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Sea Anemone *Aiptasiomorpha minuta* (Verrill, 1867) as a Possible Agent to Control Biofouling in Oyster Culture and the Optimal Conditions for Its Mass Rearing under Laboratory Conditions

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Abstract: The potential use of the sea anemone *Aiptasiomorpha minuta* as an agent for controlling biofouling on cultured oysters and the optimum culture conditions for its mass culture were evaluated. Field experiments showed that nineteen species (eight phyla), including two seaweed species, sponges, hydroids, bryozoans, mollusk/bivalve species, barnacles, and tunicates were found as biofouling assemblages on the oyster collectors. The ability of *A. minuta* to accumulate biomass on oyster collectors, thus, minimizing colonization by problem species; was also demonstrated to promote better oyster growth, condition index, and survival. Favorable mass culture conditions of *A. minuta* in laboratory trials were found at 28 °C, fed with *Artemia salina* (1000 individuals/day), and at 23 psu for the optimum temperatures, diet regimen, and salinity, respectively. These mass culture conditions could be useful for the purpose of producing enough biomass for attaching the sea anemones, *A. minuta*, to oyster collectors. The use of *A. minuta* could be a preventive strategy against biofouling that may be useful for oyster farmers; it is safe from the viewpoint of food hygiene, and is also environment-friendly.

Keywords: oyster aquaculture; biological control; biofoulers; predation; marine invertebrates

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1. Introduction

The sea anemone *Aiptasiomorpha minuta* (Verrill, 1867) is a common species found in the intertidal zones of the southern part of Honshu Island, Japan, attached to mussels, oysters, and other hard substrates. While it is considered indigenous to Japan, it is thought that its introduction to undistributed areas was the result of the species escaping through effluents from aquarium facilities as well as through the restocking of short-neck clam seeds in various coastal areas of the country [1]. Uchida and Soyama [2] described this species as having blurred vertical stripes on its column and being translucent under poor light conditions (Figure 1). It is considered a pest in aquarium tanks and by fish hobbyists because it reproduces rapidly and is difficult to remove once introduced into the facilities by using raw seawater or natural sand from the beach.

Sea anemones of the order Actiniaria, are known to reproduce both sexually and asexually by their polyps [3], although sexual reproduction has not been described in some species such as *Aiptasia pallida* (Agassiz in Verrill, 1864). Fertilization of eggs during sexual reproduction is either performed externally or internally. Asexual reproduction is common in sea anemones and occurs by transverse or longitudinal fission, pedal laceration, or autotomy of tentacles [3]. Parthenogenesis has also been described as a form of asexual reproduction in sea anemones [4]. Reproduction and growth of many sea anemone species are known to be affected by exogenous factors, i.e., temperature, food regimen, etc. Grawunder et al. [5] demonstrated that by simulating the lunar cycle with a blue wavelength light,

gametogenesis and spawning in *Aiptasia* sp. were induced under laboratory conditions. Little is known about the biology of *A. minuta*.

Figure 1. Photo of the sea anemone *Aiptasiomorpha minuta* (pedal disk diameter = 5 to 7 mm) grown under laboratory conditions fed with *Artemia salina*.

In Japan, oyster cultures are conducted in both intertidal and deep-water areas, using either raft or long-line hanging methods [6]. Farmers put their oyster spats in rafts from April to May and leave them to grow until winter (between December to March) with infrequent checking. One of the major problems faced by oyster growers is the occurrence and growth of biofouling organisms (hereafter referred to as biofoulers), which compete for space and food with cultured species [7]. Common biofoulers seen in oyster cultures include barnacles, hydroids, mussels, polychaetes, and tunicates, among others [8]. In Isahaya Bay in Nagasaki, Japan, the presence of biofoulers in oyster cultures lead to low productivity due to mortality and poor growth [7]. Oyster farmers either remove biofoulers from oysters by hand, by mechanical removal (pressure washing), or by outplanting to a beach where desiccation and scouring by wave action remove fouling organisms [9]. All of these strategies have significant drawbacks; removal by hand is labor intensive, mechanical treatments require specialized equipment and can potentially damage oyster shells, and outplanting is limited by space availability. Disposal of removed biofoulers adds to labor and cost. Therefore, oyster farmers would prefer preventive measures to control biofoulers rather than the strategies described above. One method used by farmers to prevent fouling, called "fuka-zuri", is to suspend oyster lines in deeper areas (5 to 10 m depth) during the season when fouling occurs [9]. However, in shallow sea areas such as Isahaya bay which has an average depth of 10 m [10], the method is not applicable.

Biological control or biocontrol, which is defined as the introduction by humans of natural enemies to reduce pest population [11], has long been practiced in agriculture [12]. In the marine environment, researchers have tested organisms such as periwinkles [13], hermit crabs [14], and sea urchins [14–17] to mitigate the problem of biofouling in shellfish aquaculture. Sea urchins have also been tested to control the growth of invasive kelp species in natural marine habitats [18]. In laboratory trials, Atalah et al. [19] demonstrated that the sea anemone *Anthothoe albocincta* (Hutton, 1879) substantially prevented the larval settlement of *Bugula neritina* (Linnaeus, 1758) on test substrates.

This study investigates the possible use of the sea anemone *Aiptasiomorpha minuta* as an agent to control biofouling on oysters during aquaculture. An important problem that needs to be addressed to effectively use biocontrol against biofouling is establishing the mass culture technique of the biocontrol agent, in this case, *A. minuta*. Here, a field experiment was conducted on *A. minuta* that were artificially attached to spat collectors (scallop shells) containing oyster spats and examined their effect on fouling, and on the growth, condition index, and survival of the cultured oysters. In a laboratory experiment, the population growth of *A. minuta* at different temperatures, salinities, and diet treatments was investigated by the authors in order to determine the optimum conditions for its mass culture.

2. Materials and Methods

2.1. Field Experiment

2.1.1. Source of *Aiptasiomorpha minuta*

The sea anemone (*Aiptasiomorpha minuta*) used in the experiments was originally collected from a population attached to oyster shells in Isahaya Bay (32°55′2″ N, 130°12′0″ E). Dominant sea anemone species were scraped carefully, photographed, and identified morphologically according to Uchida and Soyama [2]. Based on the morphological keys, only one species was found in oyster shells collected on that day and identified as *A. minuta*. Collected sea anemones were kept in the laboratory at Nagasaki University for years as the stock culture and were maintained in seawater (salinity 33 psu), provided with light (3.2 W/m^2), and fed with newly hatched *Artemia salina* (Linnaeus, 1758) *ad libitum* three times a week. The stock culture was propagated by pedal laceration. Sea anemones used in the subsequent experiments were propagated from animals taken from the stock culture.

