

**EFFECTS OF BIOFILM AND GASTROPOD GRAZING ON  
BARNACLE CYPRID SETTLEMENT IN A MANGROVE  
REHABILITATION SITE, SG. BESAR, SELANGOR, MALAYSIA**

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**FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

**2014**

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**WONG JIN YUNG**

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Field of Study: **MICROBIOLOGY**

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## ABSTRACT

Biofouling by barnacles is a problem commonly encountered in mangrove replanting projects. This study examined the effect of biofilm and snail grazing on settlement of cyprids and proposed solution to control biofouling. An effective identification tool for barnacle cyprids was first built to facilitate the study as the barnacle cyprids are very difficult to identify. Several species of wild-caught barnacle cyprids from Matang Mangrove Forest Reserve waters were studied. The cyprids were identified through DNA barcoding analysis. Their morphological characters, both qualitative and quantitative, were studied and used to develop a morphology-based classification model to facilitate classification on large scale. Compared to using linear measurements only, inclusion of the qualitative carapace sculpturing character greatly improved the classification model. Field experiments were conducted to test the settlement preference of barnacles on substrates without and with biofilms of different ages. Higher number of barnacle settlement was found on substrates with aged biofilms compared to substrates without or with young biofilms. Characterization of biofilm successional profiles with respect to their bacterial and microeukaryotic compositions and biofilm structure were also carried out. Significant association was found between the successional changes in microeukaryote composition and the settlement of barnacle, but not with the bacterial composition or biofilm structure. All three successional profiles (bacteria, microeukaryotes, biofilm structure) were quantitatively shown to be concordant, indicating likely interactions among them and warrant future studies. Naturally-grown mangrove trees were observed to be less prone to biofouling than artificial substrate and re-planted mangroves. Abundant snail grazers were observed in the natural mangroves and exclusion experiments were conducted. Results showed that exclusion of grazers led to settlement of large number of barnacles and higher growth of microbial biofilms,



suggesting grazing pressure, not anti-fouling activity from the trees, as the underlying factor regulating barnacle abundance on natural mangrove trees. Study on the snail behaviour was carried out to understand why grazing pressure on barnacles was not established in the re-planted mangrove plants. While strong collective movements and grazing activity of snails in tandem with the tidal cycle (to avoid submersion) were observed in natural mangrove trees, it may be difficult for such behaviors to form on replanted mangrove seedlings.

## ABSTRAK

*Biofouling* oleh teritip adalah masalah yang biasa didapati dalam projek penanaman semula bakau. Kajian ini menguji kesan biofilem dan siput terhadap pelekatan larva teritip dan memberikan cadangan untuk menyelesaikan masalah ini. Tatacara pencaman larva cypris teritip dibangunkan dahulu untuk memudahkan kajian ini kerana pengecaman larva teritip adalah amat sukar. Pelbagai larva cypris teritip liar disampel dari perairan Hutan Bakau Simpan Matang telah dikaji. Larva cypris dikenalpastikan melalui analisis *DNA barcoding*. Ciri-ciri kualitatif dan kuantitatif morfologi larva cypris telah dikaji dan digunakan untuk membangunkan model klasifikasi berdasarkan ciri-ciri morfologi untuk memudahkan klasifikasi pada skala yang lebih besar. Penambahan ciri-ciri kualitatif karapas memberikan ketepatan yang lebih tinggi apabila berbanding dengan penggunaan ukuran *linear* sahaja. Eksperimen lapangan telah dijalankan untuk menguji pilihan pelekatan teritip pada substrat tanpa biofilem dan substrat dengan biofilem yang berbeza usia. Bilangan pelekatan teritip yang lebih tinggi didapati pada substrat dengan biofilem yang berusia tua daripada substrat tanpa biofilem ataupun dengan biofilem yang berusia muda. Profil pengalihgantikan (*succession*) biofilem berkenaan dengan komposisi bakteria dan micro-eukariot serta struktur biofilms telah juga dikajikan. Korelasi yang bererti didapati antara komposisi pengalihgantikan micro-eukariot dan teritip, tetapi tidak dengan komposisi bakteria dan struktur biofilm. Ketiga-tiga profil pengalihgantikan biofilem telah juga dibuktikan secara kuantitatif adalah konkordan. Ini menunjukkan interaksi yang mungkin ada dan menjamin kajian seterusnya. Pokok bakau semulajadi telah diperhatikan sebagai kurang bermasalah dengan *biofouling* daripada anak pokok bakau yang ditanam semula. Pengesel (*grazer*) siput diperhatikan sebagai banyak pada bakau semulajadi. Eksperimen pengecualian (*exclusion experiment*) telah dijalankan. Keputusan

menunjukkan bahawa pengecualian pengesel meningkatkan nombor pelekatan teritip dan pertumbuhan biofilem mikrob, mencadangkan tekanan ragut (*grazing pressure*) sebagai faktor yang mendasari pengawalan teritip pada bakau semulajadi, kurang mungkin kerana aktiviti *anti-fouling* dari pokok bakau. Kajian tingkah laku siput telah dijalankan untuk memahami mengapa tekanan ragut tidak berkesan di bakau yang ditanam semula. Pergerakan kolektif yang kuat dan tingkah laku yang mengelakkan air pasang surut telah diperhatikan. Tingkah laku siput-siput ini mungkin mempunyai kesukaran untuk dibentuk pada pokok bakau yang ditanam semula.

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## LIST OF SYMBOLS AND ABBREVIATIONS

~	approximate
×	times (dilution/ magnification/concentration)
°C	degree Celsius
$\alpha$	significance level
AF	anti-fouling
AFSW	autoclaved filtered seawater
ANOVA	analysis of variance
$\beta$ -GlcNAc	$\beta$ -N-acetylglucosamine
bp	base pair
CD	chart datum
CI	confidence interval
CLSM	confocal laser scanning microscopy
cm	centimetre
COI	cytochrome c oxidase subunit I
CRD	completely randomised design
CSR	complete spatial randomness
DAPI	4', 6-diamidino-2-phenylindole
ddH <sub>2</sub> O	double distilled water
Df	degree of freedom
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
E	east
EDTA	ethylenediamine-tetraacetic acid

EPS	extracellular polymeric substances
HSD	honestly significant difference
ITS	internal transcribed spacer
$k$	dimension (unless otherwise stated)
K2P	Kimura 2-parameter
kb	kilo base
$\lambda$	power
LDA	linear discriminant analysis
LOESS	local regression
m	metre
MANOVA	multivariate analysis of variance
mg	milligram
MgCl <sub>2</sub>	magnesium chloride
min	minute
mL	millilitre
mM	millimolar
MMFR	Matang Mangrove Forest Reserve
mmHg	millimetre of mercury
N	north
$n$	number of observations
N/A	not available
nm	nanometre
nMDS	non-metric multidimensional scaling
OTU	operational taxonomic unit
$p$	$p$ -value; probability of observing a test statistic equal or larger than the observed value under null hypothesis

