efforts to increase product quantity and quality, improve aquaculture environments, and enhance the well-being of the community. Use of genetic breeding is one of the important strategies because genetically selected broodstock can produce well-adapted offspring or seed. For shellfish aquaculture, triploid-tetraploid technology has been widely recognized by the industry and utilized in many farming species, especially oyster species (Guo, 2004). Triploids grow fast, have better meat quality in summers, and probably resist diseases in aquaculture bivalve species (Guo et al., 2009, Yang et al., 2018). So far, farming of triploids has become an important part of the global oyster industry. For Pacific oysters, *Crassostrea gigas*, triploids account for about 50% of the production on the west coast of the United States, 100% of the hatchery seed production in France (Degremont et al., 2016), and over 2.3

billion triploid seed in China (Yang et al., 2019). For eastern oysters, *Crassostrea virginica*, triploids account for nearly 100% of the seed production in the Chesapeake Bay (Peachey and Allen, 2016) and a majority of oyster seed in the Gulf of Mexico (Wadsworth et al., 2019). For Sydney rock oysters, *Saccostrea glomerata*, triploids account for about 15% of the production in Australia (Peachey & Allen 2016).

Triploids can be produced by directly inhibiting the first or second polar body or crossing of diploids and tetraploids (Yang et al., 2018). Commercially, production of triploids is usually achieved by crossing (mating) oocytes from diploids with sperm from tetraploids in most hatcheries worldwide (Guo et al., 1996) because this method can produce 100% triploids and ease to operation by commercial hatchery staff (Yang et al., 2018). Therefore, establishment of tetraploid breeding stocks is becoming a critical factor for the application of triploid-tetraploid technology in shellfish aquaculture.

Establishment of Tetraploid Breeding Stocks

Tetraploid induction in shellfish was initiated in the 1980s and has been a challenge due to the poor survival of tetraploid larvae to beyond the metamorphosis stage (Guo et al., 2009, Yang et al., 2019). In 1994, a unique methodology for tetraploid induction was reported in Pacific oysters and the induced tetraploid larvae survived to the adult stage (Guo and Allen, 1994c) and were used for all-triploid seed production by crossing with normal diploids (Guo et al., 1996). This innovative method used oocytes from triploid females to fertilize with sperm from diploid males and was followed by inhibition of the first polar body (PB 1) (Guo and Allen, 1994c). So far, this method has been applied to several other shellfish species and produced viable tetraploids in Pearl oysters *Pinctada margaritifera* (He et al., 2000), eastern oysters *Crassostrea virginica* (Guo et al., 2002), Sumino oysters *Crassostrea ariakensis* (Allen et al., 2005), bay scallops *Argopecten irradians* (Surier et al., 2012), and Catarina scallops *Argopecten ventricosus* (Maldonado et al., 2003). Tetraploid stocks have been mostly established in oyster species by using this method and applying it to commercial triploid seed production.

Challenges for Tetraploid Production

The process to produce tetraploids using this innovative method includes the following steps: 1) produce triploids from normal diploids and culture to adult stage; 2) identify triploid individuals and find fecund triploid females; 3) collect oocytes from triploid females; 4) induce tetraploids by fertilizing oocytes from triploids with sperm from diploids and following polar body inhibition; 5) culture putative tetraploid larvae to beyond metamorphosis and adult stage, and 6) confirm tetraploid individuals by non- lethal ploidy determination as founders for breeding stocks. Each of these steps is connected to one another and problems which could occur at any step would result in failure of the tetraploid production.

During this process, the big challenges for successful tetraploid production are the infrequent occurrence of fecund triploid females, limited availability of oocytes from triploid females, and poor survival of putative tetraploid larvae to beyond metamorphosis (Yang et al., 2019).

1) Occurrence of fecund triploids.