2.1.2. Culture of Oysters in Isahaya Bay

To examine the effect of the sea anemone *Aiptasiomorpha minuta* as a possible agent to control biofouling in cultured oysters, a field experiment was conducted in an oyster culture raft off Konagai-cho port, Isahaya Bay, Nagasaki, Japan (32°55′2″ N, 130°12′0″ E) for four months from May to September, 2017 [8]. Oyster spats attached on spat collectors (scallop shells, Shell Length = 125.43 ± 6.2 mm (n = 32)) were purchased from Isahaya Bay Fishery Cooperative, Nagasaki, Japan. Collectors were cleaned using a plastic brush, and the biofoulers and dead oysters were removed. The number of oyster spats attached to the collectors was adjusted to 22 to 25 individuals/collector and the shell height of each spat was measured using a vernier caliper (precision 0.1 mm). Initial shell heights of all spats employed in the experiment ranged from ca. 30 to 50 mm. A total of 32 spat collectors were used in the experiment. Cleaned collectors were then inserted between the strands of a 2-strand nylon rope (12 mm diameter), at an interval of 60 cm between collectors, and a total of 4 collectors were inserted in each rope. Ropes with collectors were then placed in a tank with continuously flowing seawater. A day prior to the start of the experiment, half of the ropes with the collectors (4 ropes with 16 collectors, used for the treatment group) were removed from the seawater, and mass-cultured *A. minuta* were then placed on both sides of the collectors and left to dry for about 10 min to let *A. minuta* attach before the ropes were placed back to the tank. On the day of the experiment, the total number of oyster spats in each collector of the treatment and control group was counted again, and the initial number of oysters per collector was recorded. The number of *A. minuta* individuals on the collector of the treated group was adjusted to 50 individuals per collector. *A. minuta* from the stock culture were mass cultured in the laboratory for 30 days in 30 L round polycarbonate tanks containing 10 L of GF/C filtered seawater (FSW). Conditions to mass culture *A. minuta* were the same as with the condition to maintain the stock culture, except they were reared in FSW and were fed with newly hatched *A. salina* every day after the culture water was renewed. On the 15th day of mass culture, *A. minuta* were scraped off gently and transferred to new containers.

A total of 8 ropes, 4 ropes in the control group and 4 ropes in the *A. minuta* treated group, were employed in the experiment. The ropes were put inside an insulated container and brought to the experimental site (approximately 60 km away) and hung on an oyster raft in a manner such that the collector nearest the sea surface was 1 m from the surface. Detailed conditions of the field experiment are shown in Figure 2.

2.1.3. Field Data Collection and Analysis

From June 2017, at an approximately 30 days interval, one rope in each treatment was removed from the experiment area and put in a 45 L plastic container, provided with aeration, and transported to the laboratory. In the laboratory, oyster collectors were detached from the ropes, and each collector was placed on a paper towel for 5 min and weighed (Ishida, Japan; accuracy = 5.0 g) individually. Subsequently, attached sea anemones

on both sides of the collector were counted and removed with a tweezer, and biofouling taxa (i.e., barnacles, bryozoans, tubeworms, sponges, and algae) were hand-picked using a tweezer, sorted, and weighed (Shimadzu; 0.1 g precision).

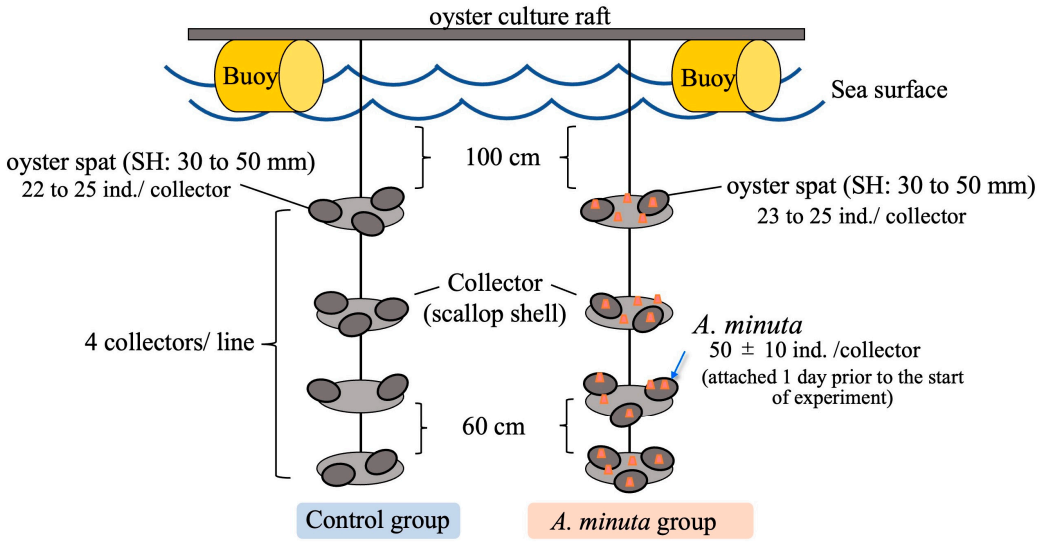

Figure 2. Oyster culture raft containing the spat collectors with either the control group or the *A. minuta* group.

After all the attached biofoulers were removed, collectors were cleaned thoroughly under running water, blotted dry, and biological parameters were measured. The number of living oysters in each collector was counted and shell height was measured using a vernier caliper (precision 0.1 mm). Shell growth of each oyster was measured as the difference in shell height from the average initial shell height of each collector. Individual weights of surviving oysters were measured using a top balance (Shimadzu, Japan), Subsequently, meat tissues of all surviving oysters were shucked and weighed. Data on the shell total wet weight and shucked tissue were used to determine the condition index (CI) according to Park et al. [20]:

$$\mathrm{CI} = \frac{\text{tissue wet weight (g)}}{\text{tissue wet weight} + \text{shell weight (g)}}$$

During the sampling, water temperature in the experimental site was monitored by directly recording the temperature at 1 m depth using a thermorecorder (TR41, T and D Corp., Matsumoto, Nagano, Japan). Water samples were also brought to the laboratory and salinity was measured using a calibrated salt meter (YK-31SA, FUSO Co. Ltd., Tokyo, Japan).

2.2. Laboratory Experiment

2.2.1. Culture of *Aiptasiomorpha minuta* under Laboratory Conditions

In this present study, three experiments were conducted to optimize the culture condition of the sea anemones for mass culture as outlined in Table 1. Sea anemones with pedal disk diameters of 5 to 8 mm were employed in the laboratory culture experiments.

Table 1. Culture conditions of *A. minuta* under different temperatures, diet, and salinity treatments.