PCR	polymerase chain reaction
Pr ( $>F$ )	$p$ -value of associated $F$ statistics
$r$	correlation coefficient
RCBD	randomised complete block design
rRNA	ribosomal ribonucleic acid
s	second
S	Svedberg
SD	standard deviation
SE	standard error
SEM	scanning electron microscopy
TAE	Tris-acetate EDTA
Taq	<i>Thermus aquaticus</i>
TRITC	tetramethylrhodamine isothiocyanate
$\mu\text{g}$	microgram
$\mu\text{L}$	microlitre
$\mu\text{m}$	micrometre
$\mu\text{M}$	micromolar
UV	ultraviolet
V	Volt
w/v	weight per volume
WGA	wheat germ agglutinin



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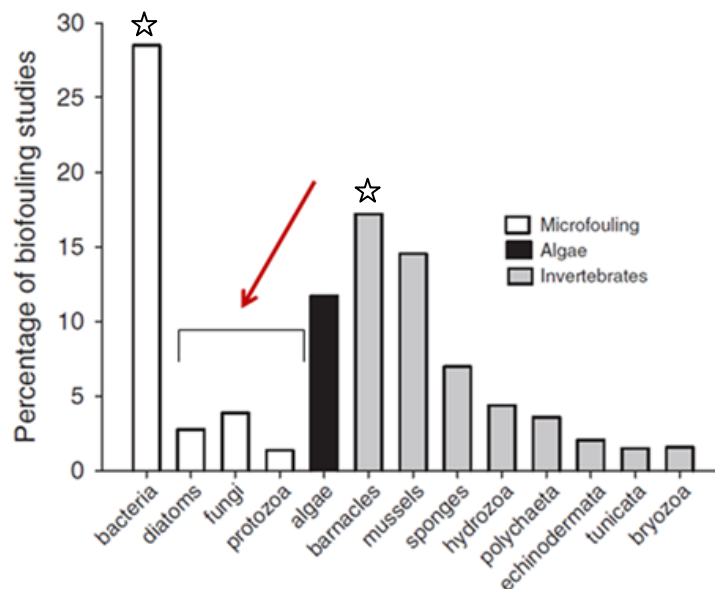
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## CHAPTER 1

### GENERAL INTRODUCTION

#### 1.1 Biofouling

Biofouling is the undesirable accumulation of organisms on artificial or biological surfaces in natural, industrial or medical environments. For marine environments, the substrates could be rocks, ship hulls, jetty pillars, fish cages, shells or body of animals, tree trunks, etc. The stages of marine biofouling include first, the adsorption of dissolved molecules; second, colonization by prokaryotes and unicellular eukaryotes (microfouling); and finally, the recruitment of invertebrate larvae and algal spores (macrofouling; Dobretsov, 2009). Fouling organisms consist of a very diverse group of micro- and macro-organisms with a sessile life style. Of these organisms, bacterial biofilm and barnacle are the most studied among micro- and macro-fouling, respectively (Figure 1.1).



**Figure 1.1:** Main organisms in biofouling studies. Bacteria and barnacles are the most popular subjects in microfouling and macrofouling studies respectively (star). Arrow emphasizes the low number of studies on eukaryotic microbes (Adapted from Dobretsov, 2009).