Triploids possess three sets of chromosomes and could have abnormal chromosome synapsis and segregations during gametogenesis. Therefore, triploids generally exhibit abnormal gonad development. To obtain fecund triploid females for tetraploid induction, a large number of triploids need to be produced and screened. For oyster species, occurrence of fecund triploids differed depending on species. For example, about one female out of 1,600 triploid eastern oysters (i.e., 0.06%) was observed (Supan, 2000), but for Pacific oysters, higher proportions (58%) of fecund females were observed in triploids (Guo and Allen, 1994a). For *Crassostrea hongkongensis*, most of the triploids exhibited polymorphic sterility with atrophic gonads and abnormal gametogenesis (Zhang et al., 2017). For *Soccostrea commercialis*, triploids were found highly retarded (Cox et al., 1996). In other shellfish groups, the occurrence of fecund triploid females also varied. For soft-shell clams, *Mya arenaria*, 77% of triploids were females (Allen et al., 1986), for Dwarf surfclams, *Mulina lateralis*, 59% of triploids were females (Guo and Allen, 1994b), and for Noble scallops, *Chlamys nobilis,* 25% of triploids were females (Komaru and Wada, 1989). Ongoing research on eastern oysters in my laboratory showed the female occurrences in one-year old triploids were 0.6% (5 out of 840), 3.5% (23 out of 630), and 1.2% (14 out of 1142) in three populations originating from different geographic locations.

Additionally, for the same species (eastern oysters), different cohorts of triploids may have significantly different occurrences of fecund triploids (Dr. John Supan, personal communication). Even for the same cohort of triploids (eastern oysters), the occurrences of fecund triploids varied greatly in different culture locations (Yang, personal observation in 2019). A comparison of diploid and triploid gametogenesis in Pacific oysters indicated that high levels (42 to 50%) of gametogenesis occurred in triploids in all nine triploid batches in different culture locations, and could be the reason for the summer heavy mortality of these triploids (Houssin et al., 2019).

For the mechanism of triploid female gametogenesis, a specific gene (Nanos-like gene) was reported to be perhaps responsible because this gene was expressed in the gonads of diploid females matching with gonad seasonal development, and was expressed significantly lower in the gonads of sterile triploids (Xu et al., 2018). Another two genes were also found to be correlated with triploid gametogenesis (Jiang et al., 2017). Observation of gametogenesis in triploids of Pacific oysters identified two types of gametogenesis: α-pattern, which corresponded to animals displaying numerous proliferating gonia and resulted in abundant gametes, and p-pattern, which was associated with locked gametogenesis (only few mature gametes at sexual maturity) with accumulation of abnormal gonia (Jouaux et al., 2010).

2) *Limited availability of oocytes from triploid females.*

Oocytes from triploid organisms usually have abnormal meiosis due to the three sets of chromosomes. Thus, the number of oocytes from triploid females is usually low (Gong et al., 2004). Efforts have been made to improve gonad development in triploids, such as applying estradiol hormones to eastern oyster triploids (Quintana, 2005). The availability of oocytes varied from 100 to 1.6 million per female in one-year-old triploids (n = 41, Yang, unpublished data), and could be related to culture locations and seasons sampled.

3) *The poor survival of induced tetraploid larvae.*

This is a major challenge for tetraploid production. In Pacific oysters, the survival of putative tetraploid larvae to beyond metamorphosis was 0% in two replicates and 0.0739% in one replicate (Guo and Allen, 1994c). Thus, to obtain viable tetraploid juveniles (after metamorphosis), it is necessary to have enough oocytes (at least 10,000) from triploid females. Additionally, tetraploid mussel larvae were found smaller than diploids and triploids in the same cohort (Scarpa et al., 1993) and the same situation was observed in Pacific oysters (Benabdelmouna and Ledu, 2015). Therefore, smaller larvae may be disposed of during larval culture. Extensive care is needed for culture of putative tetraploid larvae by saving slow growers and maintaining good culture conditions to ensure they can survive beyond metamorphosis.