	Experiment 1 (Temperature)		**Experiment 2 (Diet)**		**Experiment 3 (Salinity)**	
Container	1 L beaker		1 L beaker		1 L beaker	
Water exchange	once/day		once/day		once/day	
Light intensity and cycle	3.2 W/m^2, 12 h light: 12 h dark		3.2 W/m^2, 12 h light: 12 h dark		3.2 W/m^2, 12 h light: 12 h dark	
Aeration	yes		yes		yes	
Temperature (°C)	20, 25, 28, 30		28		28	
Salinity (psu)	33 to 34		33 to 34		8, 11, 15, 23, 33	
Diet	*Artemia salina* nauplii (ind./day)	250	unfed	N/A	*A. salina* nauplii (ind./day)	1000
			A. salina nauplii (ind./day)	250, 500, 1000		
			Fistulobalanus kondakovi nauplii (ind./day)	1000		
Culture days	10		14		10	

Optimization of Temperature Condition

To determine the optimum temperature condition for the culture of *A. minuta*, four experimental temperatures were assessed: 20, 25, 28, and 30 °C. All 1 L beaker culture containers were supplied with aerated filtered seawater at 33 to 34 psu. Sea anemones taken from the stock culture were distributed randomly among the different culture containers at a density of 5 individuals L^{-1}. The sea anemones were made to attach to the bottom of the containers before the start of the culture experiment. The sea anemones were cultured in these containers under a light intensity of 3.2 W/m^2 with 12 h light:12 h dark cycle, and were fed with *A. salina* nauplii (250 individuals/day) for 10 days culture period. The water was renewed daily prior to feeding. The cumulative number of individuals during the entire culture period in each experimental temperature was determined to assess the optimal temperature condition.

Optimization of Diet Condition

After determining the optimum temperature condition, the appropriate diet condition for *A. minuta* culture was assessed. In this experiment, five diet treatments were assessed: unfed (control), *A. salina* nauplii at varying densities of 250, 500, and 1000 individuals/day, and *Fistulobalanus kondakovi* (Tarasov and Zevina, 1957) nauplii (1000 individuals/day) for 14 days culture period. The temperature was held constant at 28 °C in all culture containers. Other culture conditions were maintained the same as previously described in the optimization of temperature conditions. The cumulative number of individuals during the entire culture period in each experimental diet was determined to assess the optimal diet condition.

Optimization of Salinity Condition

To determine the optimum salinity for mass culture, *A. minuta* was cultured at different salinity conditions: 8, 11, 15, 23, and 33 psu. The temperature and diet regimen were at 28 °C and fed with *A. salina* (1000 individuals/day), respectively, in all culture containers for 10 days culture period. Other culture conditions were maintained the same as previously described in the optimization of temperature conditions. The cumulative number of individuals during the entire culture period in each experimental salinity was determined to assess the optimal salinity condition.

2.3. Statistical Analysis

All statistical analyses were performed using JMP® Pro, version 16.0 software (SAS Institute Inc., Cary, NC, USA, 1989–2021 (https://www.jmp.com/en_us/software/predictive-analytics-software.html)) [21]. In the field experiment, monthly differences in total weight of fouling organisms, shell growth and CI of oysters between the control and *A. minuta* treated groups were assessed with the Wilcoxon matched pairs test and Student's *t*-test for the oyster survival rates. In the laboratory experiment, at the end of each culture period, data of temperature treatment groups were assessed with the Kruskal–Wallis test and followed by post hoc comparisons using the Steel–Dwass multiple comparison test to check for significantly different temperature groups. Data of the diet and salinity treatment groups were assessed using one-way ANOVA and followed by post hoc Tukey HSD to check for significantly different diet and salinity treatment groups. Differences were considered significant at $p < 0.05$. Data were presented as means ± SD.

3. Results

3.1. Field Experiment

3.1.1. Effect of *Aiptasiomorpha minuta* on Biofouling of Oyster Collectors

To investigate the efficacy of *Aiptasiomorpha minuta* as a biocontrol agent for biofouling on oyster culture, oyster collectors with or without *A. minuta* were submerged in Isahaya bay for four months. In Figure 3, a photograph comparison of oyster collectors in both control and *A. minuta* groups showed increased biofouling coverage over time. Notably, the *A. minuta* group showed fewer biofoulers compared to the control group.

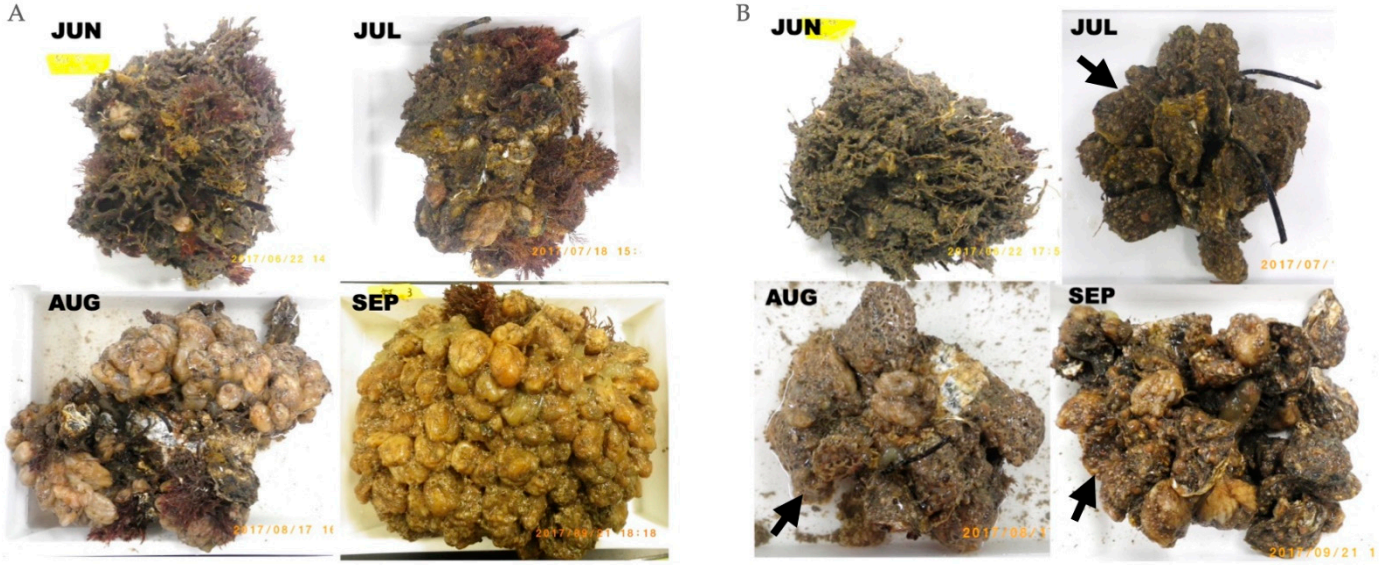

Figure 3. Photographs of oyster collectors between the (**A**) control group and (**B**) *A. minuta* group were taken for each monthly sampling. Black arrows indicate *A. minuta*-covered surfaces on oyster collectors which were dominantly observed from June to September.

