MASTER'S THESIS

Interactive effects of hypoxia and ocean acidification on biofilms and the subsequent effects on the larval settlement of the marine invertebrate Crepdiula onyx

Ho, Chun Ming

Date of Award:
2018

HONG KONG BAPTIST UNIVERSITY

Master of Philosophy

THESIS ACCEPTANCE

DATE: March 16, 2018

STUDENT'S NAME: HO Chun Ming

THESIS TITLE: Interactive Effects of Hypoxia and Ocean Acidification on Biofilms and the Subsequent Effects on the Larval Settlement of the Marine Invertebrate *Crepidula Onyx*

This is to certify that the above student's thesis has been examined by the following panel members and has received full approval for acceptance in partial fulfillment of the requirements for the degree of Master of Philosophy.

Chairman:	Dr. Ma Edmond Dik Lung Associate Professor, Department of Chemistry, HKBU (Designated by Dean of Faculty of Science)
Internal Members:	Dr. Lai Ka Man Associate Professor, Department of Biology, HKBU (Designated by Head of Department of Biology)
	Dr. Chiu Man Ying Assistant Professor, Department of Biology, HKBU
External Members:	Dr. Thiyagarajan Vengatesen Associate Professor School of Biological Sciences The University of Hong Kong Pokfulam Road, HONG KONG

Issued by Graduate School, HKBU

Interactive Effects of Hypoxia and Ocean Acidification on Biofilms and the Subsequent Effects on the Larval Settlement of the Marine Invertebrate *Crepidula onyx*

HO Chun Ming

A thesis submitted in partial fulfilment of the requirements

for the degree of

Master of Philosophy

Principal Supervisor:

Dr. CHIU Man Ying (Hong Kong Baptist University)

March 2018

DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of MPhil at Hong Kong Baptist University, and has not been previously included in a thesis, dissertation submitted to this or other institution for a degree, diploma or other qualifications.

I have read the University's current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University's Committee on the Use of Human & Animal Subjects in Teaching and Research (HASC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and the rights of the participants.

Signature: 

Date: March 2018

Abstract

Hypoxia and ocean acidification (OA) are amongst the major environmental threats to marine ecosystems worldwide. Biofilms, the signpost to guide larval settlement of many benthic invertebrates, are known to be responsive to environmental changes and thus can become the crucial factor for the response of benthic invertebrate communities. This study aimed at investigating the individual and interactive effects of hypoxia and OA on biofilms and the subsequent effects on larval settlement. Biofilms collected from two sites (clean, hypoxic) were treated with a factorial design of low dissolved oxygen and/or low pH conditions in microcosms and the bacterial cell density and viability (by LIVE/DEAD® cell viability assays) were analyzed. Larval settlement preference was tested with the marine invertebrate, *Crepidula onyx*.

The total bacterial cell densities of biofilms of the hypoxia and hypoxia and OA combination treatment were lower than that of the control biofilms for both sites. There was generally no significant difference in cell viability among control and different treatments for both sites. While the larval settlement rate on hypoxia and hypoxia and OA combination treated biofilms was significantly lower. In conclusion, this study revealed that hypoxia and OA are likely to affect larval settlement by alteration of biofilms, and this may lead to alterations in future coastal communities.

Acknowledgements

To begin with, I would like to express my deepest gratitude to my supervisor, Dr. Jill Chiu, for her guidance and encouragement throughout the project. It has been a period of intense learning for me, both in academic and personal level, which will be beneficial to me all the time.

I would like to thank my co-supervisor, Dr. Qiu, Jianwen for encouragement and support on my project. I also thank all the scientific officers at the Department of Biology for providing technical support, in particular Winnie and Wai supported me for a long period.

I also wish to thank my labmates, Beverly, Jenny, Rachelle, Ming and Jonathan, who shared research skills and helped with laboratory work. In particular Beverly and Jenny, I would not be capable of completing the work without their much assistance and support.

I thank the Hong Kong University of Science and Technology and Yung Shu O Uncle Shek for site provision for my experiment, I also thank the Coastal Marine Laboratory of the Hong Kong University of Science and Technology for seawater supply.

Finally, I wish to thank my family for their endless care and support, and my best friend, Frankie, for his selfless assistance and support.

Table of Contents

List of Table

List of Figures

Abbreviations

DO	Dissolved oxygen
OA	Ocean acidification

Chapter 1: Introduction

1.1 Research background

Ocean, covering more than 70% of the earth (NOAA 2016), is important in maintaining a stable ecosystem. For examples, it provides habitats for marine organisms, regulates climate such as wind pattern, rainfall and temperature, acts as an important component in many nutritional cycles, and involves in many chemical reactions that moderates the existing environment.

However, human activities are affecting the ocean system and changing some of its properties, two of which are hypoxia and ocean acidification (OA). These global marine environmental problems are currently affecting marine organisms in many aspects (reviewed in Gobler et al. 2016). In order to estimate the fate of marine ecosystems better, this thesis investigated the effects of hypoxia and OA on biofilms and subsequently on settlement of marine invertebrate.

1.2 Marine hypoxia

Marine hypoxia refers to a low water dissolved oxygen (DO) level that is harmful to most organisms and may lead to mass mortality and substantial changes in ecosystem composition and function (Gray et al. 2002, Díaz and Rosenberg 2011). The threshold level of DO for hypoxia is not well-defined (STAP 2011, Rabalais et al. 2010), but a level which causes fishery collapse (below 2 mg O_2 L^{-1}, Vaquer-Sunyer and Duarte 2008) has been used as a threshold of occurrence of adverse effects in most marine organisms (Rabalasis et al. 2010) among scientists and policy makers, whereas DO level of 2.8 mg O_2 L^{-1} has been commonly used for hypoxia experiments in laboratory (Wu 2002).

Hypoxia in marine environment can be caused by either natural or anthropogenic factors. The naturally occurring hypoxia areas are usually fjords, deep basins and open ocean oxygen minimum zone because they are sinks of organic matters which have very high biochemical oxygen demand from microorganisms that feed on the food resource. Stratification also contributes to such phenomenon. Surface water is usually less saline, warmer and thus less dense. This prevents the denser bottom water from mixing with the surface water and thus the replenishment of oxygen. However, the increasing occurrence of hypoxia in shallow coasts and estuaries are believed to be caused by anthropogenic activities (Díaz and Rosenberg 1995). They include the discharge of sewage, agricultural fertilizers and excessive nutrients which result in nutrient enrichment in

water (Howarth et al. 2011). The additional nutrients cause proliferation of phytoplankton that contributes to extra high biomass as well as rate of decomposition, which increases the demand of DO and results in hypoxia (Rabalais et al. 2010).

Nowadays, hypoxia occurs in both coastal and offshore marine waters all over the world (STAT 2011). More than 500 hypoxic coastal areas have been reported (Díaz and Rosenberg 2008, Isensee et al. 2016), with the area of oxygen-minimum zones (regions with the lowest oxygen saturation) reached to 4,500,000 km^2 in 2010 (Stramma et al 2010). Hypoxic zones were doubling once every ten years since 1960 (Gilbert et al. 2010, Rabalais et al. 2010).

Estuaries are particularly prone to development of hypoxia as apart from anthropogenic pollutions, they receive freshwater from rivers that encouraging stratification. Moreover, global warming, which is characterized by increase in temperature and freshwater and nutrients outflow, has been predicted to further exacerbate the intensity, duration and extent of hypoxia. As it could cause decrease in oxygen solubility of seawater, increase in respiration and oxygen consumption rates among marine organisms, and reinforcement of eutrophication and stratification (Wu 2002, Díaz and Rosenberg 2008). Due to increase usage of fertilizers and industrialization by the expanding populations, it was expected that more hypoxic systems would occur in southern and eastern Asia (Seitzinger

et al. 2002). In Hong Kong, hypoxia occurs periodically in the Tolo Harbour (Fleddum et al. 2011).

Hypoxia can initiate a spectrum of behavioural and physiological responses depending on the degree of hypoxia, individuality, stage of life and experience in exposure of the organisms (Vaquer-Sunyer and Duarte 2008, Rabalais and Turner 2001, Gray et al. 2002). For instance, mobile organisms such as crabs, fishes and shrimps would escape from the hypoxic areas (Das and Stickle 1994, Weltzien et al. 1999, Larkin et al. 2007), others such as the shrimp *Penaeus monodon* and the ophiuroid *Amphiura filiformis* showed decreased hemocyte phagocytosis (Direkbusarakom and Danayado 1998) and reduced metabolism (Calder-Potts et al. 2015) under hypoxia, respectively. Compromised larval development and increase in proportion of malformed larvae was also shown in the larvae of the tubeworm *Hydroides elegans* when exposed to hypoxia condition (Shin et al. 2013). It has been suggested that vast and prolonged existence of hypoxia may cause mass mortality and substantial changes in ecosystem composition, function and service (reviewed in Gray et al. 2002, Díaz and Rosenberg 2011).

1.3 Ocean Acidification

Emission of atmospheric carbon dioxide (CO_2), which was accelerated since the industrial revolution by anthropogenic activities including burning of fossil fuels and deforestation, is the main cause of OA. The concentration of atmospheric CO_2 has been risen from about 280 ppm before the industrial revolution to about 380 ppm nowadays (Feely et al. 2004). The rate of CO_2 emission has increased by at least an order of magnitude compared with millions of years ago (Doney and Schimel 2007), and the average surface ocean pH has been decreased by 0.1 since the early 1900s (Solomon et al. 2007).

When atmospheric CO_2 dissolves in seawater, it reacts with water molecule to form carbonate ion (CO_3^{2-}) and hydrogen ion, which decreases the pH. (Doney et. al. 2009). Models have been developed to project the level of CO_2 emission and the subsequent pH of water, showing that the pH value of surface water may drop by about 0.4 before the year of 2100 and 0.7 by 2250 (Caldeira and Wickett 2005). Such change in ocean pH would be irreversible in terms of human timescales (Royal Society 2005).

OA is harmful to marine organisms, especially the calcifiers which are highly sensitive to the alterations of water chemistry (Doney et al. 2009). For examples, linear reduction of calcification rate was resulted in the mussel *Mytilus edulis* and the oyster *Crassostrea gigas* in response to two hours of incubation to reduced pH (Gazeau et al. 2007). This led to an

approximation of 25% and 10% of projected reduction of calcification rate, respectively, in 2100 under the IPCC IS92a scenario of future CO_2 level (Gazeau et al. 2007). In addition, significant reduction in growth rate (with respect to shell length and wet weight) occurred after fourteen weeks of exposure to a pH of merely 0.03 lower than control in the juvenile of gastropod *Strombus luhuanus* (Shirayama and Thorton 2005). Apart from calcification process and growth rate, hypercapnia (the decrease in blood pH) can also cause adverse effect on marine organisms. For example, hypercapnia in *Mytilus galloprovincialis* caused reduction in metabolic rate and growth and a net degradation of protein (Michaelidis et al. 2005). OA can even induce trans-generation effects, leading to trans-generational phenotypic plasticity and genetic selection. Larvae of the mussel *Mytilus edulis* originating from CO_2-enriched habitat showed higher calcification performance than the non-adapted cohort (Thomsen et al. 2017).

1.4 Interaction of hypoxia and OA

There is growing evidence suggesting that hypoxia caused by eutrophication is often associated with low pH, because microbial degradation of organic matter also generates CO_2 in addition to consumption of oxygen (refer to session 1.2 for detail), resulting in reduction of both DO and pH (Feely et al. 2010). Close association between acidification and low oxygen level have been found in northern Gulf of Mexico (Cai et al. 2012), the Forge River Estuary in the US (Gobler et al. 2014), Belgian coastal zone (Borges and Gypens 2010), Chilean oxygen minimum zone (Paulmier et al. 2011) and Puget Sound in the US Pacific Northwest (Feely et al. 2010). Communities living in these areas are subjected to stresses imposed by a combination of hypoxia and OA. However, the impacts of such conditions to these communities have not been studied before.