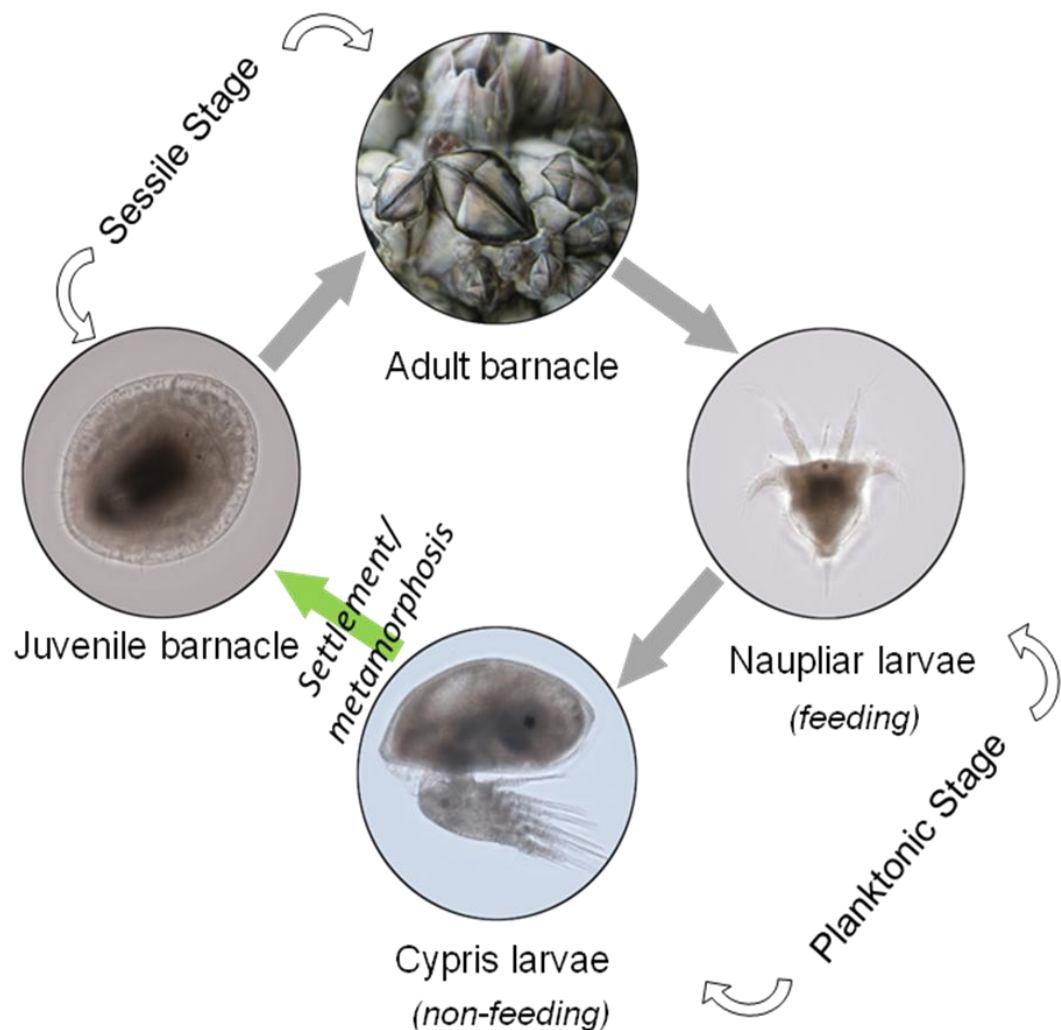
## **1.2 Biofilm**

Surface colonization by sessile microorganisms in the environments has been of interest to microbiologists for decades. Important observations of microbes attached to surfaces were made in studies carried out in the 1930's by submerging or burying glass slides in seawater (e.g. Zobell & Allen, 1935), where microorganisms were found to attach rapidly after exposure of the slides. The pioneering studies by Zobell and coworkers have remained informative even until now. They reported observations that large number of microbes in environments could adhere to surface, the physiology of attached microbes often differ from the free-swimming microbe, and that microbial film influences the attachment of subsequent larger organisms. The biofilm's importance in environmental (sustaining ecosystem functions), medical (causes of chronic infections and antibiotic resistance) and industrial (problems of biofouling) settings has led to the development of the biofilm theory (Costerton, 2007). The definition of biofilm has continuous been updated and redefined. Donlan (2002) described a biofilm as an assemblage of surface associated microbial cells that is embedded in an extracellular polymeric substance (EPS) matrix. Hall-Stoodley *et al.* (2004) used the term 'interface' instead of surface since in some occasions biofilm can form at the interface such as liquid and air. Corsterton (2007) stressed the term 'multicellular community' instead of merely aggregates of cells to reflect their co-operative nature.

## **1.3 Barnacle and biofouling**

Barnacles are crustaceans under the infraclass Cirripedia. Their life cycle is composed of both planktonic larval and sessile adult stages (Figure 1.2). The planktonic larvae include six naupliar stages and a final cyprid stage prior to settlement. Upon permanent attachment, the cyprid will metamorphose and enclose itself in highly specialized shells which give it a different appearance from many other crustaceans. 'Settlement' and

‘recruitment’ are the two common terms used in the literature to describe the successful transition from the planktonic larval stage to the sessile stage. But they are different in meanings in a strict sense. Recruitment takes post-mortality into consideration while settlement often refers to the freshly attached cyprids or young juveniles of <30 days of age (Caffey, 1985). While recruitment is important to the study the ecology of barnacles, settlement has been emphasized much more in biofouling studies, as prevention/ disruption of settlement is arguably the best means for biofouling control.



**Figure 1.2:** Life cycle of barnacle.

#### 1.4 Barnacle on mangrove

Barnacles are commonly reported as part of the faunal diversity of mangrove forest, including Malaysian mangroves (Sasekumar, 1974). They are common on the surface of roots, trunks and leaves of mangrove plants, fallen propagules and plant debris, and shells of crustaceans and mollusks. Despite its common presence in mangrove forests, mangrove barnacles are less studied than barnacles from rocky shores. Nonetheless, many species of barnacles have been found on mangroves and studied in various aspects. Examples include *Amphibalanus amphitrite* (= *Balanus amphitrite*) (Litulo, 2007), *Amphibalanus inexpectatus* (Starczak *et al.*, 2011), *Balanus eburneus* (Bacon, 1971), *Balanus kondakovi* (Rainbow *et al.*, 1989), *Balanus littoralis* (He, 2002), *Balanus thailandicus* (Puspasari *et al.*, 2001), *Amphibalanus patellaris* (= *Balanus patelliformis*; Puspasari *et al.*, 2000), *Amphibalanus reticulatus* (= *Balanus reticulatus*; Demopoulos & Smith, 2010), *Balanus trigonus* (Werner & William, 1967), *Chthamalus proteus* (Demopoulos & Smith, 2010), *Chthamalus sinensis* (Li *et al.*, 1998), *Elminius adalaidae* (Bayliss, 1993), *Elminius covertus* (Ross, 1996), *Euraphia eastropacensis* (Laguna, 1987), *Euraphia withersi* (Coates & McKillup, 1995; He, 2002), *Fistulobalanus albicostatus* (Chan & Leung, 2007), *Fistulobalanus citerosum* (Beasley *et al.*, 2010), *Fistulobalanus pallidus* (= *Balanus pallidus stutsburi*; Sandison & Hill, 1966), *Fistulobalanus sumbawaensis* (Prabowo & Yamaguchi, 2005), *Hexaminius foliorum* (Anderson *et al.*, 1988), *Hexaminius popeiana* (Coates & McKillup, 1995) and *Microeuraphia permitini* (Shahdadi & Sari, 2011). They are found on a variety of mangrove species, including *Rhizophora apiculata*, *Rhizophora mangle*, *Rhizophora stylosa*, *Avecennia marina*, *Kandelia obovata*, *Kandelia candel*, *Aegiceras corniculatum*, *Sonneratia alba* (Crona *et al.*, 2006; Maxwell & Li, 2006; He *et al.*, 2008; Li *et al.*, 2009; Starczak *et al.*, 2011). These species could also be found on other substrates or in other habitats, although some of them are more often found in mangrove areas. Some

studies also reported preference of barnacle settlement on certain species over others, but the reasons can be attributed to different factors (Maxwell & Li, 2006; Rani *et al.*, 2010).

#### **1.4.1 Positive impact of barnacles on mangrove: ecological roles**

Thoracican barnacles are important filter feeders in the mangrove food web (Fry & Smith, 2002). They feed on the plankton including the larvae of other marine invertebrates, which could have a role in regulating their supply (Young & Gotelli, 1988). On the other hand, they are food for predators such as mangrove whelks (Bayliss, 1982). Larvae of barnacles make up a large part of plankton samples from mangrove waters (Chew, 2012), suggesting possibly an important role as food sources for other organisms. Apart from their importance in the food web and ecosystem functioning, barnacles also play a role in the filtration function of mangroves (Soares-Gomes *et al.*, 2010). As a result they have been used as bioindicators for pollution in mangrove areas (Garrity & Levings, 1993; e Silva *et al.*, 2006). Recently, it was also shown that the diversity of epibionts on the mangrove roots (which include barnacles) is positively correlated with the fish diversity in the mangroves (MacDonald & Weis, 2013), suggesting that epibionts enhance mangrove habitats for use by fishes.