The water parameters of the test site and the composition of biofoulers that were recorded in the oyster collectors between the control and *A. minuta* groups for each month are found in Table 2. Water temperatures at the test site were 24 °C at the beginning of the test period, reached 29.3 °C in late August, and slightly dropped to 26.5 °C at the end of the test period. Water salinity varied in the range of 30 to 34 psu. The composition of biofoulers that appeared each month from May to September included two types of seaweeds (two groups) and 17 types of primary attaching organisms (six groups). For seaweeds, species of the Order Ceremiales appeared only in May until mid-July in both control and *A. minuta* groups while species of the Order Ulvales also appeared in the same period as those in seaweeds from Order Ceremiales but were only found in the control group.

Table 2. Water temperature and salinity of the test site, and occurrence of attaching organisms on the control and *A. minuta* collectors during the test period.

		Submerged Period							
		May 25 to Jun 22		May 25 to Jul 18		May 25 to Aug 17		May 25 to Sep 21	
Water Temperature (C°)		23.5	24.2	27		29.3		26.5	
Salinity (psu)		33	34	33		32		30	
Phylum/Class	**Organism**	**Occurrence**							
		Control	***A. minuta***	**Control**	***A. minuta***	**Control**	***A. minuta***	**Control**	***A. minuta***
Rhodophyta	Ceremiales	±	+	±	±				
Chlorophyta	Ulvales	±		±					
Porifera	*Halichondria japonica* (Kadota, 1922)		±						
	H. okadai (Kadota, 1922)		±						
	unidentified sponges		±					±	±
Cnidaria	Tubulariidae		⧺		±	±	±		
Ectoprocta	*Bugula californica* Robertson, 1905	+		±	±	±	±	±	±
	Bugula neritina (Linnaeus, 1758)	⧺	+	+	+	±	±	±	±
	Bugulidae		±	±					
	Membraniporidae	±		±	±			±	±
Mollusca/Bivalvia	*Modiolus nipponicus* (Oyama, 1950)					±	±	±	±
	Musculita senhousia (Benson, 1842)					±	±	±	±
	Mytilus galloprovincialis Lamarck, 1819	±						±	
	other bivalves					±	±	±	±
Arthropoda	*Fistulobalanus kondakovi* (Tarasov and Zevina, 1957)					±	±		
	Small barnacles (AD < 4 mm)			±	±			±	±
Chordata	*Ciona intestinalis* (Linnaeus, 1767)			±		±	±	±	±
	Styela plicata (Lesueur, 1823)			+	+	⧺	⧺	⧺	⧺
	Solitary ascidians (BL < 5 mm)	±	±						

Water temperature and salinity were measured from 1.0 m below the sea surface each time of sample collection. Water temperature was measured at around 12:00 noon during each sampling collection. Occurrence of each species: ±, weight < 10% of total weight; +, weight was 10 to 50% of total weight; ⧺, weight > 50% of total weight was recorded following the method of Satuito et al. [8]. AD, aperture diameter; and BL, body length.

Among the 17 primary attaching organisms, hydroids, bryozoans, and tunicates were among the observed dominant biofoulers throughout the May to September sampling period. The presence of several barnacles and mollusk species occurred from the late-June to late September and late July to late September, respectively.

The wet weight of each biofoulers in oyster collectors was mainly dominated by tunicates (mostly by *Styela plicata*) which continued to increase over time, the highest of which was recorded in August and September (Figure 4A). Interestingly, in comparison to the control group, the number of biofoulers attached to the oyster collectors from the *A. minuta* group significantly decreased from June to August ($p < 0.05$; Table 3). In August, the wet weight of biofoulers in the *A. minuta* group was approximately 6-times lower compared to the control collectors. Collectors which initially had *A. minuta* on them became covered with *A. minuta* and an increased population growth trend of *A. minuta* was observed as shown in Figures 3 and 4B, indicating that the increase in their number effectively suppressed other biofoulers from attaching to the collectors.

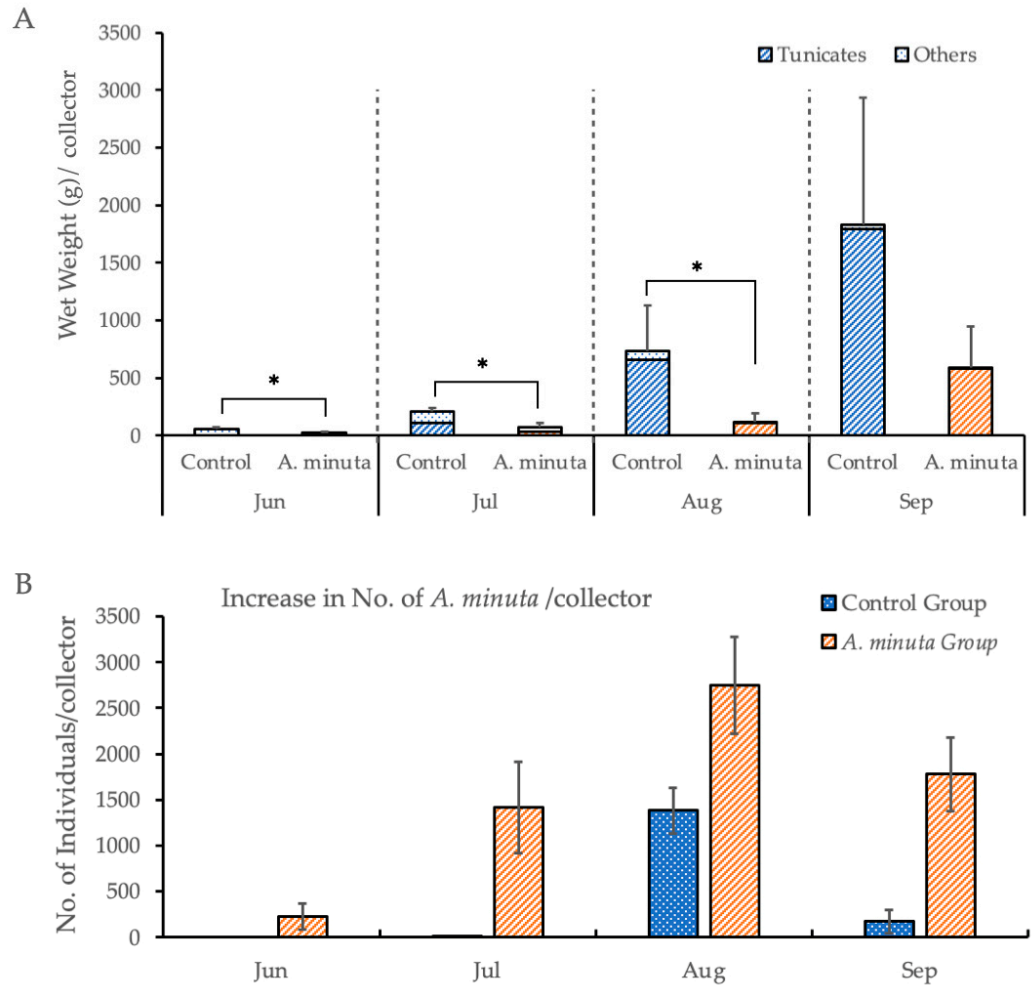

Figure 4. Composition of biofoulers on the collectors in each monthly sampling during the oyster culture period. (**A**) Wet weight of biofoulers attached to collectors submerged at the survey site. Diagonal striped boxes indicate tunicates; dotted boxes, others. (**B**) Population growth of *A. minuta* on each collector between the control and *A. minuta* groups. Data are expressed as the mean ± SD (n = 4). Values with an asterisk "*" are significantly different ($p < 0.05$).