Laboratory studies have found additive and synergistic adverse effects of hypoxia and OA on the fitness of invertebrates at early life stage. For instance, although juveniles of the hard clam *Mercenaria mercenaria* were resistant to hypoxia or OA individually, when they were exposed to both stressors significant reduction in growth rates was resulted (Gobler et al. 2014). Furthermore, alterations in the proteome and phosphorylation status in response to hypoxia, acidification and their combination were resulted in the larvae of *H. elegans* (Mukherjee et al. 2013).

1.5 Biofilms

Biofilms are defined as “complex agglomerates of bacteria, diatoms, protozoa and fungi existing on virtually all submerged substrata” (Wieczorek and Todd 1998). Biofilms produce chemical cues which affect settlement preference of marine invertebrates (Steinberg et al., 2001), the effects can be both inductive and inhibitive (review in Chung et al. 2010). It is proved that many marine invertebrates across different phyla metamorphose in response to cues from biofilms (reviewed in Pawlik 1992, Wieczorek and Todd 1998, Holmstrom and Kjelleberg 2000, Maki et al. 2000, Hadfield and Paul 2001, Steinberg et al. 2001).

Biofilms have characteristic variations such as microbial density, composition, biomass and chemical contents. The variations are determined by a series of environmental factors such as availability, diversity and physiology of colonizing microbial species, age of biofilms, nature of substratum, latitude, depth, tidal level, season, temperature, salinity, illumination, nutrient supply, and water chemistry including DO and pH (reviewed in Wieczorek and Todd 1998, Lidbury et al. 2012, Cheung et al. 2013), as well as some artificial factors such as the presence of nutrients, pollutants, antibiotics, and exposure of UV light (Chiu et al. 2007, Chiu et al. 2012, Lawes et al. 2016). These factors would alter the attractiveness of biofilms on different species of marine invertebrates for their settlement, and thus, biofilms can be signpost for larvae to distinguish between biofilms and

metamorphose into habitats that are benefit to their growth on the later life stages (reviewed in Chiu et al. 2007).

1.6 Individual and interactive effect of hypoxia and OA on biofilms

Bacterial composition of biofilms often governs the substratum preference and settlement of larvae of many marine invertebrates (Wieczorek and Todd 1998, Qian et. al. 2007). The bacterial communities of biofilms are sensitive in changes of environmental condition (Tolker-Nielsen and Molin, 2000). Biofilms that have undergone chemical treatments or stresses had altered bacterial density and community and was found to alter settlement preference of marine invertebrates (Chiu et. al. 2012, Cheung et. al 2014).

Existing literatures regarding effects of OA on biofilms in general show that OA can alter the microbial community by boosting their productivity, increasing the abundance and diversity of primary producer such as diatoms, and altering the bacterial composition (Lidbury et al. 2012, Baragi et al. 2016). Knowledge on the effect of hypoxia on biofilms is relatively scarce. Hypoxia has been shown to reduce biofilm bacterial cell density and change bacterial community structure, and the alteration increased with longer exposure period (Cheung et al. 2014). However, how both hypoxia and OA affect biofilm bacterial communities is still unclear.

1.7 Effects of hypoxia or OA manipulated biofilms on settlement of marine invertebrates

Marine benthic invertebrates play a pivotal role in maintaining the normal functions of coastal ecosystems and support commercial fisheries worldwide (Costanza et al. 1997). It is known that the bacterial composition of biofilms can be altered by certain stress, and the alteration can affect the preference of settlement of marine invertebrates (Chiu et al. 2012, Cheung et. al. 2014).

Hypoxia manipulated biofilms were shown to alter the benthic community by reducing the number of settling individuals and settler diversity (Cheung et al. 2014, Lagos et al. 2016). The effect on settlement is species-specific. While some species, for instance, *Schizoporella* sp. showed indistinguishable preference between normoxic and hypoxic-treated biofilms (Cheung et al. 2014), *H. elegans* and *Bugula neritina* showed reduced settlement rate (Shin et al. 2013 , Lagos et al. 2016) which may lead to a change in coastal ecosystem.

There is lack of literature showing the direct relationship between the OA manipulated biofilms on settlement of marine invertebrates. Nevertheless, low pH water on CO_2 vent was shown to reduce number of taxa, taxonomic evenness and biomass of a rocky reef benthic community (Kroekera et al. 2011).

1.8 The studied organism, *Crepidula onyx*

Crepidula onyx (phylum Mollusca, class Gastropoda) is a slipper limpet which has been recognized for being a common marine fouling species since 1935 at the intertidal zone and subtidal water in the coastline of southern California (Coe 1935). The distribution of this species ranged from the Pacific coast of North and South America to Asia (Huang et al. 1984). It was recorded as an invasive species in Hong Kong in 1970s, and their distribution was around the Victoria Harbour in the 1980s. (Huang et al. 1984, Morton 1987). Up to 2014, *C. onyx* has been recorded around the Victoria Harbour and Hong Kong Island in Hong Kong (Astudillo et al. 2014). However, two juveniles *C. onyx* were found at the hypoxic site of this study in Yung Shue O, Sai Kung on 28th June, 2017, meaning this species has already expanded their distribution to the Tolo Channel in Hong Kong.

C. onyx is a benthic marine invertebrate which has a complex life cycle. They have pelagic larval stage which is beneficial for their dispersal and colonization of new habitats (Thorson 1950; Pechenik 1999). After the pelagic larvae become competent, they undergo settlement, which is a two-steps-process that involves behavioral search for and attachment to suitable substratum, and metamorphosis (Hadfield et al. 2001).

C. onyx has been used for studies involving environmental stress (such as Zhao and Qian 2002, Chiu et al. 2007, Li and Chiu 2013). For example, hypoxia was found to reduce juvenile growth rates and delay metamorphosis

on *C. onyx* (Li and Chiu 2013). OA has also been demonstrated to exert no effect on larval mortality and respiration rate, but reduction in shell length, growth rate and shell quality on *C. onyx* (Maboloc and Chan 2017). Furthermore, interaction between OA and diet of algae cultured under low pH level encouraged settlement of *C. onyx* larvae (Maboloc and Chan 2017). Settlement rate of *C. onyx* was also shown to have a positive relationship with the bacterial density (Chiu et al. 2012) but unknown relationship with the bacterial community composition on biofilms, which are the two common factors governing the settlement rate of many marine invertebrates.

Thus, this species was chosen as the target species because 1) it is a common marine invertebrate which is distributed widely, 2) it is easily cultured in laboratory and their eggs can be easily observed and monitored, and 3) its settlement rate was shown to be sensitive to alterations of biofilms (such as Chiu et al. 2007, Chiu et al. 2012).

1.9 Research objectives

Considerable number of studies have been carried out to investigate the effects of hypoxia on marine organisms and as well as the effects of OA on sensitive groups (e.g. foraminifera, coccolithophores, pteropods, urchins, corals and molluscs), using single stressor approach. In the natural environments, however, marine populations are simultaneously exposed to both hypoxia and OA. Surprisingly there is a lack of multi-stressor studies in marine climate change research. Multi-stressor studies are important to properly evaluate the environmental risks when organisms are subjected to more than one stressor simultaneously, as it is well known that additive, synergistic or antagonistic interactions may occur (e.g. Gobler et al. 2014).

Using *C. onyx* as a model species, this study set out to test the hypothesis that hypoxia, OA and their combination will alter the characteristic of biofilms, and the altered biofilm bacterial communities will subsequently affect the settlement preference of marine invertebrates. The individual and interactive effects of hypoxia and OA on settlement of *C. onyx* by means of modified biofilms were investigated. A factorial experiment comprising microcosms with two levels of DO and pH (control and future levels) was employed to observe changes in biofilms bacterial community and the subsequent effects on settlement choice of *C. onyx* on these biofilms. Bacterial density and viability on biofilms were employed as parameters to determine the variation on biofilms. The experiments were conducted with

biofilms developed from a clean and a hypoxic site in order to test if the conclusion is applicable in waters with varied DO background.

In this study, pH 7.6 and 1.5 mg O_2 L^{-1} were used to simulate the OA and hypoxia conditions respectively. pH 7.6 is the predicted annual average seawater pH value for the year of 2100 (Caldeira and Wickett 2005) and the same pH level has been measured in naturally acidified coastal systems (e.g. Cai et al. 2012). It is within the range of pH *C. onyx* may encounter in where the adult *C. onyx* was caught for larvae production in this study (Tai Miu Wan) (EPD 2015). This pH level has been applied in some studies that tested OA effects on marine invertebrates (such as Wong et al. 2011, Mukherjee et al. 2013). The reason for choosing 1.5 mg O_2 L^{-1} is that it is below 2.0 or 2.8 mg O_2 L^{-1} which is generally considered as hypoxia (Vaquer-Sunyer and Duarte, 2008). It is also a possible range of DO that *C. onyx* may encounter in the natural environment, for example, the hypoxic site of this study, Yung Shue O, had a lowest measured record for 0.18 mg O_2 L^{-1} on 8^{th}, August 2017. This value was used in various hypoxia experiments for marine invertebrates (Cheung et. al. 2014, Leung et. al. 2013, Li and Chiu 2013).

Chapter 2: Methods

2.1 Biofilms development

Two sites from the eastern water of Hong Kong were selected for biofilms development. The clean site was in Port Shelter accessible from the pier of the Hong Kong University of Science and Technology (HKUST, 22º34'N, 114º27'E). No obvious pollution source was around the site. The hypoxic site was a fish farm in Yung Shue O located in the Three Fathoms Cove, Tolo Channel (22º43'N, 114º28'E). Hypoxia occasionally occurs in that area in summer according to the Environmental Protection Department, Hong Kong (EPD 2015). There were 3 individual repeats of the clean site experiment carried out from April to May, 2017, and 3 individual repeats of hypoxic site experiment carried out from May to August, 2017. Physical parameters (pH, DO, water temperature) were measured on the beginning and ending days for the clean site and at an interval of four to five days during the development period for hypoxic site. For the hypoxic site, the DO levels of all measurement days of repeat 1 and 3 and two out of four measurement days of repeat 2 were lower than 4 mg O_2 L^{-1}, within, the DO levels of one measurement day of repeat 2 and all the measurement days of repeat 3 were lower than 2 mg O_2 L^{-1} (Table 2.1).

Petri dishes (Falcon 301006, 50 x 9 mm) were used as the substrate for biofilms development. They were fixed on plastic trays (31cm x 40cm) by cable tie and submerged in subtidal zone with nylon mesh bag (80 μm mesh

size) in order to prevent unwanted larval settlement. The length of time for biofilms development was 13 days except in repeat 2 of clean site (11 days) due to the availability of *Crepidula onyx* larvae. Petri dishes coated with biofilms were transferred to laboratory with minimum sunlight and air exposure.