#### **1.4.2 Negative impact of barnacles on mangrove: biofouling**

Barnacles may be considered as biofouling organisms because their colonization can be detrimental to the mangroves too. Barnacle settlement on the stems and leaves can result in mortality or reduced fitness of mangrove, especially to the seedlings (Perry, 1988; Li & Chan, 2008; Li *et al.*, 2009; but see Satumanatpan & Keough, 1999). Although the biofouling problem for mangroves is not much a focus as compared to the industrial biofouling problem, it has been getting more attention due to the increased efforts in

mangrove planting projects. In fact, barnacle infestation on natural or re-planted mangrove seedlings is recognized as one of the important problems in mangrove conservation or rehabilitation projects in many countries including Malaysia (Zamora, 1987; Maxwell, 1995; Angsupanich & Havanona, 1996; Lee *et al.*, 1996; Li *et al.*, 1998; Tam, 2003; Jagtap & Nagle, 2007; Primavera & Esteban, 2008; Rani *et al.*, 2010; Macintosh *et al.*, 2012; Jusoff, 2013; Tan, 2013). Control of biofouling by barnacles on mangroves has since attracted a lot of research interests, such as the application of natural antifouling (AF) compounds (Lin *et al.*, 2009) or application of pesticides on mangrove seedlings (He *et al.*, 2008; Tan, 2013).

### **1.5 Problem statement**

At the mangrove rehabilitation site at Kg. Sg. Haji Dorani, mangrove seedlings were reported to have been severely infested with biofouling organisms (dominated by barnacles), to the extent that it retarded the growth or caused death of the seedlings (Tan, 2013). Tan (2013) found that while both physical and chemical approaches were useful in reducing barnacle settlement the chemical approach using chemicals such as chlorine, ivermectin and neguvon, also affected the natural population of other crustaceans. Although Tan (2013) had studied the effects of macrofouling on mangrove seedlings and how to control it (albeit with limited success), the complexity of the biofouling process requires more studies before the biofouling problem could be effectively addressed. For instance, the microfouling stage that precedes the macrofouling stage, i.e. the development of the microbial biofilm, may underpin the whole biofouling problem, and understanding and resolving it may provide a better solution.

## **1.6 Significance of present study**

Although governments, NGOs and the private sector have invested much money and effort on mangroves rehabilitation or ‘afforestation’ projects (Erftemeijer & Lewis III, 1999), the success rate of these projects leaves much to be desired. There are many reasons for their failure (Macintosh *et al.*, 2012) but one of them is due to poor seedling survival, in part due to the biofouling problem. From 2005 to 2012, Malaysia had spent RM40.1 million on mangrove rehabilitation programmes implemented by the Forestry Department (Anonymous, 2013). Many of such programmes are also assisted by NGOs and private sector as part of their corporate social responsibility (CSR) programmes. Failures in various sites such as in Meruntum, Putatan, and Lahad Datu (Jusoff, 2013) have been attributed to serious barnacle infestation problems, thus hindering rehabilitation efforts. Until now, much attention has been paid to mangrove replanting methods to improve seedling attachment and rooting on to soft mud. Unfortunately, no study on the biofouling problem on seedling growth has ever been studied apart from the work of Tan (2013) who had partly addressed the macrofouling problem. Tan (2013)’s work clearly demonstrates the need to also look at alternative control methods, particularly those research that will address the problem at the microfouling level, as well as to use natural control measures that are not harmful to the environment. The present study aimed to further contribute to the understanding of barnacle fouling and its control.

## **1.7 Objectives and scope**

The primary objectives of this thesis were:

- (i) To elucidate the link between microfouling (biofilm development) and macrofouling (barnacle larvae settlement)

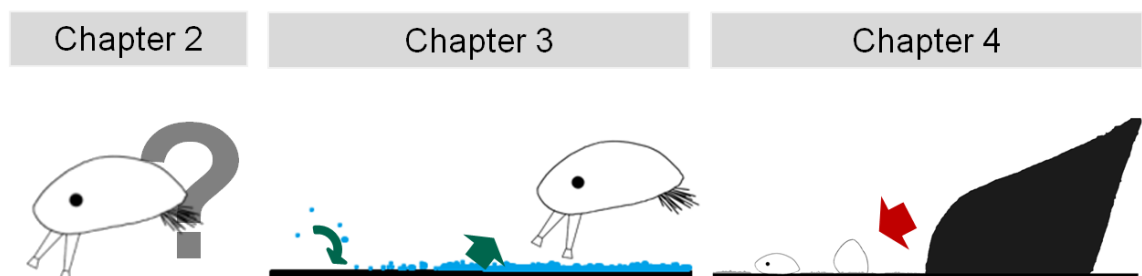


- (ii) To determine a way to reduce the mangrove biofouling problem at both the microfouling and macrofouling levels.

In order to achieve the objectives, three studies were conducted. They were as follows:

- (i) The Identification tool for barnacle cypris larvae (chapter 2);
- (ii) Succession of microbial communities and morphology of biofilm in relation to the settlement of barnacles (chapter 3);
- (iii) Effects of mangrove snails (littorinids) on development of biofilm and recruitment of barnacles (chapter 4).

The first study was necessary to identify the barnacle larvae at the study site and other mangrove waters. This also served to address a long-standing problem of difficulty in larval identification which has hampered biofouling studies. The second study sought to establish the link between the biofilm and barnacle, and to provide an explanation of the mechanism underlying the biofilm-barnacle relation. The third and final study investigated the effect of mangrove snails on both biofilm and barnacle, which could be beneficially applied to solving the biofouling problem (Figure 1.3). From experimental manipulation and observation on mangrove snails on natural mangrove trees, site comparisons and recommendations can better made for successful mangrove rehabilitation.



**Figure 1.3:** The three main focus of this thesis: identification of barnacle cyprids (Chapter 2), effect of biofilm on cyprid settlement (Chapter 3) and effect of grazer on cyprid settlement (Chapter 4).

## CHAPTER 2

### IDENTIFICATION TOOL FOR BARNACLE CYPRIS LARVAE

#### 2.1 INTRODUCTION

The distribution of the barnacle cyprids in the water column is patchy on spatial and temporal scales (Pineda, 2000) which can affect the subsequent recruitment dynamics of adults (Grosberg, 1982; Pineda *et al.*, 2002), including those that inhabit the mangrove ecosystem (Ross & Underwood, 1997; Satumanatpan *et al.*, 1999; Ross, 2001; Satumanatpan & Keough, 2001). In replanted mangroves at Ban Don Bay, Thailand (Angsupanich & Havanona, 1996), as well as in Haji Dorani, Malaysia (Tan, 2013), the pulse recruitment of barnacle cyprids is often intense, resulting in rapid cover by barnacles on the replanted mangrove. The supply-side ecology of barnacle cyprids is, therefore, important to understanding the distribution and larval settlement processes of barnacles in mangroves. However, the remarkable similarity of cyprid morphology among species (Elfimov, 1995) and lack of detailed morphological descriptions of larvae of many species make identification difficult, posing a major obstacle to the study of barnacle supply-side ecology.