Table 3. Monthly comparison of the total weight of fouling organisms and each biological parameter between the control and *A. minuta* groups in the field experiment. Treatments with a *p*-value < 0.05 were considered significant and highlighted in bold and with an asterisk "*".

Biological Parameter	Month	*p*-Value	Statistical Test
Total weight of fouling organisms	**JUN**	**0.0304 ***	Wilcoxon matched pairs test
	JUL	**0.0304 ***	
	AUG	**0.0304 ***	
	SEP	0.1124	
Shell Growth	JUN	0.4893	Wilcoxon matched pairs test
	JUL	0.0724	
	AUG	**0.0258 ***	
	SEP	**0.0012 ***	
Condition Index	JUN	0.1872	Wilcoxon matched pairs test
	JUL	**0.0004 ***	
	AUG	**0.0207 ***	
	SEP	**<0.0001 ***	
Oyster Survival	JUN	0.1587	Student's *t*-test
	JUL	0.1473	
	AUG	0.2247	
	SEP	**0.003 ***	

3.1.2. Effect of *A. minuta* on the Growth and Survival of Cultured Oysters

The shell growth and condition index of cultured oysters were significantly higher in the *A. minuta* group compared to the control group in the months of August–September and July–September, respectively ($p < 0.05$, Figure 5A,B, Table 3). By the end of the four-month experiment, the average oyster shell growth was approximately 31 mm in the control group and 44 mm in the *A. minuta* group. The condition index (CI) that was observed in both the control and *A. minuta* groups decreased as the temperature rose over time (Figure 5B). Condition index (CI) is usually used as a parameter to evaluate the physiological condition in bivalves. The decrease in CI in both treatment groups may be because oysters became weaker during the period of high water temperature [7]. Although, in this study, CI was lower in the control than in the *A. minuta* group. The condition index (CI) of oysters in September was 0.18 and 0.23 in the control and *A. minuta* groups, respectively. Moreover, the survival rate of oysters in the *A. minuta* group was significantly higher compared to the control group by the end of the test period ($p < 0.05$, Figure 5C, Table 3). These results indicate that the presence of *A. minuta* on collectors led to better oyster growth and condition index and had no adverse effect on oyster survival.

3.2. Laboratory Experiment

3.2.1. Temperature

The cumulative number of sea anemone individuals increased over time at different temperatures (Figure 6). By the end of the culture period, the population growth of *A. minuta* was at 11 ± 3, 34 ± 10, 56 ± 15, and 45 ± 6 in 20, 25, 28, and 30 °C treatments, respectively. Steel-Dwass multiple comparison test indicated that the population growth of *A. minuta* was significantly increased when cultured at 25 °C and higher, with the highest population at 28 °C ($p < 0.05$, Table 4). Hence, after determining the optimum temperature condition, the temperature was held constant at 28 °C when the appropriate diet condition for *A. minuta* culture was assessed.

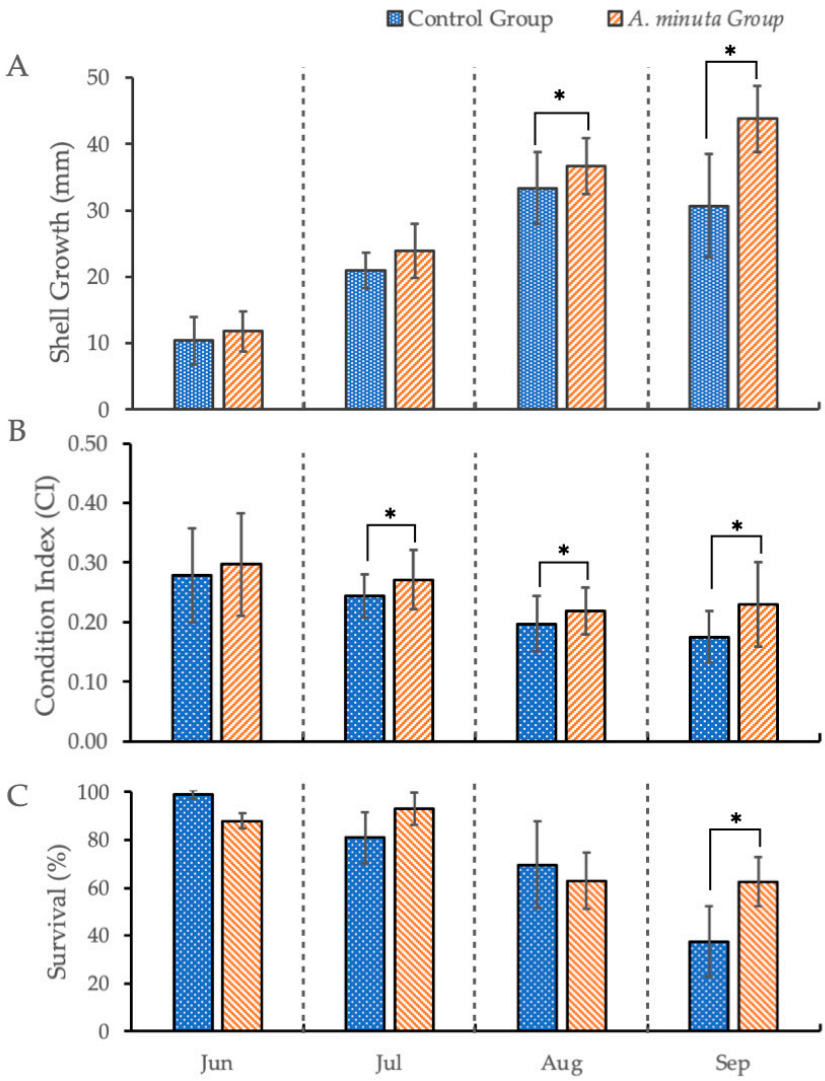

Figure 5. Monthly variation in the (**A**) shell growth, (**B**) condition index, and (**C**) survival rate of oysters per collector (n = 4) during the culture period. Values with "*" are significantly different ($p < 0.05$).