Site (Repeat)	Biofilms development period	Measurement record date	Water parameter records			
			pH	DO (mg O_2 L^{-1})	Temperature (^oC)	Salinity (psu)
Clean site repeat 1	31/3 - 13/4	31/3	8.14	6.09	19.4	29
		13/4	8.27	6.78	21.3	29
Clean site repeat 2	15/4 - 27/4	15/4	8.25	6.68	21.7	29
		27/4	8.17	6.19	22.5	N/A
Clean site repeat 3	28/4 - 9/5	28/4	8.2	6.27	22.7	N/A
		9/5	8.16	6.45	26.6	N/A
Hypoxic site repeat 1	25/5 - 7/6	25/5	7.96	3.63	25.5	31*
		29/5	7.9	3.81	26.3	31*
		2/6	7.86	2.01	26.5	31
		7/6	7.8	3.93	27.5	31
Hypoxic site repeat 2	15/6 - 28/6	15/6	8.11	2.25	29.0	30
		19/6	7.92	1.04	28.6	30
		24/6	7.7	4.55	27.3	30
		28/6	8.01	5.20	26.3	31
Hypoxic site repeat 3	26/7 - 8/8	26/7	7.75	1.42	28.7	27
		31/7	7.57	0.63	27.9	30
		4/8	7.65	0.28	25.3	32
		8/8	7.56	0.18	24.6	32

Table 2.1. Summary of experimental days, biofilms age, measured pH, DO, temperature and salinity of the experimental repeats. All the experiments were done in the year of 2017. The 2 measurements of clean repeats were done on the first and the last days of the biofilm deployment period. The 4 measurements of hypoxic site repeats were done on the first, last days and additional 2 middle days of the biofilm deployment period. N/A: data not available. *: Readings of Yung Shue O bottom water, EPD “Red Tide Information Network” mobile application.

2.2 Exposure of biofilms to lower DO and pH levels in microcosms

Biofilmed dishes were incubated in the four microcosms of two levels of DO and pH which combined as a 2 x 2 factorial experiment. The four microcosms for biofilms treatment were filled with 10 L of filtered seawater (pore size 0.22 μm) obtained from Port Shelter. Water temperature was maintained at 25ºC (± 1ºC).

The desired pH and DO levels were achieved by constantly bubbling the filtered seawater with carbon dioxide (CO_2) and nitrogen (N_2) gas respectively (Figure 2.1). Flow controllers (Aalborg GFC-17) were used to control the amount of gases entering the system. Air was mixed with CO_2 before being distributed into the system to increase the gas volume for evener gas distribution. Gas distributors were used to adjust the right amount of gas to reach each microcosm. Control condition (6 mg O_2 L^{-1} and pH 8.1) was bubbled with air only. Hypoxia-OA combination condition (1.5 mg O_2 L^{-1} and pH 7.6) received mixture of N_2, CO_2 and air. Hypoxia condition (1.5 mg O_2 L^{-1} and pH 8.1) also received N_2, air and a small proportion of CO_2. CO_2 in this case was used to compensate the effect of increased pH caused by administration of N_2 gas, a process which initiated sparging and removed both dissolved O_2 and CO_2 from water by increasing the gas-liquid interface (Gobler et. al. 2014). OA condition (6 mg O_2 L^{-1} and pH 7.6) received mixture of CO_2 and air. Gases were mixed either in mixing tank or by gas distributor before reaching the microcosms. All of the microcosms were

covered with plastic films to create a closed environment for the water to reach the desired conditions at equilibrium. All DO levels were maintained within ± 0.2 mg O_2 L^{-1} throughout the experiment. They were monitored regularly using an optical DO meter (YSI ProODO). While all pH levels were maintained within pH ± 0.1. The pH levels were monitored with a pH meter (HANNA pHep®5 pH/Temperature Tester - HI98128) regularly.

On the day of collection from field (day 0, before exposure), and after 2 and 3 days of exposure in microcosms, except for repeat 2 of clean site where the exposure period was 3 and 4 days due to the availability of *C. onyx* larvae, biofilmed dishes were collected for bacterial cell viability assay (Section 1.3) and larval settlement assay (Section 1.5).

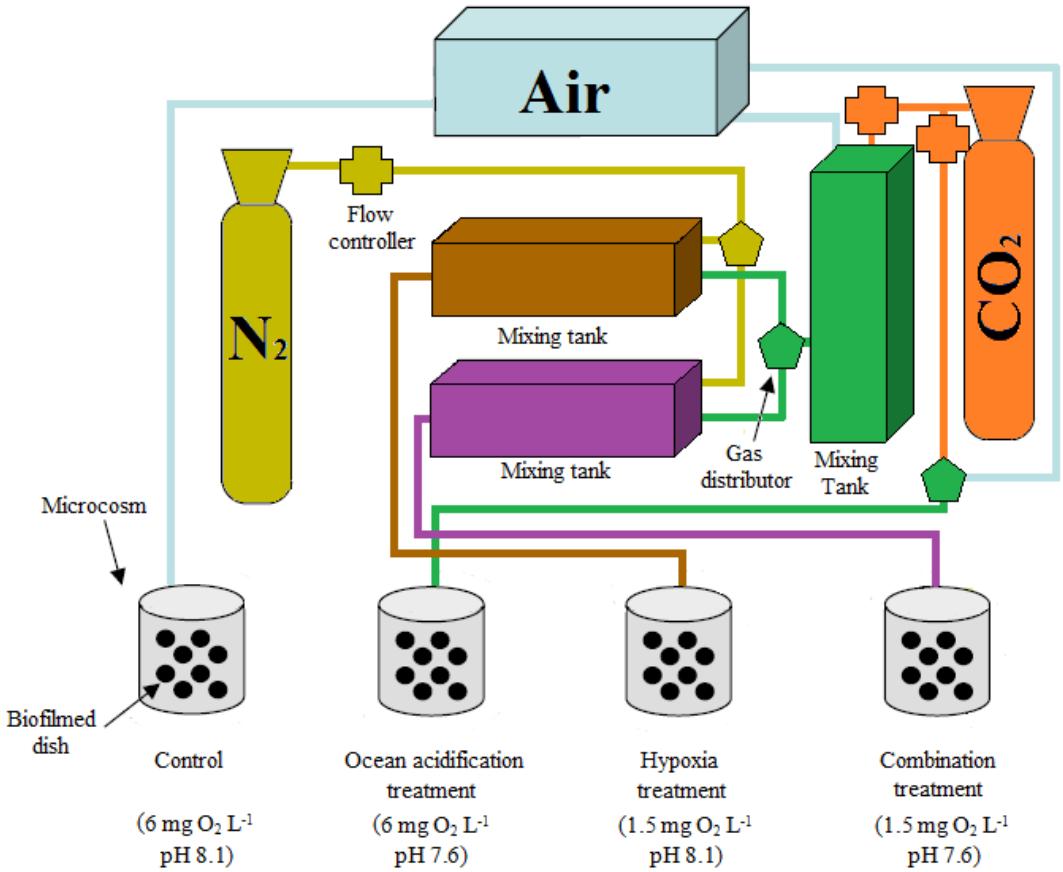

Figure 2.1: Experimental set-up of the microcosms for biofilms treatment. Conditions (pH and DO) in microcosm tanks for control, hypoxia and OA combination, hypoxia, and OA treatments were adjusted by pumping in mixtures of air, carbon dioxide (CO_2) and nitrogen (N_2) gas.

2.3 LIVE/DEAD cell viability assays for bacterial density and viability

Six replicate dishes, except for repeat 1 of clean site which consisted of three replicate dishes, were assayed for each time point of experiment (described in Section 2.2). Biofilms were stained with LIVE/DEAD™ BacLight™ Bacterial Viability Kit, for microscopy & quantitative assays, following the protocol from the manufacturer. Briefly, 5 µl of stain was pipetted onto the biofilms which was then covered by a glass coverslip. After 30 minutes of incubation in darkness, live and dead cells were counted by ImageJ after photos were taken from an epifluorescence microscope at a magnification of 1000× in 5 randomly chosen fields. The results from two days of post-treatment cell viability assays (except for repeat 1 of clean site where only biofilms after 3 days treatment were analyzed) were combined for reporting and statistical analysis.

2.4 Studied organism

Adult *C. onyx* were sampled from low intertidal zone in Tai Mui Wan (22°16'N, 114°17'E), Hong Kong. They were transferred and cultured in the laboratory conditions (temperature: 25 ± 1°C, DO: 6.5 ± 0.2 mg O_2L^{-1}, and pH: 8.1 ± 0.1) with artificial seawater (salinity: 30 ± 1 ‰) that was changed once per week. They were fed with the microalgae *Isochrysis galbana* and acclimatized for over 6 months before the experiments began.

Newly hatched larvae were collected by removing capsules carrying completely developed embryos (by observation under microscope) from the brooding chamber of females. Larvae were reared in a density of 1 individual per mL of 0.22 μm filtered seawater with temperature control to 25 ± 1°C. They were fed with microalgae *I. galbana* in a concentration of 2×10^5 cells mL^{-1} and the medium was changed while dead individuals were removed daily. Larvae of *C. onyx* become competent after 8 - 9 days when geometry of shell growth is shifting from spiral form characteristic of larvae to the linear form characteristic of adults (Pechenik 1980).

2.5 Larval settlement assay

Larval settlement assays were performed using biofilms from each time point of experiment (described in Section 2.2) for each repeat. The collection (from clean site and hypoxic site) and treatments of biofilms were in parallel with those for the determination of bacterial cell density and viability.

There were six polypropylene experimental chambers (dimension of chamber: 150 × 90 × 45 mm) for larval settlement in each of the four combinations of DO and pH conditions: control, hypoxia, OA, and hypoxia and OA combination. They were submerged in microcosms of 10 L of 0.22 μm filtered seawater at 25 ± 1°C under the corresponding DO and pH conditions. Experimental set-up for these conditions was described in Section 2.2. 150 morphologically competent larvae were transferred into each chamber. In order to equalize the DO and pH levels between the chambers and the microcosm, and to prevent the larvae inside the chambers from escaping to the microcosm, one of the sides of the chambers was an opening with 150 μm nylon mesh (130 × 75 mm). Each of the chamber served as a pseudoreplicate. For day 0 experiment, each chamber contained two fresh biofilmed dish and one clean petri dish as negative control. While for post-treatment experiment, each chamber contained three types of petri dish: a treated biofilmed dish which was subjected to the corresponding combinations of DO and pH levels, a positive control biofilmed dish which was subjected to normal DO and pH levels (6 mg O_2 L^{-1} and pH 8.1), and a

clean petri dish as negative control. The settlement assays were performed on orbital shakers continuously rotating at 100 rpm. This was proven to encourage the larvae to swim (Zhao and Qian, 2002) and to stimulate settlement on dish (Figure 2.2).

After 24 hours, the numbers of individuals that settled on the either of the biofilmed dishes, negative control dish, or on the wall of the chamber, were counted. Larvae with significant reduction in velar lobes, occurrence of shell brims and appeared to be crawling with foot were regarded as settled individuals (Pechenik 1980). The results from two days of post-treatment settlement assays were combined for reporting and statistical analysis.

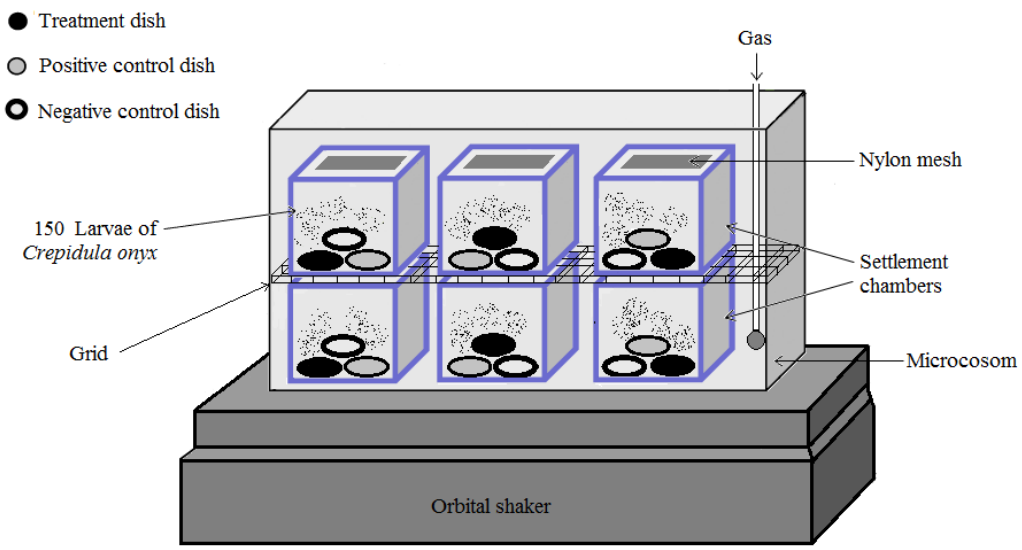

Figure 2.2: Experimental set-up of larval settlement assay. The DO and pH levels of the microcosm were adjusted to the corresponding levels of the biofilms treatment though pumping of air, carbon dioxide (CO_2) and nitrogen (N_2) gas as in Figure 2.1. The dish arrangements of three replicate settlement chambers together formed a Latin square. Each settlement chamber contained 150 larvae.