At present, descriptions of barnacle cyprids are mostly dependent upon laboratory-reared larvae. There are very few morphological keys for the identification of wild caught barnacle cyprids. Such keys are often limited in their usefulness. For instance, the guide developed by Standing (1980) pertains to only the cyprids of Oregon waters in U.S.A. A guide has yet to be developed for barnacle cyprids for any particular region in the tropics. Moreover, larval culture itself poses several challenges in terms of

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Wong, J. Y., Chen, H.- N., Chan, B. K. K., Tan, I. K. P., & Chong, V. C. (2014). A combined morphological and molecular approach in identifying barnacle cyprids from the Matang Mangrove Forest in Malaysia: essentials for larval ecology studies. *Raffles Bulletin of Zoology*, 62, 317-329.

the need for suitable larval food and rearing conditions to ensure sufficient larval survival.

Molecular techniques which enable accurate species identification could dispense with the need for larval culture. For example, DNA barcoding has been extensively used for species identification in recent years. By matching a chosen region of DNA fragments from the specimen with known reference specimens, identification can be achieved (Hebert *et al.*, 2003). The method is very useful for the identification of larvae of species in which the adult can be confidently identified by morphology. Chen *et al.* (2013) have shown that DNA barcoding based on mitochondrial COI sequences is suitable for identifying wild-caught barnacle cyprids including those from possible invasive species. Other markers used to resolve barnacle taxonomic problems and biodiversity surveys include the 12S and 16S rRNA genes and nuclear ITS1 region (e.g. Chan *et al.*, 2007a, 2007b, 2007c, 2009; Tsang *et al.*, 2009; Chen *et al.*, 2012; Cheang *et al.*, 2012).

### **2.1.1 Objectives**

- (i) To describe barnacle cypris larvae from tropical mangroves
- (ii) To construct an identification key for cypris larvae of tropical mangrove barnacles based on morphology and molecular characters.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Specimen collection sites**

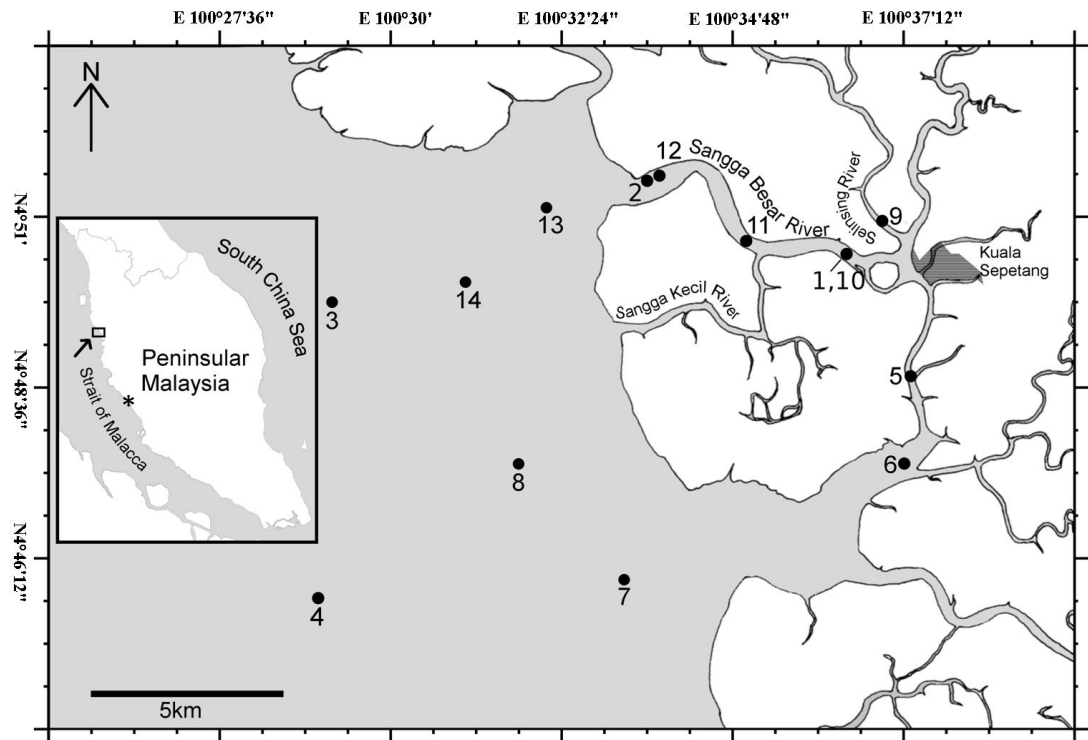
Preliminary investigations, by way of examining the adult barnacle fauna, showed a preponderance of mainly one species *Amphibalanus amphitrite* in Kg. Sg. Haji Doraini, where most of the biofouling experiments were to be carried out. This being the case, it

was thought that it would not be beneficial to confine the sampling of larval cyprids in the waters off Kg. Sg. Haji Dorani, since the cyprid fauna was not expected to be diverse enough to enable the construction of a more encompassing and useful taxonomic key for the Malaysian barnacle cyprids. Thus, it was decided that cyprid samplings were to be made in the very large estuarine system of the Matang Mangrove Forest Reserve (MMFR), located 140 km north of Kg. Sg. Haji Dorani (Figure 2.1).

Matang Mangrove Forest Reserve (MMFR), located in the state of Perak, was chosen as the site for sampling a more diverse fauna of cyprid larvae. MMFR is the largest mangrove forest in the Peninsular Malaysia and has attracted extensive ecological and scientific interest (Shaharuddin *et al.*, 2007). The numerous creeks and channels with diverse water conditions, from the upper estuary to near shore waters, provide suitable habitats for barnacle colonization on the fringing mangrove vegetation as well as on the numerous fish stakes, jetty pilings and floating fish cages. Barnacle diversity in the MMFR waters has not been reported before except for one biofouling study on floating fish cages, where *Amphibalanus amphitrite* (= *Balanus amphitrite*) was indicated (Madin *et al.*, 2009).