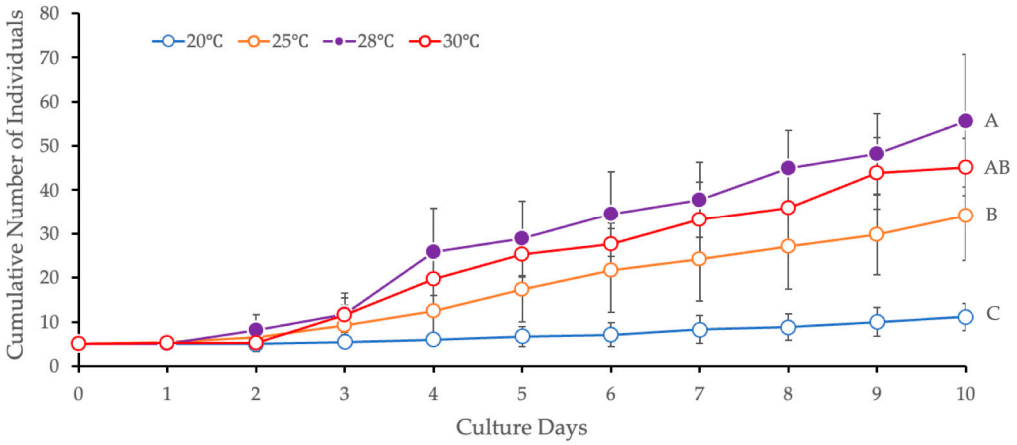

Figure 6. Population growth of *A. minuta* at different temperatures over a 10-day culture period. Data are expressed as means ± SD; each of the temperature treatments started with five anemones at day 0. Letters beside the 10th day of the dotted line graph denote significant differences in relation to different temperature treatments ($p < 0.05$, n = 5–19, A > B > C).

Table 4. Statistical comparisons of the effect of temperature, diet, and salinity conditions on the population growth of sea anemone *A. minuta* during the 10–14-day culture period. Treatments with a *p*-value < 0.05 were considered significant and highlighted in bold and with an asterisk "*".

Experiment	Groups Compared	*p*-Value	Statistical Test
1 (Temperature)	**20 vs. 25**	**<0.0001 ***	Steel-Dwass multiple comparison test
	20 vs. 28	**0.0007 ***	
	20 vs. 30	**0.0115 ***	
	25 vs. 28	**0.0025 ***	
	25 vs. 30	0.1221	
	28 vs. 30	0.6875	
2 (Diet)	not fed vs. *A. salina* 250	0.5457	Tukey-HSD
	not fed vs. *A. salina* 500	0.1525	
	not fed vs. *A. salina* 1000	**0.0273 ***	
	not fed vs. *F. albicostatus* 1000	0.2728	
	F. albicostatus 1000 vs. *A. salina* 250	0.9752	
	F. albicostatus 1000 vs. *A. salina* 500	0.993	
	F. albicostatus 1000 vs. *A. salina* 1000	0.5665	
	A. salina 250 vs. *A. salina* 500	0.8539	
	A. salina 250 vs. *A. salina* 1000	0.2868	
	A. salina 500 vs. *A. salina* 1000	0.7925	
3 (Salinity)	15 vs. 23	0.0605	Tukey-HSD
	15 vs. 33	0.1785	
	23 vs. 33	**0.0032 ***	

3.2.2. Diet Regimen

Population growth of *A. minuta* increased over time at different diet treatments (Figure 7). Although an increase in population growth was observed in the unfed treatment, it plateaued from day 8 until the end of the culture period. Without a food supply for 1 week, *A. minuta* still reproduced by pedal laceration, but division eventually stopped after a week, indicating that clone production was suppressed when the energy supply through diet was stopped. Interestingly, no sea anemone in the unfed treatment died during the 14-day culture period. The cumulative number of *A. minuta* individuals increased by the end of the 14-day culture period from the initial 5 individuals to 42 ± 16, 70 ± 30, 88 ± 19, 108 ± 20, and 80 ± 23 in unfed, *Artemia salina* 250, *A. salina* 500, *A. salina* 1000, and *Fistulobalanus kondakovi* 1000, diet treatments, respectively. Post hoc testing indicated that sea anemones fed with *A. salina* at 1000 individuals/day led to a significant increase in population growth compared to other diet treatments ($p < 0.05$, Table 4). Hence, temperature and diet were held constant at 28 °C and fed with *A. salina* (1000 individuals/day), respectively, when the appropriate salinity condition for *A. minuta* culture was assessed.

3.2.3. Salinity

Population growth of *A. minuta*, increased in number from 5 individuals to 60 ± 9, 79 ± 14, and 47 ± 5 in 15, 23, and 33 psu, respectively, by the end of the 10-day culture period (Figure 8). Mortality of sea anemones was observed in the 8 psu and 11 psu salinity treatments, and no reproduction by pedal laceration was observed in these two treatment groups. Moreover, all individuals died in 8 psu after 2 days. Thus, 8 psu and 11 psu treatment groups were excluded from comparisons of the cumulative number of individuals on the 10th day of culture (Table 4). After 10 days of culture, *A. minuta* in the 23 psu treatment showed a significantly higher increase in the number of individuals as compared to the 33 psu ($p < 0.05$, Table 4) treatment group. Hence, the determined optimum condition for *A. minuta* culture was observed at a salinity of 23 psu which resulted in the highest increase in population growth.

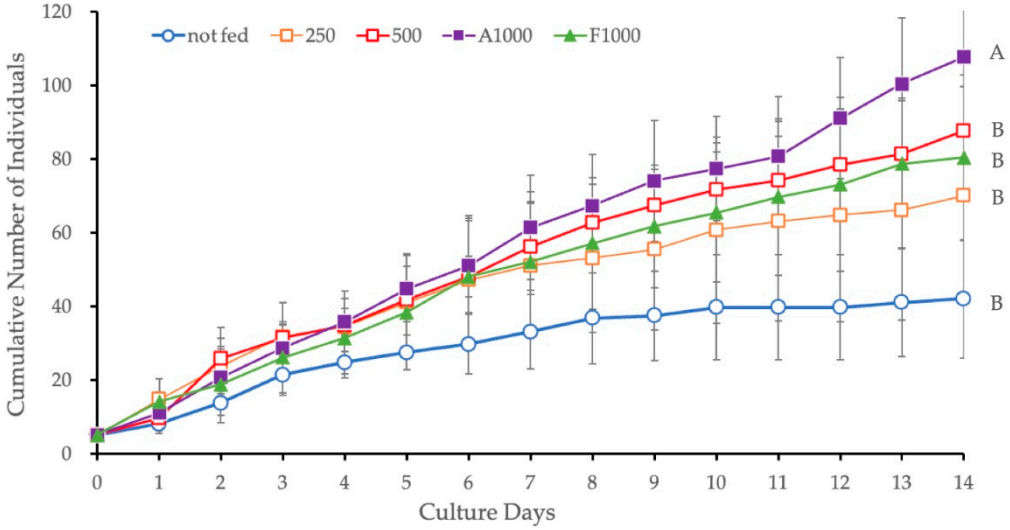

Figure 7. Population growth of *A. minuta* fed different diet treatments over a 14-day culture period. Data are expressed as means ± SD; each of the diet treatments started with five anemones at day 0. Letters beside the 14th day of the dotted line graph denote significant differences in relation to different diet treatments ($p < 0.05$, n = 3, A > B). Abbreviations: unfed, control; 250, 250 individuals/day of *Artemia salina*; 500, 500 individuals/day of *A. salina*; A1000, 1000 individuals/day of *A. salina*, and F1000, 1000 individuals/day of *Fistulobalanus kondakovi*.