2.6 Statistical analysis

All percentage data were arcsine-transformed before analysis. Data of bacterial density and viability and settlement were checked for the normality and homogeneity by Shapiro-Wilk test and Equal Variance test respectively.

For bacterial cell density and viability assay, if data passed the normality and homogeneity tests, comparison between multiply treatments were checked for any significant difference by one-way ANOVA. Post-hoc Tukey's HSD test was used to identify differences among treatments once significant difference was found, while T-test was used for comparison with two treatments in this case. If the normality and homogeneity tests were failed, data were checked for any significant difference with their counterpart by Kruskal-Wallis test, post-hoc Tukey's HSD test was also used to identify differences among treatments once significant difference found, while Mann-Whitney U test was used for comparison with two treatments in this case. The significance level for the tests was 0.05.

For larval settlement assay, if data passed the normality and homogeneity tests, significant difference was checked by repeated measures one-way ANOVA. Post-hoc Tukey's HSD test was used to identify differences among treatments once significant difference was found. If the normality and homogeneity tests were failed, data were checked for significant difference by Friedman test, post-hoc Tukey's HSD test was also used once significant difference was found. The significance level for the tests was 0.05.

Chapter 3: Results and discussion

3.1 Physical parameters of clean and hypoxic sites

In clean site, the average water temperature and salinity were 22.37 ± 2.38°C and 29.00 ± 0.00 psu, respectively. The mean water DO level were 6.44 ± 0.49, 6.44 ± 0.35 and 6.36 ± 0.13 mg O_2 L^{-1} for repeat 1, 2 and 3 respectively, while the mean water pH were 8.21 ± 0.09, 8.21 ± 0.06 and 8.18 ± 0.03 for repeat 1, 2 and 3 respectively (Table 2.1).

In hypoxic site, the average water temperature and salinity were 26.96 ± 1.44°C and 30.50 ± 1.31 psu, respectively. The mean water DO level were 3.35 ± 0.90, 3.26 ± 1.95 and 0.63 ± 0.56 mg O_2 L^{-1} for repeat 1, 2 and 3 respectively, while the mean water pH were 7.88 ± 0.67, 7.94 ± 0.17 and 7.63 ± 0.09 for repeat 1, 2 and 3 respectively (Table 2.1).

3.2 Bacterial cell density and viability

Clean site

For the clean site, the mean bacterial density of the biofilms before exposure ranged from 26.41 – 54.20 × 10^3 cells mm^{-2}. After exposing to various levels of pH and DO, the mean bacterial density ranged from 24.51 – 83.76 × 10^3 cells mm^{-2} (Figure 3.1). When the control dish was compared with that before exposure, significantly higher bacterial density was resulted in two out of three repeats (T-test, $t_{(16)}$ = -4.41 and -4.27, $p < 0.001$ for repeat 2 and 3, respectively.)

The bacterial density of treated biofilms was different among the control and treatment groups in all repeats. The bacterial density of the combination treatment was consistently and significantly lower than that of the control (Tukey's test, $p < 0.05$ for all repeats). The bacterial density of hypoxia treatment generally lower than that of control, with significant difference occurred in two (repeat 2 and 3) out of three repeats (Tukey's test, $p < 0.001$). For ocean acidification treatment, while significantly lower bacterial density was resulted when compared with control in one of the repeats (repeat 2), no difference was found in other repeats.

For OA treatment, while significantly lower bacterial density was resulted when compared with control in one of the repeats (repeat 2), no difference was found in other repeats.

Comparing among OA, hypoxia and combination treatments, there was a generally lower bacterial density in both the hypoxia and combination treatment than that of OA treatment. Significantly, the bacterial density in the hypoxia and combination treatment was lower than that in the OA in repeat 3. No significant difference was found between hypoxia and combination treatments in all repeats.

The mean percentage of live bacterial cells on biofilmed dish before exposure were 51.72 ± 7.86%, 62.14 ± 5.67% and 45.40 ± 9.57% for repeat 1, 2 and 3 respectively and that after exposure were 40.78 ± 10.62%, 48.57 ± 7.98% and 49.85 ± 8.57% for repeat 1, 2 and 3 respectively (Figure 3.2). The percentage of live bacterial cell of control after exposure was generally the same as that before exposure, and only one repeat (repeat 2) showed significant reduction (T-test, $t_{(16)} = 5.39$, $p < 0.001$). There was generally no difference in the percentage of live bacterial cells among treatments, although a significant reduction was found in the OA biofilms compared with the hypoxia biofilms in repeat 2.

Hypoxic site

For the hypoxic site, the mean bacterial density of the biofilms before exposure ranged from 14.35 – 93.04 × 10^3 cells mm^{-2}. After exposure in laboratory, the mean density was ranged from 9.36 – 73.04 × 10^3 cells mm^{-2} (Figure 3.1). When the control dish was compared with that before exposure,

a significant reduction in bacterial density was found in one out of three repeats (repeat 1) (T-test, $t_{(16)} = 2.49$, $p = 0.024$).

The total bacterial density of treated biofilms was different among the control and treatment groups in all repeats. The hypoxia and combination treatments were significantly lower than that of the control in all repeats (Tukey's test, $p < 0.001$). However, there was no significant difference in total bacterial density between OA treatment and control, with an exception in repeat 3 where significantly lower bacterial density was resulted in OA treatment.

Comparing among OA, hypoxia and combination treatments, similar to the clean site, there was a generally lower bacterial density in hypoxia and combination treatments, which remained similar between themselves. Significantly lower bacterial density was found in hypoxia and combination treatments versus OA treatment in repeat 1, and in combination treatment versus OA treatment in repeat 2 (Tukey's test, $p < 0.05$).

The mean percentage of live cells for biofilmed dish before exposure were 60.51 ± 4.39%, 58.94 ± 4.88% and 60.00 ± 10.14% for repeat 1, 2 and 3 respectively and that after exposure were 54.47 ± 9.70%, 47.94 ± 10.72% and 33.94 ± 12.78% for repeat 1, 2 and 3 respectively (Figure 3.2). The percentage live cell of control after exposure was generally lower than that before exposure, however, one repeat (repeat 3) showed significant

reduction (T-test, $t_{(16)} = 4.86$, $p < 0.001$). There was no significant difference among control and treatments in all repeats.

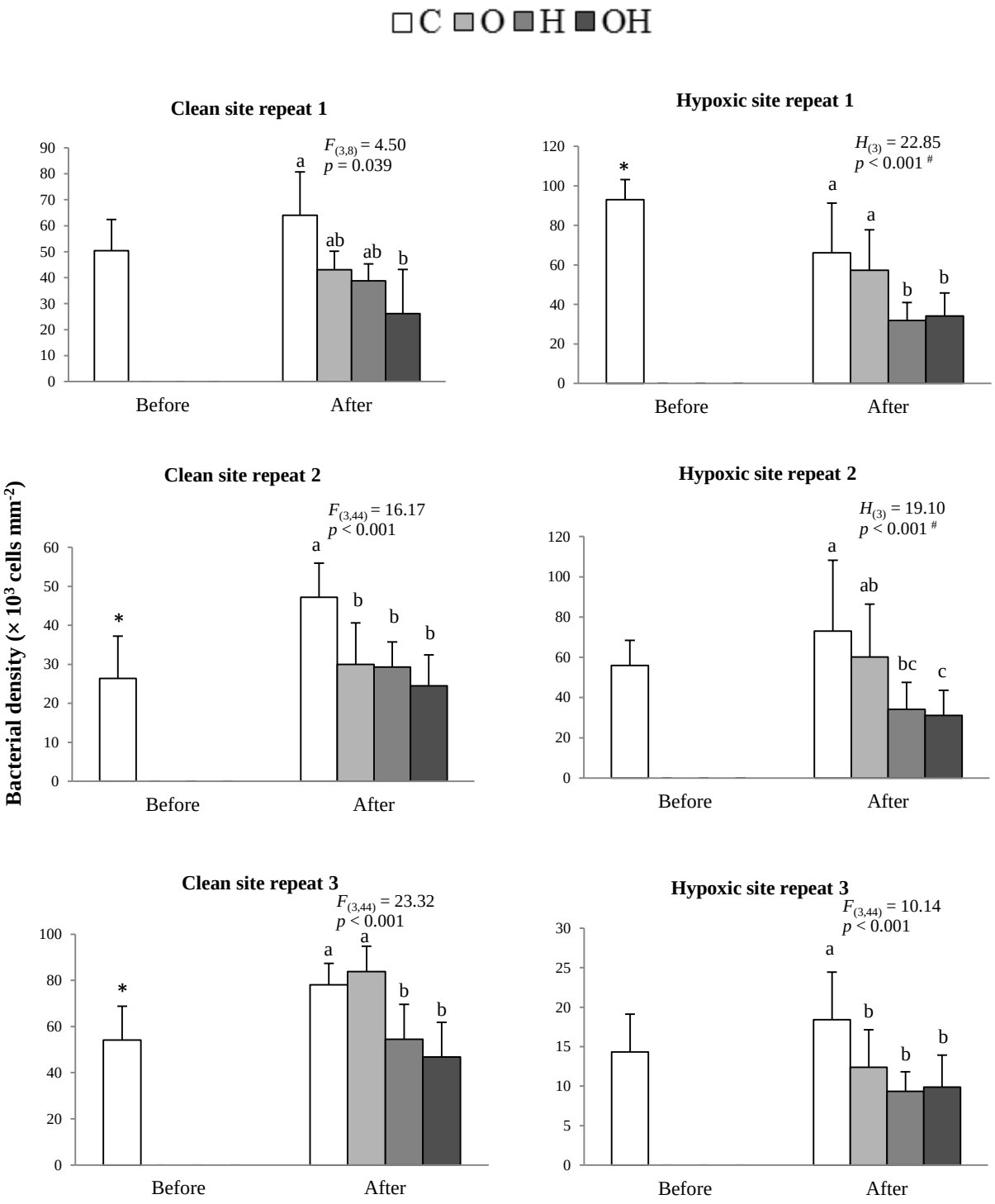

Figure 3.1: Total bacterial cell density of biofilms before and after exposure to combinations of two levels of pH (normal: 8.1, lower: 7.6) and DO (normal: 6 mg O_2 L^{-1}, hypoxia: 1.5 mg O_2 L^{-1}) in four microcosms conditioned as control (C) and treatments of OA (O), hypoxia (H), and OA and hypoxia combination (OH). Biofilms from a clean sites and a hypoxic site were investigated for three times (repeats). Each bar represents the mean

(+ SD) of six replicates (but three replicates for clean site repeat 1). Statistical values of One-way ANOVA (or Kruskal-Wallis test specified with $^{\#}$) are shown above the bars. Significantly different means (t-test, $p < 0.05$) between the unexposed biofilms and the biofilms after control exposure (white bars) are represented by an asterisk (*), while the significantly different means after treatments (Tukey's test, $p < 0.05$) are represented by different alphabets above each bar.