### **2.2.2 Field collection of barnacle cyprids and adults**

Barnacle cyprid collections were made from the upper estuary (MMFR as far as the coastal waters (< 12 km offshore) on two separate sampling occasions, one on 21-20 April 2011 and the other on 25-26 June 2012 (see Figure 2.1 for the location of sampling sites). Additional cyprid samples were obtained from Haji Dorani in August 2011 (Detailed map and description of Kg. Sg. Haji Dorani given in figure 3.2.1 and section 3.2.1). For MMFR samplings, multiple surface plankton samples were collected by a standard plankton net (45cm mouth diameter) of 160µm net mesh size towed for either 5 or 10 min each. For Haji Dorani samplings, passive plankton traps (Todd *et al.*,



**Figure 2.1:** Map of sampling locations at Matang Mangrove Forest Reserve (MMFR) in Perak, Malaysia. Sampling was carried out in April 2011 at sites 1- 8 and in June 2012 at sites 9- 14. Sampling was also carried out at Haji Dorani in August 2011 (\*; inset; refer to Figure 3.2.1 for detailed map of this site)

2006) were tied onto wooden sticks, deployed and left in the field for 3 days before they were collected. No plankton tows were made here because of the very shallow intertidal water (0-1m depth). Adult barnacles were collected as species references, using a hammer and chisel to detach the animals from their substrates, which included mangrove tree trunks and roots, and buoys for oyster culture. All collected specimens were immediately preserved in 95% ethanol in the field and kept in the Biotechnology Laboratory, University of Malaya, before subsequent analyses.

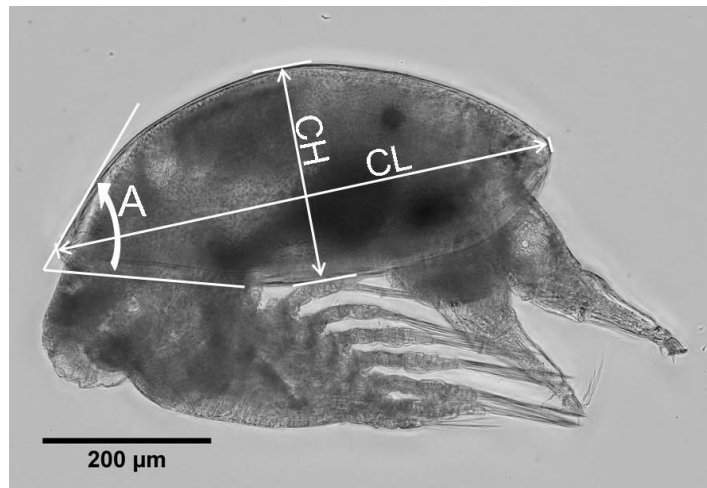
### 2.2.3 Laboratory analyses

The preserved samples of cyprids and adults were analysed in the Coastal Ecology Laboratory, Academia Sinica, Taiwan, during my visit there under a student research

fellowship. These materials were analysed in conjunction with additional barnacle materials collected from MMFR on 21-20 April 2011 earlier analysed by Professor Benny Chan K. K. of the Coastal Ecology Laboratory.

### **2.2.3.1 Morphological analyses**

The adult barnacles were identified to species level based on their morphology and served as the adult reference collection for subsequent study. All barnacle cyprids were first sorted out from the plankton samples under a stereo microscope (Olympus SZX7). Approximately 250 cyprids were analyzed in order to cover the entire range of observed morphological variations. Photos of the lateral view of the selected set of cyprids were taken under normal bright field of a compound microscope (Zeiss Axio Scope A1) equipped with a camera (Panasonic Lumix G1). A series of photos at differential focus were taken for each larva and integrated into an extended- focus image using the iSolution Lite image processing software (i-Solution Inc., Vancouver, Canada) for optimal viewing and measurement. Morphometric measurements of the carapace of each cyprid were then taken from the extended-focus images using ImageJ (version 1.44; Schneider *et al.*, 2012). The measurements included carapace length (maximum distance between anterior and posterior margin), carapace height (maximum distance between dorsal and ventral margin), posterior carapace angle (angle formed by extension of dorsal and ventral margin), and calculated ratio of length-to-height (Figure 2.2; also see Chen *et al.*, 2013). Carapace sculpturing was examined, described and recorded in addition to the morphometric measurements. Since not all the cyprids had their antennules and thoracic appendages extended, measurements were restricted to the carapace only.



**Figure 2.2:** Lateral view of cypris larvae of barnacle showing measurements used for morphometric analysis. CL: carapace length; CH: carapace height; A: posterior carapace angle. Ratio of CL/CH was also calculated.

#### 2.2.3.2 Scanning electron microscopy (SEM)

Morphology and carapace sculpturing patterns of cyprids initially observed under light microscopy were further observed by scanning electron microscopy (SEM). Cyprids preserved in 95% ethanol were transferred into acetone, critical point dried, and coated with gold palladium before observation with a FEI Quanta 200 Scanning Electron Microscope (methods follows Chan & Leung, 2007). Measurements related to the carapace sculpturing pattern were made on SEM images. Maximum feret diameter (largest distance between two parallel planes restricting an object) was used to measure the size of the ultrastructures if the use of diameter was not appropriate.

#### 2.2.3.3 DNA extraction, PCR and sequencing

Total genomic DNA from adult and - larval tissue was extracted using DNeasy blood and tissue extraction kit (Qiagen GmbH, Germany) after the cirripedes were identified and morphological measurements made. A faster alternative extraction method using extraction buffer containing 5% (w/v) Chelax<sup>®</sup>-100 resin (Bio-Rad, California, USA) was used only for cyprids DNA extraction (Walsh *et al.*, 1991). For DNA extraction using the tissue extraction kit, soft tissue (~25mg) of adult barnacle or whole barnacle

cyprids were used for DNA extraction following the manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify a region of the mitochondrial 12S-rRNA gene from the DNA using forward primer 5'-GACCGTGCTAAGGTAGCATAATC-3' (Tsang *et al.*, 2009) and reverse primer 5'-CCGGTCTGAACTCAAATCGTG-3'. Amplification was performed using reaction mixture containing 2 µL of template DNA, 12µL Taq master mix (1.5mM MgCl<sub>2</sub> type; Ampliqon, Denmark), 0.05 µM of each primer, and ddH<sub>2</sub>O to a total volume of 20 µL. PCR conditions were set as follows: 2 min and 30 s at 94°C for initial denaturation, then 30 cycles of 30 s at 95°C, 30 s at 48°C, and 1 min at 72°C, with final extension for 5 min at 72°C. The amplicons were sequenced at Genomics BioSci & Tech Ltd. Sequencing was performed using an ABI 3730 XL DNA analyser with BigDye terminator cycle sequencing reagents kit (Applied Biosystems, California, USA).