SUMMARY

Triploid-tetraploid technology has been applied to commercial oyster aquaculture in many countries for years. Establishment of tetraploid breeding stocks is the key for the application of this technology, but many challenges exist. For successful tetraploid production, great efforts are needed to address the key challenges, especially further investigations on triploid gametogenesis, which can increase the occurrence of triploid females for oocyte collection for tetraploid production.

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Tetraploid production for all-triploid seed production

Tetraploid production has been a challenge in shellfish due to their poor survival to spat and adult stages despite strong efforts on this research topic since the 1980s. In 1994, Guo and Allen reported a unique methodology for tetraploid induction in the Pacific oyster. The tetraploids survived to an adult stage and were used for all-triploid seed production by crossing with normal diploids (Guo et al., 1996). This innovative method for viable tetraploid spat and adult production uses oocytes from triploid females to fertilize with haploid sperm, followed by inhibition of the first polar body (PB 1) (Guo and Allen 1994). Later, this method was applied to other oyster species and led to the production of viable tetraploids in the Pearl oyster *Pinctada margaritifera*, eastern oyster *Crassostrea virginica*, and Somonie oysters *Crassostrea ariakensis* (He et al. 2000, Guo et al. 2002, Allen et al. 2005). This unique methodology has been patented by Rutgers University (US5824841A) and adopted by a private company. So far, tetraploid breeding stocks have been successfully established mostly in oyster species (including Pacific oysters, eastern oysters, Sydney rock oysters, and Suminoe oysters) by using this method and applied for commercial triploid seed production.

McCombie, H., C. Ledu, P. Phelipot, S. Lapegue, P. Boudry, and A. Gerard. 2005. A complementary method for production of tetraploid *Crassostrea gigas* using crosses between diploids and tetraploids with cytochalasin B treatments. Marine Biotechnology*,* 7**:** 318-330.

An alternative method for tetraploid production by using tetraploid founders

Complementarily, another novel approach has been reported in the Pacific oyster using tetraploid founders (McCombie et al. 2005) to produce viable tetraploids by fertilizing oocytes from diploids with sperm from tetraploids followed by inhibition of the polar body 2 (PB2). This method offers an alternative way for producing more tetraploids after production of initial founders and avoids the use of oocytes from triploid females, which is always a big challenge. This is an approach to diversify, change, and enrich the genetic background of tetraploid populations (50% from mother in the 1st generation, and probably increased to 100% from mother in the second generation, depending on the chromosome segregation in sperm formation).

Guo, X., Y. Wang, Z. Xu, and H. Yang. 2009. Chromosome Set Manipulation in Shellfish. Pages 165-194. in G. Burnell & G. Allen editors. New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management. Woodhead Publishing Limited, Abington, Cambridge.

Reviews and summaries

This book chapter systematically reviewed shellfish polyploid breeding, including triploid and tetraploid production, concepts, mechanisms, history, updates, and future application for the aquaculture industry. In this publication, two comprehensive tables are included to summarize triploid production, growth, and performance, and tetraploid induction and breeding. In addition, there are several figures included in this review to describe the chromosome segregation mechanism for triploid, tetraploid and aneuploid production.

Yang H., N. Simon, L. Sturmer. 2018 Production and performance of triploid oysters for aquaculture. Electronic Data Information Source - UF/IFAS Extension, http://edis.ifas.ufl.edu/fa208.

Yang H, X. Guo, and J. Scarpa. 2019. Induction and establishment of tetraploid oyster breeding stocks for triploid oyster production. Electronic Data Information Source - UF/IFAS Extension. https://edis.ifas.ufl.edu/fa215.

Updated reviews in extension-style publications for oyster triploid-tetraploid technology

These two publications summarized the history, mechanisms, procedures, and updates about oyster triploid-tetraploid technology. Different from other journal publications, these two publications were written in extension style with glossaries as appendixes that are understandable for general public and industry communities. Download of these two publications is free through the website links stated above from the Electronic Data Information Source, Institute of Food and Agricultural Sciences Extension, University of Florida.

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