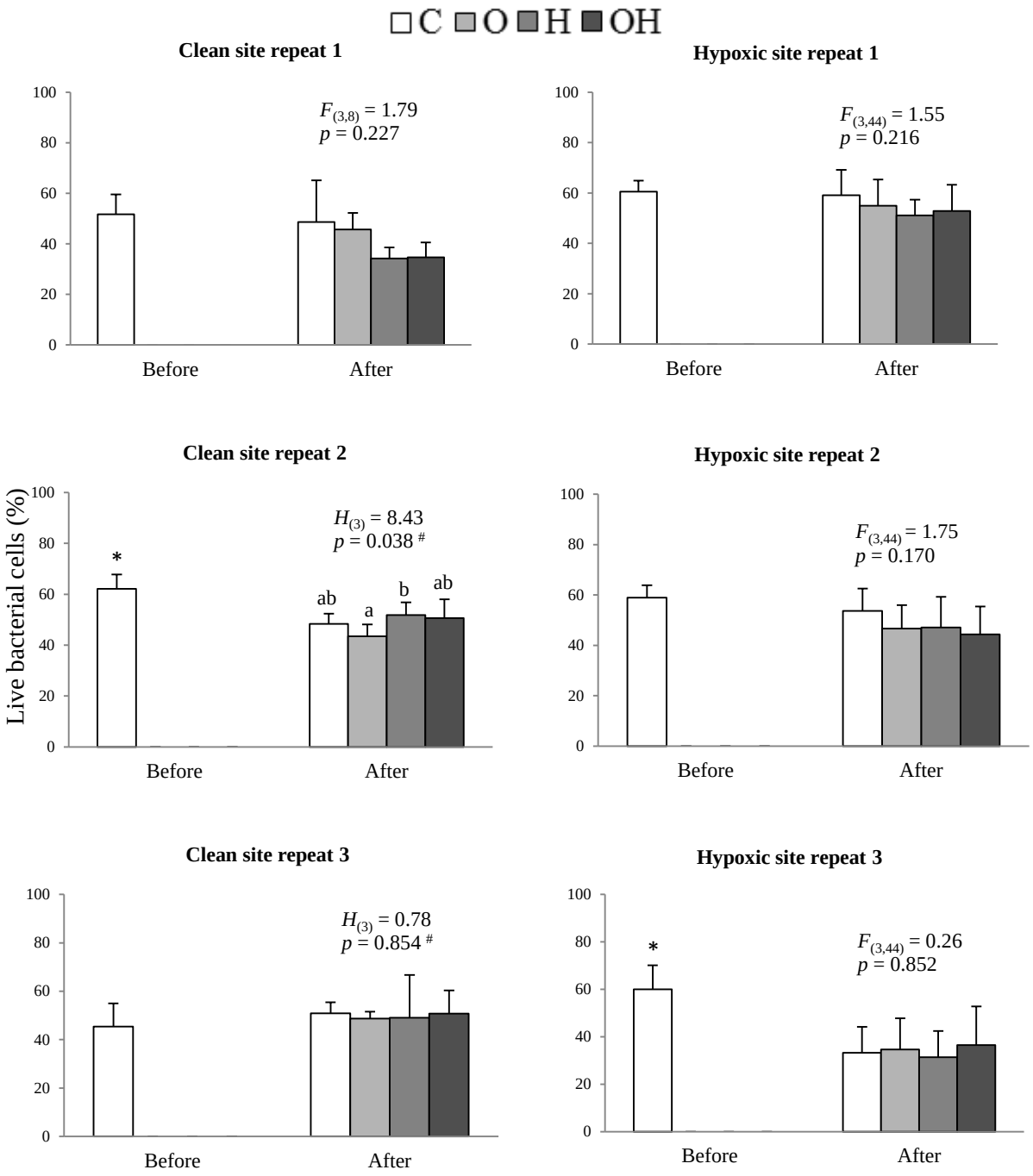

Figure 3.2: Percentage of live bacterial cells on biofilms before and after exposure to combinations of two levels of pH (normal: 8.1, lower: 7.6) and DO (normal: 6 mg O_2 L^{-1}, hypoxia: 1.5 mg O_2 L^{-1}) in four microcosms as control (C) and treatments of OA (O), hypoxia (H), and OA and hypoxia combination (OH). Biofilms from a clean sites and a hypoxic site were investigated for three times (repeats). Each bar represents the mean (+ SD) of six replicates (but three replicates for clean site repeat 1). Statistical values for One-

way ANOVA (or Kruskal-Wallis test specified with $^{\#}$) are shown above the bars. Significantly different means (t-test, $p < 0.05$) between the unexposed biofilms and the biofilms after control exposure (white bars) are represented by an asterisk (*), while the significantly different means after treatments (Tukey's test, $p < 0.05$) are represented by different alphabets above each bar.

3.3 Larval settlement assay

Before exposure of biofilms to treatments

Clean site

For the clean site, there was no significant difference in the settlement rate of *C. onyx* larvae on the two biofilmed dishes for all the treatments and repeats (Figure 3.3).

The mean proportion settlement on the negative control dish was always significantly lowest (Tukey's test, $p < 0.05$) in the control, OA and combination treatments of all repeats. However, in the hypoxia treatment of repeat 3, the settlement rate of negative control had no significant difference with that of the biofilmed dishes, although the mean settlement rate of negative control was still the lowest.

Hypoxic site

For the hypoxic site, the settlement rates of larvae on the two biofilmed dishes were the same for all repeats and water conditions. The larval settlement rate on the negative dish was the lowest in all but repeat 3 in which there was no difference among all dishes (Figure 3.3).

After exposure of biofilms to treatments

Clean site

For the clean site, the proportion that settled on the treatment dish was significantly lower than the positive control dish (Tukey's test, $p < 0.05$) in all of the repeats of both hypoxia treatment and combination treatment (Figure 3.4). The differences in that proportion ranged from 16.95% to 19.79% and 28.43% to 33.77% for hypoxia and combination treatments, respectively. Nonetheless, there was no such difference in control and OA treatment. The larval settlement rate on the negative control dish was always the lowest.

Hypoxic site

For the hypoxic site, there was no significant difference on the settlement rate of larvae of *C. onyx* on the treatment dish and positive control dish in control and OA treatment. The settlement rate on treatment dish was significantly lower than that on positive control dish (Tukey's test, $p < 0.05$) in all repeats except one repeat of hypoxia (repeat 3, this might due to the extra low bacterial density on the biofilms which caused an overall reduction in settlement rate and large standard deviation among the data which encouraged insignificant statistical comparisons) of both hypoxia treatment and combination treatment. The difference in proportion of larvae ranged from 26.84% to 45.66% and 19.00% to 40.94% for hypoxia and combination treatments, respectively.

The larval settlement rate on the negative dish was the lowest in most treatments with some exceptions. These included that the negative control dish being similar in proportion settlement as the treatment dishes in combination treatment of all repeats and the hypoxia treatment of repeat 1, and that in repeat 3 the settlement percentage on negative control was not significantly different from other dishes other than the positive control dishes of hypoxia treatment.

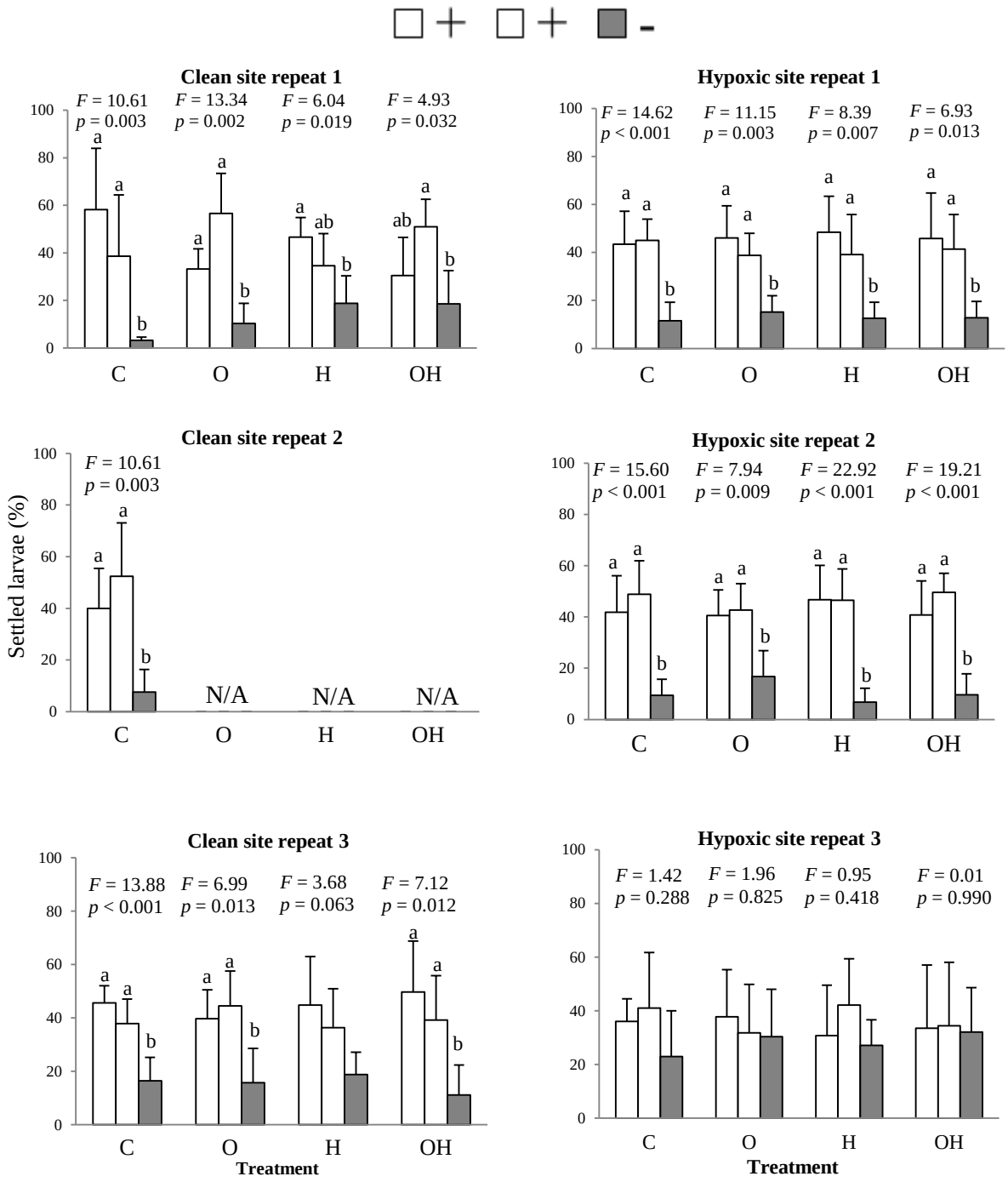

Figure 3.3: Settlement choice assays of *Crepidula onyx* larvae on biofilms before treatment (on the day the biofilms were freshly collected from the clean site and hypoxic site). Each bar shows the mean proportion (+ SD) of settled larvae that were found on one of three choices of dishes (two biofilmed dishes (+) and one negative control clean dish (–)) from 6 replicate chambers, each with 150 competent larvae, after 24 hours. Chambers

were submerged into filtered seawater with four different conditions during the assays: Control (C: 6 mg O_2 L^{-1} and pH 8.1); OA treatment (O: 6 mg O_2 L^{-1} and pH 7.6); hypoxia treatment (H: 1.5 mg O_2 L^{-1} and pH 8.1); and OA and hypoxia combination treatment (OH: 1.5 mg O_2 L^{-1} and pH 7.6). Statistical values of repeated measures One-way ANOVA (*df* = 2, residual degree = 10) are shown above the bars and means which are different significantly (Tukey's test, $p < 0.05$) are represented by different alphabets above each bar. N/A: data not available.