#### **2.2.3.4 Sequence analyses**

Cyprids were identified through comparison of their 12S DNA sequences with that of the identified adult barnacles. All sequences (including 207 cyprid sequences successfully obtained from the selected set, 16 adult sequences and 3 outgroup sequences from GenBank) were first aligned using MUSCLE (Edgar, 2004) using the default settings, and these were then manually inspected. The three outgroups used were *Verruca laevigata* (JX083933.1), *Metaverruca recta* (JX083931.1) and *Rostratoverruca krugeri* (JX083932.1). A neighbour-joining tree was constructed from the aligned sequences using MEGA 5.05 (Tamura *et al.*, 2011), with a Kimura 2-parameter (K2P) model used to compute the genetic distances. Bootstrapping was conducted with 1000 replicates to estimate the reliability of the inferred tree. When the sequences of cyprids and adult references formed a “monophyletic” clade with high bootstrap support, it was considered to be the same species. Monophyletic groups that failed to cluster with any

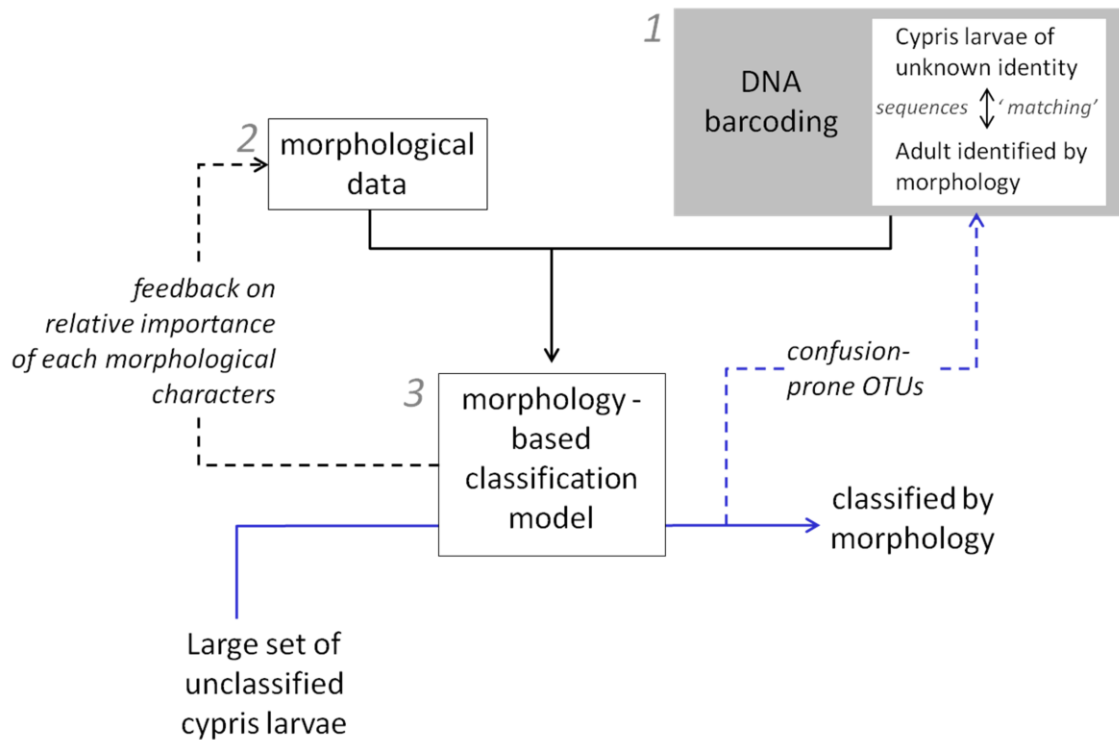


adult references were then considered as an operational taxonomy unit (OTU). To assess the strength of the current 12S sequence fragments for DNA barcoding purposes, the pair-wise genetic distances of all of the sequences (except outgroups) computed from the K2P model were also summarized to show the between- and among- clade genetic divergence.

#### **2.2.4 Statistical analyses and construction of morphology-based classifier**

Of 207 sequenced cyprids from the selected set, only 183 were used for the morphological analysis due to the exclusion of cyprids with low quality images. With species identity determined from the DNA barcoding analysis, morphology-based classification models were then constructed. The classification models would later be used for quick preliminary classification for remaining cyprids based on morphology, achieving one of the primary purposes of the study, i.e. building tool for large scale identification (Figure 2.3). The classification tree algorithm method was chosen over LDA (linear discriminant analysis) because it can handle mixed inputs of predictor variables (both quantitative and qualitative variables), and is easier to interpret (De'ath & Fabricius, 2000). Furthermore, classification trees are not limited by the number of samples used in each group (i.e. species), whereas LDA requires the number in each group to be not less than the number of variables. This is a problem for the present study as the specimen numbers of *A. amphitrite*, OTU 1 and OTU 2 were low in the training dataset.

Two models of classification tree were constructed and compared, i.e. one with only quantitative morphological characters, and the other with both quantitative and qualitative (carapace sculpturing) morphological characters. This was done to show the effect of adding carapace sculpturing as a morphological variable to differentiate cyprids. The performance of the tree classifiers was evaluated using multiple runs of 5-



**Figure 2.3:** The intended use of the morphology-based classification model. By combining DNA barcoding analysis and morphological analysis on the cyprids, the model could be used as efficient tool for large scale identification. Dotted lines represent possible feedback mechanisms.

fold cross validation. Within each run, the dataset of the selected cyprids was randomly partitioned into 5 subsets; four subsets were used as training sets and one subset was used as a validation set. This process was repeated until each subset had been used once as a validation set. The cross validation was then repeated for 100 runs and the misclassification rate of the classifier was estimated from the average over the 100 runs. All graphical representations of data and statistical analyses described above were performed using R (version 2.15.0; R Development Core Team, 2012). R package ‘tree’ was used for classification trees (Ripley, 2011).

#### 2.2.4.1 Application of morphology-based classifier

The decision tree classifier was then utilized to aid the classification of the remaining cyprid specimens to give a preliminary view of the species composition of cyprids at different locations in MMFR. To achieve this, the remaining collection was identified

and counted under a compound microscope. Photos were taken as certain species/ OTU need measurements before a decision on species identity could be made. The decision on species identity was assisted by the tree classifier.