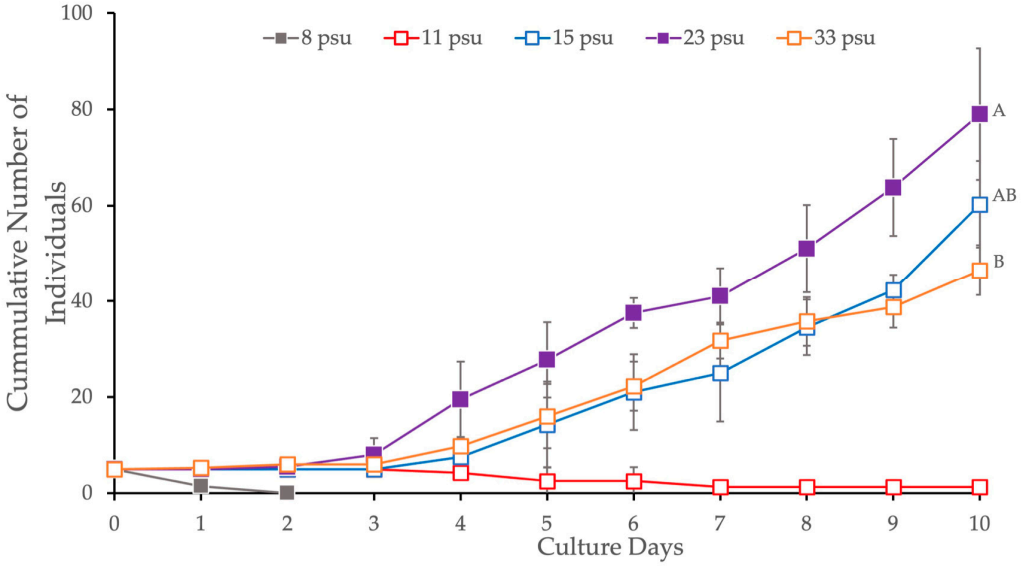

Figure 8. Population growth of *A. minuta* at different salinities over a 10-day culture period. Data are expressed as means ± SD; each of the salinity treatments started with five anemones at day 0 (n = 4–7). Letters beside the 10th day of the dotted line graph denote significant differences in relation to the other salinity treatments ($p < 0.05$, n = 4, A > B).

4. Discussion

This study reports the potential use of the sea anemone *Aiptasiomorpha minuta* as an agent for controlling biofouling on cultured oysters and the optimum temperature, diet, and salinity conditions for its mass culture.

The occurrence and growth of biofoulers on cultured oysters, which compete for space and food with cultured species, has been reported to be one of the causes of oyster mortality, a major problem for oyster farmers in Isahaya bay, Nagasaki, Japan [7,8]. In this present study, a field experiment was conducted to evaluate the efficacy of *A. minuta* as a

biocontrol agent for biofouling. The results of this study showed that 19 species (8 phyla), including two seaweed species, sponges, hydroids, bryozoans, mollusk/bivalve species, barnacles, and tunicates were found as biofouling assemblages on the experimental oyster collectors (Figure 3, Table 2). A comparison of species occurrence by month showed that there was a transition in species occurrence from month to month which may be due to changes in environmental factors such as seawater temperature and salinity [8]. The total wet weights of biofoulers on the collectors increased with increasing water temperature wherein oyster collectors were heavily fouled in the summer months from August to September. The occurrence and variation in abundance of fouling organisms in this present study were also found similar to a previous report by Satuito et al. [8] on a survey of fouling organisms in oyster farms of Isahaya bay. Notably, oyster collectors that were initially inoculated with the sea anemone, *A. minuta*, significantly reduced the settlement, accumulation, and structure of biofouling assemblages on cultured oysters over time (Figures 3 and 4, Table 2). A significant six-fold reduction in biofouling assemblages was found on collectors inoculated with anemones in August, the peak month of summer. This result has significant implications for preventing biofoulers such as barnacles that have been reported to appear in large numbers in August [8]. The observed increase in population growth of sea anemones over time coincided with the decrease in the settlement, accumulation, and structure of biofouling assemblages on cultured oysters. This indicates that *A. minuta* could effectively suppress the attachment of other biofoulers. One possible mechanism underlying such an effect of anemones to prevent attachment of other biofoulers is its ability to multiply and colonize a given substrate, thus mitigating the negative impacts of biofouling [19]. Sea anemones are also considered a natural enemy, as a predator, to several fouling organisms such as bryozoans and barnacles [19]. A similar report by Atalah et al. [19] demonstrated the ability of the sea anemone, *Anthothoe albocincta*, to reduce biofouling on marine artificial structures. Moreover, the ability of *A. minuta* to accumulate biomass in oyster collectors, thus, minimizing colonization by problem species; was also demonstrated in this study to have no adverse effects on oyster growth, condition index, and survival (Figure 5, Table 3). A reason for this is that sea anemones and oysters do not compete for food; oysters are filter feeders while sea anemones catch their zooplankton prey by their tentacles. These properties of sea anemones to act as a natural predator of other biofoulers and their ability to monopolize space on oyster collectors shows the high potential of this species as a biocontrol agent for biofouling [15].

From the results of the field study, while sea anemones significantly reduced biofouling, they did not completely suppress fouling on cultured oysters. However, the biocontrol method using sea anemones as the agent can be used in the field because less biofouling will result in less labor time in removing the biofoulers from the oyster shells. This was experienced when the authors removed the biofoulers from the oyster shells for analysis. Moreover, less biofouling can lead to less biomass to be disposed of after cleaning the oyster shells. Furthermore, after the enumeration of the sea anemones on the oysters, the authors were able to easily remove the animals from the shells by gentle brushing and washing of the oysters. According to local oyster farmers, the soft bodies of the sea anemones can be easily removed from the oyster shells by brushing and washing, in contrast to scraping the hard shells of biofoulers such as barnacles using metal tools and scraping machines.