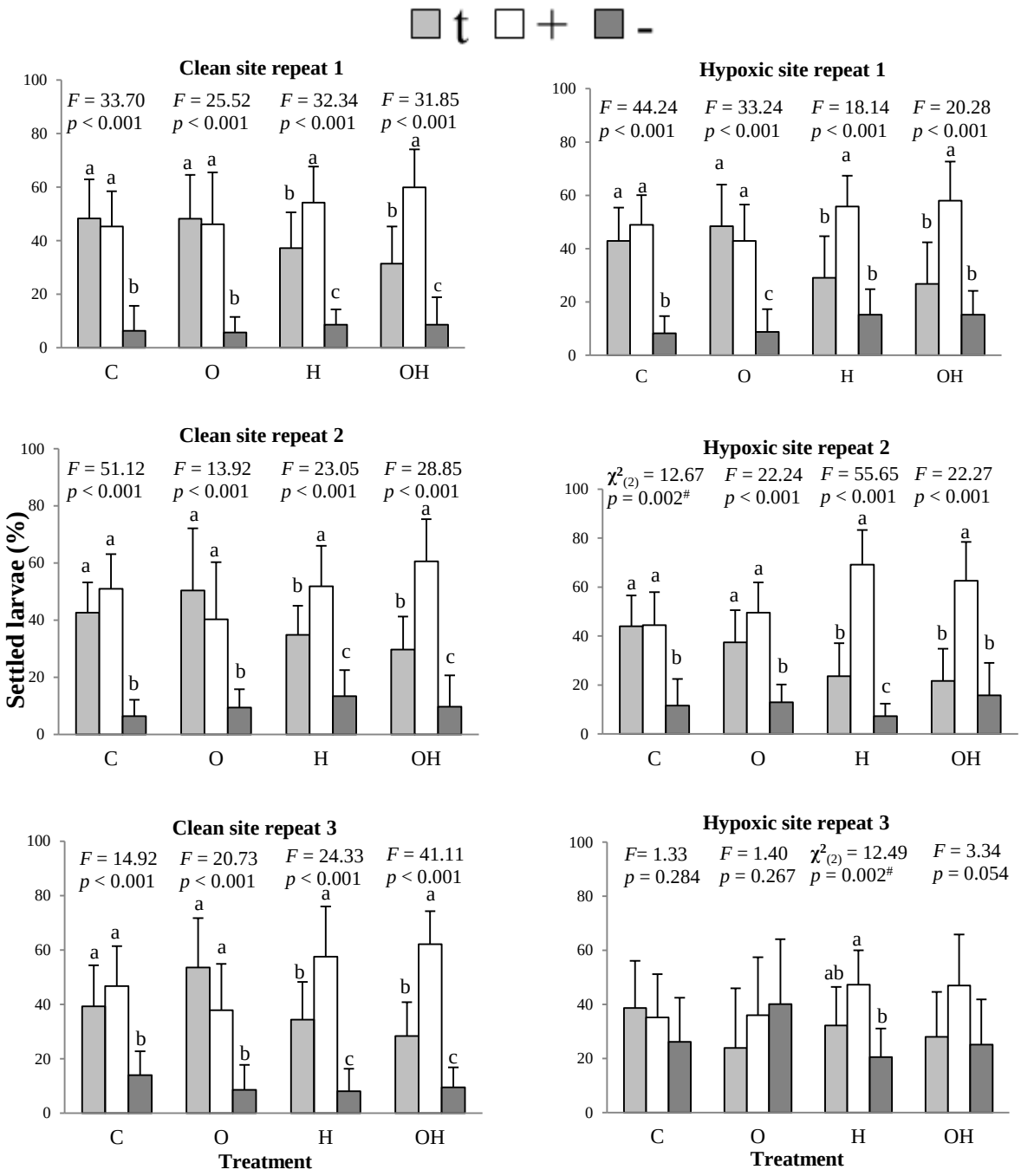

Figure 3.4: Settlement choice assays of *Crepidula onyx* larvae on treated biofilms (originated from clean site or hypoxic site) which were submerged in four conditions: Control (C: 6 mg O_2 L^{-1} and pH 8.1); OA treatment (O: 6 mg O_2 L^{-1} and pH 7.6); hypoxia treatment (H: 1.5 mg O_2 L^{-1} and pH 8.1); and OA and hypoxia combination treatment (OH: 1.5 mg O_2 L^{-1} and pH 7.6) for two to three days. Each bar shows the mean proportion (+ SD) of settled larvae that were found on one of three choices of dishes (treated biofilmed

dish (t), positive control biofilmed dish (+), and negative control clean dish (–)) from 12 replicate chambers, each with 150 competent larvae, after 24 hours. Chambers were submerged into filtered seawater with the corresponding treatments during the assays. Statistical values of repeated measured One-way ANOVA (df = 2, residual degrees = 22 in all repeats, except where Friedman test was used which were specified with $^{\#}$) are shown above the bars and means which are different significantly (Tukey's test, $p < 0.05$) are represented by different alphabets above each bar.

3.4 Correlation between bacterial density and percentage settlement

There was significant positive correlation between percentage settlement on treatment dish and cell density among all repeats for both sites (Linear Regression, $F(1,22) = 12.15$, $p = 0.002$, $R^2 = 0.356$) (Figure 3.4).

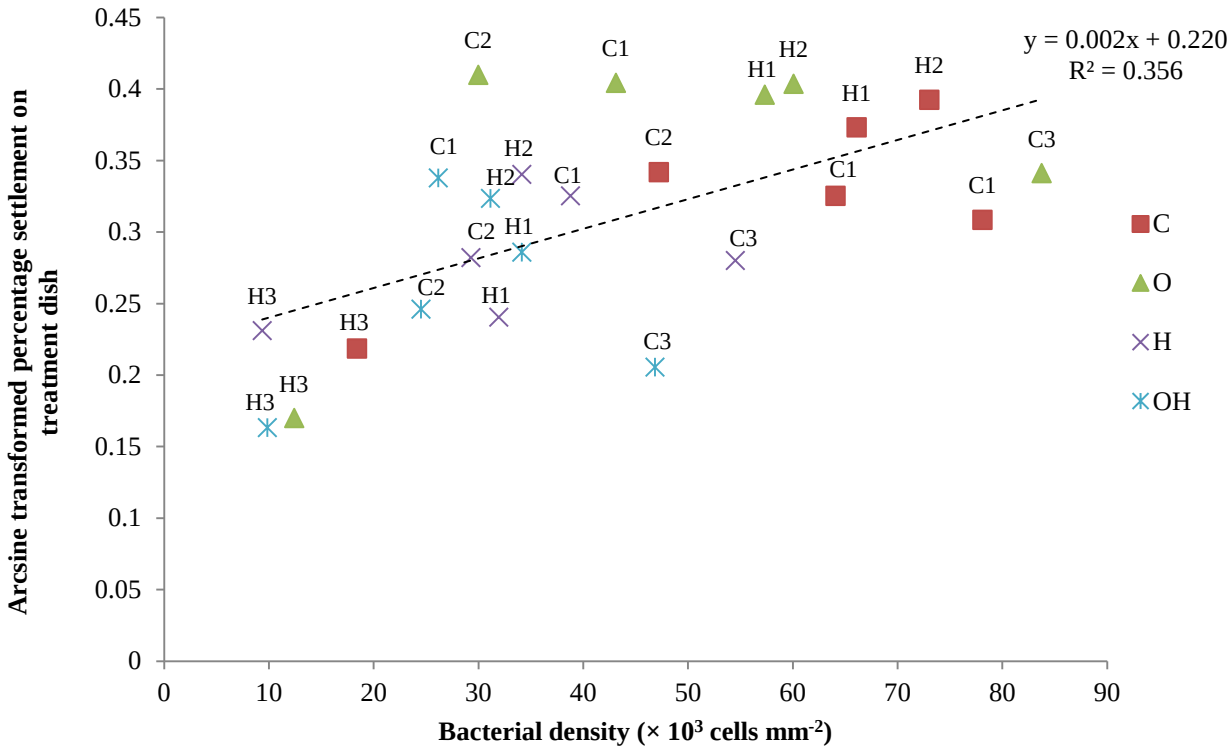

Figure 3.5: Correlation between bacterial cell density and percentage settlement on treated biofilmed dishes among all repeats of clean and hypoxic site. Alphabets in legend: C: control; O: OA treatment; H: hypoxia treatment; OH: OA and hypoxia combination treatment; alphabets above each point: C: clean site repeat; H: hypoxic site repeat.

Chapter 4: Discussion, conclusion and implications

4.1 Discussion

This study is the first study that attempts to investigate the individual and interactive effects of hypoxia and OA on biofilms and the subsequent effects on settlement of *Crepidula onyx*. Variations in settlement preference of *C. onyx* on biofilms which undergone different pH and DO level treatments were explicitly evident. Reduction of seawater pH and DO have also altered the bacterial cell density in biofilms.

An extra low DO during the biofilms development period of repeat 3 of hypoxic site (Table 2.1) had probably resulted in biofilms with very low bacterial density (Cheung et al. 2014), and subsequently an overall lower settlement rate (Chiu et al. 2012). Owing to this, data from this repeat contributed to a larger variation among repeats that brought to abnormal distributions and unequal variation and subsequently insignificant statistical comparisons. Thus, this repeat was omitted when drawing conclusion about the settlement preference among dishes in choice assay.

Larval settlement on biofilms before treatment

The percentage of larval settlement on the two biofilmed dishes showed no difference to each other among settlement chambers of control and all

treatments (Figure 3.3). This showed that the natural variation occurs on each biofilmed dish is not enough to cause a differentiation on settlement preference among the dishes. Moreover, this further showed the background DO and pH level had no effect on the settlement preference on the two biofilmed dishes. Thus, the difference in settlement preference after biofilms treatment could be assumed to be caused only by the alterations in biofilms.

After biofilms treatment

Control

Bacterial density

Biofilms from clean site were generally higher in bacterial density after a maximum of three days of laboratory incubation. It was expected as the DO and pH levels were similar to that of the field, only minimum stress was encountered by the bacterial community. This could also prove that the microcosm for incubating biofilms provided suitable environment that did not cause major mortality on bacteria. For hypoxic site biofilms, no significant trend found. This could be explained by the resilience of bacteria which favoured normoxia and ambient pH accompanied with the die off of bacteria which favoured hypoxia and/or low pH. Subsequent emergence of opportunistic species might also contribute to this.

Settlement

The same settlement rate on positive control and treatment dish perfectly demonstrated no significant variation regarding larval settlement preference occurred among biofilms under the same condition during the 2 to 3 days of laboratory exposure. This further supported the assumption that any difference in settlement preference among biofilms would be caused by the biofilms treatments.

OA

Bacterial density

The bacterial density of OA treated biofilms was generally lower than that of control for both sites. Some literatures showed that bacterial abundance was resistant to OA (Takeuchi et al. 1997, Grossart et al. 2006 and Oliver et al. 2014). Different from this study, Takeuchi et al. (1997) studied on free living cultured bacteria using artificial cultivation approach, which was in contrast to the attached bacteria from natural environment in this study. While Grossart et al. (2006) and Oliver et al. (2014) studied on bacteria in mesocosms located in natural coasts with addition of nutrients to stimulate algal bloom aiming to simulate a nature OA system, in this study the nutrient was unenriched. A more similar laboratory-based study with OA treatment on biofilms showed that the bacterial density of biofilms decreased after 10 days exposure, suggesting a lower pH level increases the respiration and in turn the metabolic and energy cost among the bacteria (Baragi et al. 2016).

The general reduction in bacterial density in the current study might be explained by the same reason, but the insignificant result might be due to the shorter exposure period (3 days).

Settlement

Settlement rate of most of the marine invertebrates is mainly governed by the bacterial density and community (Shin et al. 2013). For example, settlement rate of tubeworm *Hydroides elegans* is correlated to the bacterial density (Hadfield et al. 1994) and *Pseudoalteromonas luteoviolacea* can induce settlement of *H. elegans* (Hadfield 2011). For *C. onyx*, their settlement rate was shown to have a positive relationship with the bacterial density. (Chiu et al. 2012). However, it is still not known whether there is and if so, which bacterium governs the settlement of *C. onyx*.

In most repeats of this study, OA exerted no effect on both bacterial density and settlement. Based on the unchanged bacterial density, OA characterized community should have no effect on settlement. For specific repeats with reduction in bacterial density, settlement rate was also unaffected, there were two possible explanations. The first explanation was that the OA bacterial community might conditionally encourage settlement of *C. onyx* when the overall bacterial density is reduced. In this case, another factor should have attracted settlement so that the effect from decrease in bacterial density was balanced off. It is proposed that the composition in bacterial community might have been changed and resulted to an unchanged overall settlement

rate. The second possible explanation was that since the mean reduction in the bacterial density was smaller (in comparison with that of hypoxia and combination of hypoxia and OA treated biofilms), it might not be sufficient to reduce the settlement of *C. onyx*.

Hypoxia

Bacterial density

The bacterial density of biofilms was reduced after hypoxia exposure compared with the control biofilms for both sites. An unusual result where there was no significant difference in repeat 1 of clean site, nevertheless the mean was lower in hypoxia exposed biofilms, may due to a low number of replicates (3 replicates, 3 replicates from 1 time point) compared with other repeats (12 replicates, 6 replicates from 2 time points). Hypoxia-induced reduction in biofilms bacterial density was also shown by previous study (Cheung et al 2014). A possible explanation was the reduction in oxygen, the key electron acceptor, limited aerobic respiration among bacteria, this resulted in reduction in energy production and, subsequently, cell proliferation. For hypoxic site, although some species which was associated with low DO environments might be benefited by hypoxia treatment, reduction in energy production might still limited the overall cell proliferation rate. However, further study is needed to investigate the

mechanism of hypoxia on the respiration and growth of bacteria in order to prove this.

Settlement

The settlement rate on hypoxia treated biofilms was lower than that of control biofilms for both sites. This study suggests that biofilms density was a factor contributing to the reduction in settlement of *C. onyx*. Similarly, reduction of bacterial density caused by other factor (organic pollutant) has also reduced settlement preference of *C. onyx* (Chiu et al. 2012).

Combination of hypoxia and OA

Bacterial density:

The bacterial density was reduced compared with the control and was similar to that of the hypoxia-treated biofilms for both sites. As there was no change in density in OA-treated biofilms in most repeats, and as there was no interaction between OA and hypoxia, it indicated that the reduction in cell density was due to exposure to hypoxia.

It has been predicted that OA may compensate the effect of hypoxia on bacterial density by replenishment of oxygen. The mechanism suggested was that lower pH level in seawater can stimulate the growth of algal species and shift the biofilms community into an algal dominant community (such

as Baragi et al. 2016, Russell et al. 2009), as algal species is benefited by the reduced energetic cost and improved resource allocation through the down-regulated Carbon Concentrating Mechanisms under such condition (Trimborn et al. 2009, Hopkinson et al. 2011). This led to a higher overall photosynthetic rate and more oxygen would be produced.

However, this prediction was not true in this study, this might be explained by the following. Firstly, nutrient-load hypothesis explains that the boost the growth of algae it needs both nutrients and carbon, and growth of algae is restricted if there is a lack of either one of the factors (Verspagen et al. 2014). The sediment particles associated with biofilms, being the only source of nutrient in the whole microcosm, might have been insufficient to support the growth of algae. Secondly, light which is required by photosynthesis might also be a limiting factor as only low background lighting from the culture room was provided. Finally, three days exposure time might be too short to accumulate enough algae and oxygen.

Settlement:

Having a similar settlement pattern of *C. onyx* in hypoxia treated biofilms, the settlement rate on combination treated biofilms was lower than that of the positive control biofilms. The study suggests there was no overall interaction between hypoxia and OA on settlement of *C. onyx*.

4.2 Conclusion

In conclusion, the effects exerted by OA, hypoxia and their combination on biofilms and the subsequent settlement of *C. onyx* were revealed. Only hypoxia and combination conditions had reduction in total bacterial abundance and subsequently in settlement preference of *C. onyx*, while OA only tended to reduce bacterial density on biofilms. There was no interaction between hypoxia and OA on settlement rate and bacterial density. However, there were variations on the biofilms bacterial community among different treatments (unpublished work by labmates, 16S rRNA gene sequencing, Illumina MiSeq sequencing). Further analysis on the settlement response of *C. onyx* under biofilms from different treatments with adjustment to same density is needed, in order to clarify if the change in community composition by different DO and pH levels also affects the settlement preference.

4.3 Implications

Reduction in settlement rate on hypoxia and hypoxia and OA combination treated biofilms implied that larvae of *C. onyx* were able to distinguish and avoid unfavourable environments. This tendency might reduce the abundance of the sensitive species in hypoxic and combination of hypoxic and OA environments by reduction in recruitment rate, and thus, overgrowth of tolerant species that could be resulted from reduced competition. If most invertebrate species response similarly, changes in ecological balance by alterations of important processes such as prey-predator relationship and trophic transfer of energy could occur. These may finally lead to significant changes in ecological balance and services and shifts in coastal ecosystem. Studying with more invertebrate models may be needed to elucidate the response of the invertebrate community.

List of References

Astudillo JC, Wong JCY, Dumont CP, Bonebrake TC, Leung KMY. 2014. Status of six non-native marine species in the coastal environment of Hong Kong, 30 years after their first record. *BioInvasions Records*. 3(3): 123–137.

Baragi LV, Anil AC. 2016. Synergistic effect of elevated temperature, pCO_2 and nutrients on marine biofilm. *Marine Pollution Bulletin,* 105(1):102-109.

Borges AV, Gypens N. 2010. Carbonate chemistry in the coastal zone responds more strongly to eutrophication than to ocean acidfication. *Limnology and Oceanography*. 55(1):346–353.

Bray RJ, and Curtis JT. 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological Monographs. 27:325-249.

Cai Y, Guo X, Aldson KM, Donald GR. 2012. The source and distribution of dissolved and particulate organic matter in the Bay of St. Louis, northern Gulf of Mexico. Estuarine, *Coastal and Shelf Science.* 96:96-104.

Caldeira K, Wickett ME. 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *Journal of Geophysical Research*.110:C09S04.

Calder-Potts R, Spicer JI, Calosi P, Findlay HS, Widdicombe S. 2015. A mesocosm study investigating the effects of hypoxia and population density on respiration and reproductive biology in the brittlestar *Amphiura filiformis*. *Marine Ecology Progress Series.* 534:135-147.

Cheung SG, Chan CYS, Po BHK, Li AL, Leung JYS, Qiu JW, Ang PO, Thiyagarajan V, Shin PKS, Chiu JMY. 2014. Effects of hypoxia on biofilms and subsequently larval settlement of benthic invertebrates. *Marine Pollution Bulletin*. 85(2):418-424.

Chiu JMY, Po BHK, Chan CYS, Lam MHW, Qian PY, Wu RSS. 2012. Polybrominated Diphenyl Ethers (PBDEs) Alter Larval Settlement of Marine Intertidal Organisms across Three Phyla via Reducing Bacterial Abundance on the Biofilms. *Environmental Science and Technology*. 46(14):7772-7781.

Chiu JMY, Thiyagarajan V, Pechenik JA, Hung OS, Qian PY. 2007. Influence of bacteria and diatoms in biofilms on metamorphosis of the marine slipper limpet *Crepidula onyx*. *Marine Biology*. 151:1417-1431.

Chung HC, Lee OO, Huang YL, Mok SY, Kolter R, Qian PY. 2010. Bacterial community succession and chemical profiles of subtidal biofilms in relation to larval settlement of the polychaete *Hydroides elegans. The ISME Journal*. 1-12.

Clarke KR. 1993. Non-parametric multivariate analysis of changes in community structure. Australian Journal of Ecology. 18:117-143.

Clarke KR, Warwick RM. 1999. The taxonomic distinctness measure of biodiversity: weighting of step lengths between hierarchical levels. Marine Ecology Progress Series. 184:21-29.

Coe WR. 1935. Sexual phases in prosobranch mollusks of the genus *Crepidula*. *Science*. 81:570-572.

Costanza R, d'Arge R, De Groot RS, Farber S, Grasso M, Hannon B, Limburg K, Naeem S, O'Neil RV, Paruelo J, Raskin RG, Sutton P, van den Belt M. 1997. The value of the world's ecosystem services and natural capital. *Nature*. 387:253-260.

Das T, Stickle WB. 1994. Detection and avoidance of hypoxic water by juvenile *Callinectes sapidus* and *C. similis*. *Marine Biology*. 120:593-600.

Díaz RJ, Rosenberg R. 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioral responses of benthic macrofauna. *Oceanography and Marine Biology*. 33:245-303.

Díaz RJ, Rosenberg R. 2008. Spreading Deadzones and Consequences for Marine Ecosystems. *Science.* 321:926-929.

Diaz RJ, Rosenberg R. 2011. Introduction to environmental and economic consequences of hypoxia. International Journal of Water Resources Development. 27:71–82.

Direkbusarakom S and Danayadol Y. 1998. Effect of oxygen depletion on some parameters of the immune system in black tiger shrimp (*Penaeus monodon*). In TW Flegel (ed), *Advances in Shrimp Biotechnology* (pp. 147-149), National Center for Genetic Engineering and Biotechnology, Bangkok.

Doney SC, Fabry VJ, Feely RA, Kleypas JA. 2009. Ocean Acidification: The Other CO2 Problem, *Annual Review of Marine Science*. 1:169–192.

Doney SC, Mahowald N, Lima I, Feely RA, Mackenzie FT, Lamarque J-F, Rasch PJ. 2007. Impact of anthropogenic atmospheric nitrogen and sulfur deposition on ocean acidification and the inorganic carbon system. *Proceedings of the National Academy of Sciences*. 104(37):14580–14585.

Doney SC, Schimel DS. 2007. Carbon and climate system coupling on timescales from the Precambrian to the Anthropocene. *Annual Review of Environment and Resources*. 32:31–66.

Edgar RC. 2013. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature Methods. 10(10):996-998.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27:2194-2200.

EPD. 2015. Marine Water Quality Data [Internet]. Environmental Protection Department, Hong Kong; [cited 2017 March 18]. Available from http://epic.epd.gov.hk/EPICRIVER/marine/?lang=en

Feely RA, Alin SR, Newton J, Sabine CL, Warner M, Devol A, Krembs C, Maloy C. 2010. The combined effects of ocean acidification, mixing, and respiration on pH and carbonate saturation in an urbanized estuary. *Estuarine, Coastal and Shelf. Science*. 88(4):442–449.

Feely RA, Sabine CL, Lee K, Berelson W, Kleypas J, Fabry VJ, Millero FJ. 2004. Impact of anthropogenic CO2 on the CaCO3 system in the oceans. *Science*. 305:362–366.

Fleddum A, Cheung SG, Hodgson P, Shin PKS. 2011. Impact of hypoxia on the structure and function of benthic epifauna in Tolo Harbour, Hong Kong. *Marine Pollution Bulletin*. 63(5-12):221-229.

Gazeau F, Quiblier C, Jansen JM, Gattuso JP, Middelburg JJ, Heip CHR. 2007. Impact of elevated CO2 on shellfish calcification. *Geophysical Research Letters*. 34:L07603.

Gilbert D, Rabalais NN, Díaz RJ, Zhang, J. 2010. Evidence for greater oxygen decline rates in the coastal ocean than in the open ocean. *Biogeosciences*. 7: 2283-2296.