Establishing the mass culture method of a potential biocontrol agent, in this case, *A. minuta* is a crucial issue that needs to be addressed in order to apply biocontrol against biofouling effectively. The sea anemone, *A. minuta*, has been reported to be one of the commonly occurring biofoulers found in oyster farms in Isahaya bay [8]. Although little is known about the biology of *A. minuta*, this study provides a preliminary assessment of the optimum conditions for their mass culture. Under laboratory conditions, population growth of *A. minuta* under different experimental temperatures showed a significant increase when cultured at 25 °C and higher, with the highest recorded population at an optimum temperature of 28 °C ($p < 0.05$, Figure 6, Table 4). Results under laboratory conditions matched with those observed in the field experiment wherein the number of

sea anemone individuals increased significantly in the months of July to September with a recorded approximate seawater temperature range from 26 to 29 °C (Figure 4B, Table 4). The ability of *A. minuta* to thrive in higher water temperatures is in contrast to that of the Mediterranean sea anemone *Actinia equina* (Linnaeus, 1758) [22]. The *A. equinia* was found to grow best at lower temperatures (15 and 20 °C) while their biomass was reduced at higher temperatures [22]. Temperature is one of the factors that could influence the geographical distribution of sea anemones [22]. In this present study, the ability of *A. minuta* to thrive at high water temperatures in both laboratory and field experiments indicates their advantage as a biocontrol agent to mitigate biofouling, especially during the summer season which coincides with the oyster culture and the heavy fouling seasons [8].

Diet regimen effects on the population growth of *A. minuta* were examined by comparing the cumulative number of individuals at each feeding regime. Population growth was observed in the unfed treatment, although their increase in number plateaued from day 8 until the end of the culture period (Figure 7). One possible reason for this trend could be the presence of endosymbiotic algae on *A. minuta*. Some studies have shown that some cnidarians, such as sea anemones including *Aiptasia pallida*, contain zooxanthellae that could enhance the growth and survival of sea anemone individuals even during periods of starvation [23]. However, whether or not *A. minuta* contains endosymbiotic algae, remains to be clarified. It is also noteworthy that *A. minuta* can thrive in unfed conditions for an extended period (in this study, 14 days); no mortality was observed in this treatment. Furthermore, *Artemia salina* [19,23] is often used as food to maintain sea anemone cultures. Likewise, *A. minuta* fed with *A. salina* (1000 individuals /day) significantly increased their population growth (Figure 7, Table 4). In this study, evidence is also provided to show that *A. minuta* could ingest *Fistulobalanus kondakovi* and could contribute to the anemone's population growth. The barnacle *F. kondakovi* is also one of the recorded biofoulers in the field experiment of this study and in another study reported by Satuito et al. [8]. These findings provide support to the hypothesis in the field experiment that predation on other biofoulers is one mechanism by which *A. minuta* could reduce the settlement, accumulation, and structure of biofouling assemblages on cultured oysters. On the other hand, one possible limiting factor in this laboratory experiment was that the amount of individuals/day in the diet treatments of sea anemones fed with *A. salina* and *F. kondakovi* were fixed throughout the culture period. An adjustable feeding rate or a mixed diet regime might be an interesting approach to exploring higher sea anemone population growth yields.

Population growth of *A. minuta* under different salinity treatments favorably increased at salinities of 15 psu and higher, with 23 psu as the optimum condition for *A. minuta* culture. Findings in this study showed that a higher salinity range from 15 to 30 psu was found conducive for the sea anemone *A. minuta* culture which was close to the reported salinity of the field experiment in this study and those reported in the field survey of Isahaya bay [8]. These laboratory trials also confirm that the generally accepted lower limit of salinity for species under Actiniaria is between 15 to 20 psu as is reported in other sea anemone species such as *Metridium senile* (Linnaeus, 1761) [24]. Glon et al. [24] have demonstrated that exposure of the sea anemone *M. senile* to both high (45 psu) and low salinity (5 psu) trigger a physical response that impacts critical physiological functions such as contraction of tentacles, lowers oxygen consumption, increased mucous secretions that lead to mortalities. This could be one underlying reason why *A. minuta* mortality was observed at 8 and 11 psu salinity treatments in this study.

In all laboratory experiments performed in this study, there was no observation of the release of egg mass or planula larvae by *A. minuta* in the containers. Whether or not *A. minuta* reproduces sexually warrants further investigation. On the other hand, understanding the factors that control the sexual reproduction of this species can find application in the development of new mass culture methods that is more intensive and will require less space. The mass culture method employed in this study relied on *A. minuta*'s division by pedal laceration which required a greater surface area of the culture container for asexual reproduction. Being able to induce and control the sexual reproduction of

A. minuta will be beneficial because egg and planula larvae production is not reliant on the surface area of the container, and more eggs and planula larvae can be produced in less amount of time.

These present findings suggest that *A. minuta* possesses qualities as a potential agent for controlling biofouling on cultured oysters. Sea anemones, such as *A. minuta*, do not accumulate high biomass, do not leave marks on the surface of oysters, and are easy to remove [19]. *A minuta* is safe from the viewpoint of food hygiene and is also environment-friendly. It is also worth mentioning here that the authors did not observe any release of egg mass or planula larvae in the laboratory cultures. Understanding the sexual reproduction of this species can lead to mass production by sexual reproduction which will occupy less space than production by pedal laceration. Further development of management strategies together with the use of sea anemones as an anti-fouling agent could be explored to control biofouling assemblages in the aquaculture industry.

5. Conclusions

This study demonstrates the potential use of the sea anemone *Aiptasiomorpha minuta* as an agent for controlling biofouling on cultured oysters and the optimum culture conditions for its mass culture. In the field experiment, results showed that 19 species (8 phyla), including two seaweed species, sponges, hydroids, bryozoans, mollusk/bivalve species, barnacles, and tunicates were found as biofouling assemblages on the experimental oyster collectors. Initial attachment of *A. minuta* on oyster collectors successfully mitigated the fouling of several commonly found biofoulers in Isahaya bay. The ability of *A. minuta* to monopolize the surface area of oyster collectors via competition for space with other biofoulers and predation, resulted in lower total wet weights of fouling organisms and higher oyster shell growth, condition index, and survival rate. In the laboratory experiment, the conditions favorable for the mass culture of *A. minuta* were found at 28 °C, fed with *Artemia salina* (1000 individuals/day), and at 23 psu for the optimum temperatures, diet regimen, and salinity, respectively. These mass culture conditions could be useful for the purpose of producing enough biomass for attaching the sea anemones, *A. minuta* to oyster collectors. The use of *A. minuta* could be a preventive strategy against biofouling that may be useful for oyster farmers, it is safe from the viewpoint of food hygiene, and is also environment-friendly.

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