Gobler CJ, Baumann H. 2016. Hypoxia and acidification in ocean ecosystems: coupled dynamics and effects on marine life. *Biology Letters*. 12:20150976.

Gobler CJ, DePasquale EL, Griffith AW, Baumann H. 2014. Hypoxia and acidification have additive and synergistic negative effects on the growth, survival, and metamorphosis of early life stage bivalves. *PLoS One*. 9(1):e83648.

Gray JS, Wu RSS, Or YY. 2002. Effects of hypoxia and organic enrichment on the coastal marine environment. *Marine Ecology Progress Series*. 238:249–279.

Grossart HP, Allgaier M, Passow U, Riebesell U. 2006. Testing the effect of CO_2 concentration on the dynamics of marine heterotrophic bacterioplankton. *Limnology and Oceanography*. 51(1):1–11.

Hadfield MG. 2011. Biofilms and marine invertebrate larvae: what bacteria produce that larvae use to choose settlement sites. Annual Review of Marine Science. 3:453–470.

Hadfield MG, Unabia CC, Smith CM, Michael TM. 1994. Settlement preferences of the ubiquitous fouler *Hydroides elegans*. In Thompson MF, Nagabhushanam R, Sarojini R, Fingerman E (eds), *Recent Developments in Biofouling Control*. Oxford and IBH, New Delhi.

Hadfield MG, Carpizo-Ituarte EJ, del Carmen K, Nedved BT. 2001. Metamorphic competence, a major adaptive convergence in marine invertebrate larvae. *American Zoologist*. 41(5):1123–1131.

Hadfield MG, Paul VJ. 2001. Natural chemical cues for settlement and metamorphosis of marine invertebrate larvae. In McClintock JB, Baker BJ (eds), *Marine chemical ecology* (pp 431–461), CRC Press, Boca Raton Florida.

Holmstrom C, Kjelleberg S. 2000. Bacterial interactions with marine fouling organisms. In Evans LV (ed), *Biofilms: recent advances in their study and control* (pp 101–115), Harwood Academic Publisher, Amsterdam.

Hopkinson BM, Dupont CL, Allen AE, Morel FM. 2011. Efficiency of the CO_2- concentrating mechanism of diatoms. *Proceedings of the National Academy of Sciences*. 108:3830-3837.

Howarth R, Chan F, Conley DJ, Garnier J, Doney SC, Marino R, Billen G. 2011. Coupled biogeochemical cycles: eutrophication and hypoxia in temperate estuaries and coastal marine ecosystems. *Frontiers in Ecology and the Environment.* 9:18-26.

Huang ZG, Morton B, Yipp MW. 1984. The distribution and ecological biological features of *Crepidula onyx* in Hong Kong. *Acta Oceanologica Sinica*. 5:827-839.

Isensee K, Levin LA, Breitburg D, Gregoire M, Garçon V, Valdés L. 2016. The Ocean is Losing its Breath. *OCEAN AND CLIMATE, 2016 – Scientific Notes*, Second edition, Tome 2. 20-32 [Internet]. [Cited 2018 March 6]. Available from: www.ocean-climate.org

Kroekera KJ, Micheli F, Gambib MC, Martzc TR. 2011. Divergent ecosystem responses within a benthic marine community to ocean acidification. *Proceedings of the National Academy of Sciences*. 108(35):14515-14520.

Lagos ME, White CR, Marchall DJ. 2016. Biofilm history and oxygen availability interact to affect habitat selection in a marine invertebrate. *Biofouling*. 32(6):645-655.

Larkin G, Closs GP, Peake B. 2007. Tolerance and behaviour of the mysid shrimp *Tenagomysis novae-zealandiae* to low dissolved oxygen. *New Zealand Journal of Marine and Freshwater Research*. 41(3):317-323

Lawes JC, Neilan BA, Brown MV, Clark GF, Johnston EL. 2016. Elevated nutrients change bacterial community composition and connectivity: high throughput sequencing of young marine biofilms. *The Journal of Bioadhesion and Biofilm Research.* 32(1):57-69.

Li A and Chiu JMY. 2013. Latent effects of hypoxia on the gastropod *Crepidula onyx. Marine Ecology Progress Series*. 480:145–154.

Lidbury I, Johnson V, Hall-Spencer JM, Munnb CB, Cunliffe M. 2012. Community-level response of coastal microbial biofilms to ocean acidification in a natural carbon dioxide vent ecosystem. *Marine Pollution Bulletin*. 64(5). 1063–1066.

Maboloc EA, Chan KYK. 2017. Resilience of the larval slipper limpet *Crepidula onyx* to direct and indirect-diet effects of ocean acidification. *Scientific Reports*.7:12062.

Magoc T, Salzberg S. 2011. FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 27(21):2957-2963.

Maki JS, Ding L, Stokes J, Kavouras JH, Rittschof D. 2000. Substratum/bacterial interactions and larval attachment: Films and exopolysaccharides of *Halomonas marina* (ATCC 25374) and their effect on barnacle cyprid larvae, *Balanus amphitrite* Darwin. *Biofouling.* 16:159–170.

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO. 2005. Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Marine Ecology Progress Series*. 293:109–118.

Morton B. 1987. Recent marine introductions into Hong Kong. *Bulletin of Marine Science*. 41(2):503-513.

Mukherjee J, Wong KKW, Chandramouli KH, Qian PY, Leung PTY, Wu RSS, Thiyagarajan V. 2013. Proteomic response of marine invertebrate larvae to ocean acidification and hypoxia during metamorphosis and calcification. *The Journal of Experimental Biology*. 216:4580-4589.

NOAA 2016. How are satellites used to observe the ocean? [Internet]. National Ocean Service, National Oceanic and Atmospheric Administration, U.S. Department of Commerce; [cited 2016 Nov 10]. Available from http://oceanservice.noaa.gov/facts/satellites-ocean.htm

Oliver AE, Newbold LK, Whiteley AS, van der Gast CJ. 2014. Marine bacterial communities are resistant to elevated carbon dioxide levels. *Environmental Microbiology Reports*. 6(6):574–582.

Paulmier A, Ruiz-Pino D, Garcon V. 2011. CO_2 maximum in the oxygen minimum zone (OMZ). *Biogeosciences.* 8:239–252.

Pawlik JR. 1992. Chemical ecology of the settlement of benthic marine invertebrates. *Oceanography and Marine Biology: An Annual Review*. 30:273-335.

Pechenik JA. 1980. Growth and energy balance during the larval lives of three prosobranch gastropods. *Journal of Experimental Marine Biology and Ecology*. 44:1−28.

Pechenik JA. 1999. On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Marine Ecology Progress Series*. 177:269–297.

Qian PY, Lau SCK, Dahms HU, Dobretsov S, Harder T. 2007. Marine biofilms as mediators of colonization by marine macroorganisms: implications for antifouling and aquaculture. *Marine Biotechnology*. 9:399-410.

Rabalais NN, Díaz RJ, Levin LA, Turner RE, Gilbert D, Zhang J. 2010. Dynamics and distribution of natural and human-caused hypoxia. *Biogeosciences*. 7:585–619.

Rabalais NN, Turner RE (Eds.). 2001. Coastal Hypoxia: Consequences for living resources and ecosystems. In Coastal and Estuarine Studies vol.58 (p 454). American Geophysical Union, Washington, DC.

Royal Society. 2005. Ocean acidification due to increasing atmospheric carbon dioxide. Royal Society [Internet]. [cited 2017 March 18]. Available from https://royalsociety.org/~/media/Royal_Society_Content/policy/publications/2005/9634.pdf

Russell BD, Thompson JAI, Falkenberg LJ, Connell SD. 2009. Synergistic effects of climate change and local stressors: CO_2 and nutrient-driven change in subtidal rocky habitats. *Global Change Biology*. 15:2153–2162.

Seitzinger SP, Kroeze C, Bouwman AF, Caraco N, Dentener F , Styles RV. 2002. Global patterns of dissolved inorganic and particulate nitrogen inputs to coastal systems: Recent conditions and future projections. *Estuaries*. 25(4b):640-655.

Shin PKS, Leung JYS, Qiu JW, Ang PO, Chiu JMY, Thiyagarajan V, Cheung SG. 2013. Hypoxia induces abnormal larval development and affects biofilm–larval interaction in the serpulid polychaete *Hydroides elegans*. *Marine Pollution Bulletin*. 76(1-2):291-297.

Shirayama Y, Thorton H. 2005. Effect of increased atmospheric CO2 on shallow water marine benthos. *Journal of Geophysical Research*. 110:C09S08.

Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (eds.). 2007. *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.* Cambridge University Press, Cambridge. United Kingdom and New York, NY, USA.

Steinberg PD, Nys RD, Kjelleberg S. 2001. Chemical cues for surface colonization. *Journal of Chemical Ecology*. 28:1935–1951.

STAP. 2011. Hypoxia and Nutrient Reduction in the Coastal Zone. Advice for Prevention, Remediation and Research. A STAP Advisory Document. Global Environment Facility, Washington, DC.

Takeuchi K, Fujioka Y, Kawasaki Y, Shirayama Y. 1997. Impacts of High Concentration of CO2 on Marine Organisms; a Modification of CO2 Ocean Sequestration. Energy Conversion and Management. 38:S337-S341.

Thomsen J, Stapp LS, Haynert K, Schade H, Danelli M, Lannig G, Wegner KM, Melzner F. 2017. Naturally acidified habitat selects for ocean acidification–tolerant mussels. *Science Advances*. 3(4):e1602411.

Thorson G. 1950. Reproductive and larval ecology of marine bottom invertebrates. *Biological Reviews* 25:1–45.

Tolker-Nielsen T, Molin S. 2000. Spatial Organization of Microbial Biofilm Communities. Microbial Ecology. 40(2):75-84.

Trimborn S, Wolf-Gladrow D., Richter K-U, Rost B. 2009. The effect of pCO_2 on carbon acquisition and intracellular assimilation in four marine diatoms. Journal of Experimental Marine Biology and Ecology. 376:26-36.

Vaquer-Sunyer R, Duarte CM. 2008. Thresholds of hypoxia for marine biodiversity. *Proceedings of The National Academy of Sciences*. 105(40):15452-7.

Verspagen JM, Van de Waal DB, Finke JF, Visser PM, Huisman J. 2014. Contrasting effects of rising CO2 on primary production and ecological stoichiometry at different nutrient levels. *Ecology Letters*.17:951-960.

Weltzien FA, Doving KB, Carr WES. 1999. Avoidance reaction of yolk-sac larvae of the inland silverside *Menidia beryllina* (*Atherinidae*) to hypoxia. *Journal of Experimental Biology*. 202:2869–2876.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology. 73:5261-5267.

White JR, Nagarajan N, Pop M. 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLOS Computational Biology. 5:e1000352.

Wieczorek SK, Todd CD. 1998. Inhibition and facilitation of settlement of epifaunal marine invertebrate larvae by microbial biofilm cues. *Biofouling.* 12:81–118.

Wong KKW, Lane AC, Leung PTY, Thiyagarajan V. 2011. Response of larval barnacle proteome to CO_2-driven seawater acidification. *Comparative Biochemistry and Physiology, Part D.* 6:310-321.

Wu RSS. 2002. Hypoxia: from molecular responses to ecosystem responses. *Marine Pollution Bulletin*. 45:35–45.

Zhao B, Qian PY. 2002. Larval settlement and metamorphosis in the slipper limpet *Crepidula onyx* (Sowerby) in response to conspecific cues and the cues from biofilm. *Journal of Experimental Marine Biology and Ecology*. 262: 39-51.

CURRICULUM VITAE

Academic qualifications of the thesis author, Mr. HO Chun Ming:

- Received the degree of Bachelor of Science (Honours) in Applied Biology (Second Class Division One) from Hong Kong Baptist University, November 2015.

March 2018