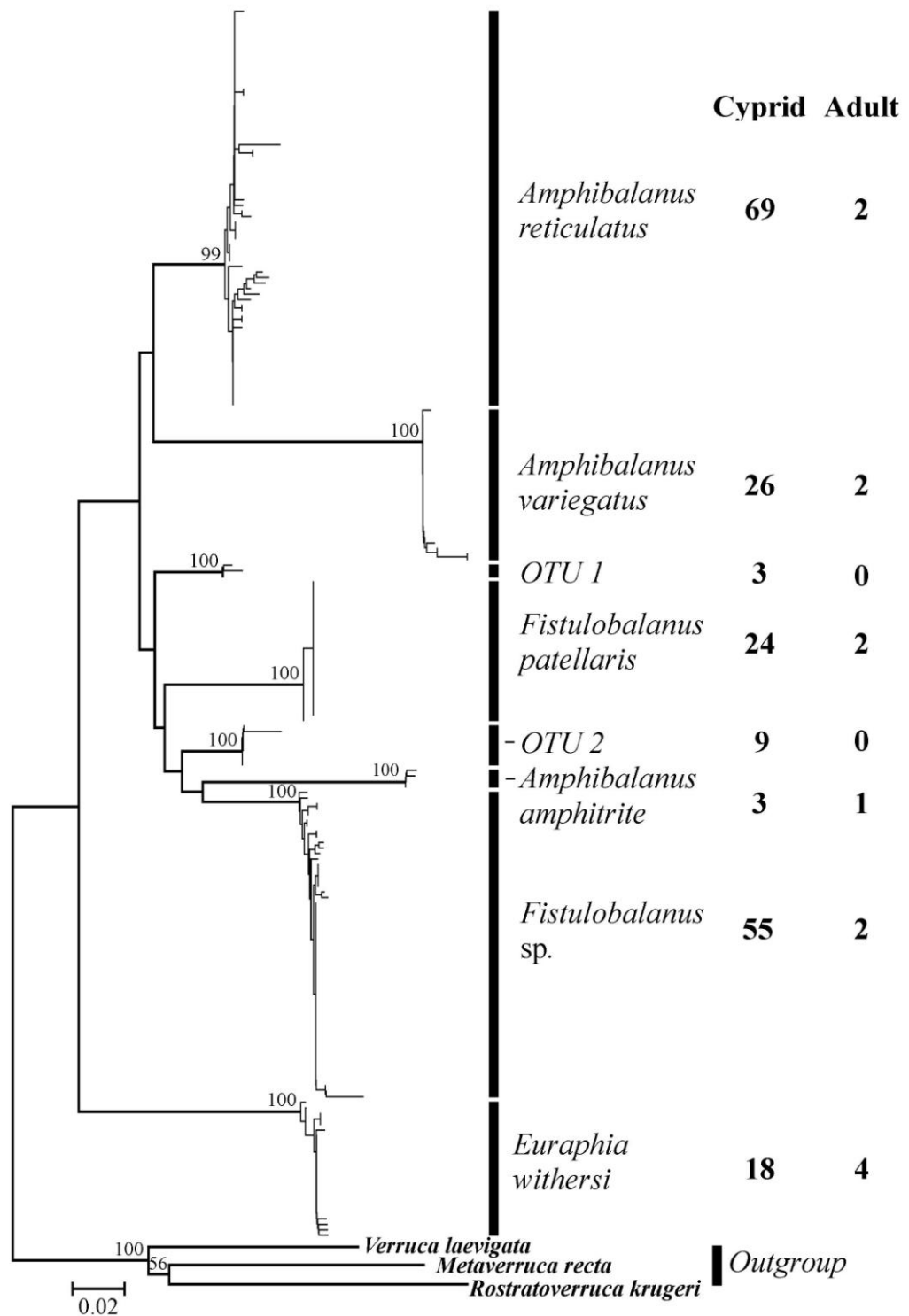
## **2.3 RESULTS**

### **2.3.1 Identification of adult barnacle**

Six species of adult barnacle from MMFR were identified to species level, namely *Fistulobalanus patellaris*, *Fistulobalanus* sp. (an undescribed species), *Amphibalanus reticulatus*, *Amphibalanus variegatus*, *Amphibalanus amphitrite* and *Euraphia withersi*.

### **2.3.2 Molecular analyses**

Partial sequences of 12S-rRNA gene were successfully obtained from 207 individuals of cyprids and 16 individuals of barnacle adults. A neighbour-joining tree constructed from the sequences is shown in Figure 2.4. Eight distinct clades were observed and six clades (including 195 cyprids sequences) had the sequences from the identified adult references. Two of the clades (comprising 12 of the cyprid sequences) with no matching adult sequence were designated as Operational Taxonomic Unit or OTU 1 and OTU 2. The mean within-species pairwise K2P distance was 0.6% (ranged from 0 % to 3.5 %) while the mean between-species distance was 13.5 % (ranged from 5.4 % to 25%). The non-overlapping ('barcode gap') of frequency distribution of pairwise K2P distance for within- and between-species suggests the suitability of the approach for barcoding purposes (Appendix A).



**Figure 2.4:** Neighbour-joining tree constructed from partial 12S-rRNA gene fragment sequences of cypris larvae and adults of barnacle. The sequences were clustered into eight clades, and species name were labelled at the clades containing sequence(s) of identified adult of barnacle. Clades with no sequence of identified barnacle adult clustered within were designated as OTU (Operational Taxonomic Unit). Number of sequences in each clade were also shown. Scale bar denotes 0.02 base substitution per site.

### 2.3.3 Morphological analyses

In the present study, the range of carapace length from all cyprids collected was 439  $\mu\text{m}$  to 685  $\mu\text{m}$ , and the range of carapace height was 199  $\mu\text{m}$  to 329  $\mu\text{m}$ . The variations in the four quantitative morphometric characters of the carapace, namely length, height, angle and length-to-height ratio among species/ OTU are shown in Table 2.1. The carapace length and height data were also compared to those previously reported in the literature (Table 2.1). The sculpturing patterns were categorized into five types (details in Table 2.2). These carapace sculpturing patterns were not observable under a dissecting microscope and only at  $>100\times$  magnification under a compound microscope (henceforth referred to as CM). SEM which provided morphological details of high resolution confirmed the pattern classification based on the type of sculpturing pattern (Figure 2.5-2.7).

Four barnacle species (*Fistulobalanus* sp., *Fistulobalanus patellaris*, *Euraphia withersi* and *Ampibalanus variegatus*) showed diagnostic carapace sculptures (Table 2.2). However, four other taxa (*A. amphitrite*, *A. reticulatus*, OTU1 and OTU2) showed no sculpturing pattern (i.e. smooth carapace). The honeycomb pattern of type A (*Fistulobalanus* sp.; Figure 2.5A-D) is readily identifiable under CM. Type B pattern (*Fistulobalanus patellaris*; Figure 2.5E-J) was not very apparent under CM, but was revealed under SEM. Due to their larger size, lunular pores on the ventral side were easier to observe under CM (Figure 2.5G) compared to the punctae on the dorsal side (Figure 2.5F). For type C pattern (*Ampibalanus variegatus*, Figure 2.6A-H), the punctate pattern was observed on the ventral aspect of the carapace (Figure 2.6C) but was absent on its dorsal aspect. Differentiation between the punctae of type C and the lunules of type B on the ventral aspect could only be identified under SEM (Figure 2.6G & 2.6C, respectively). However, under CM, type C can be differentiated from type B

**Table 2.1:** Quantitative morphological characters and comparison to other studies

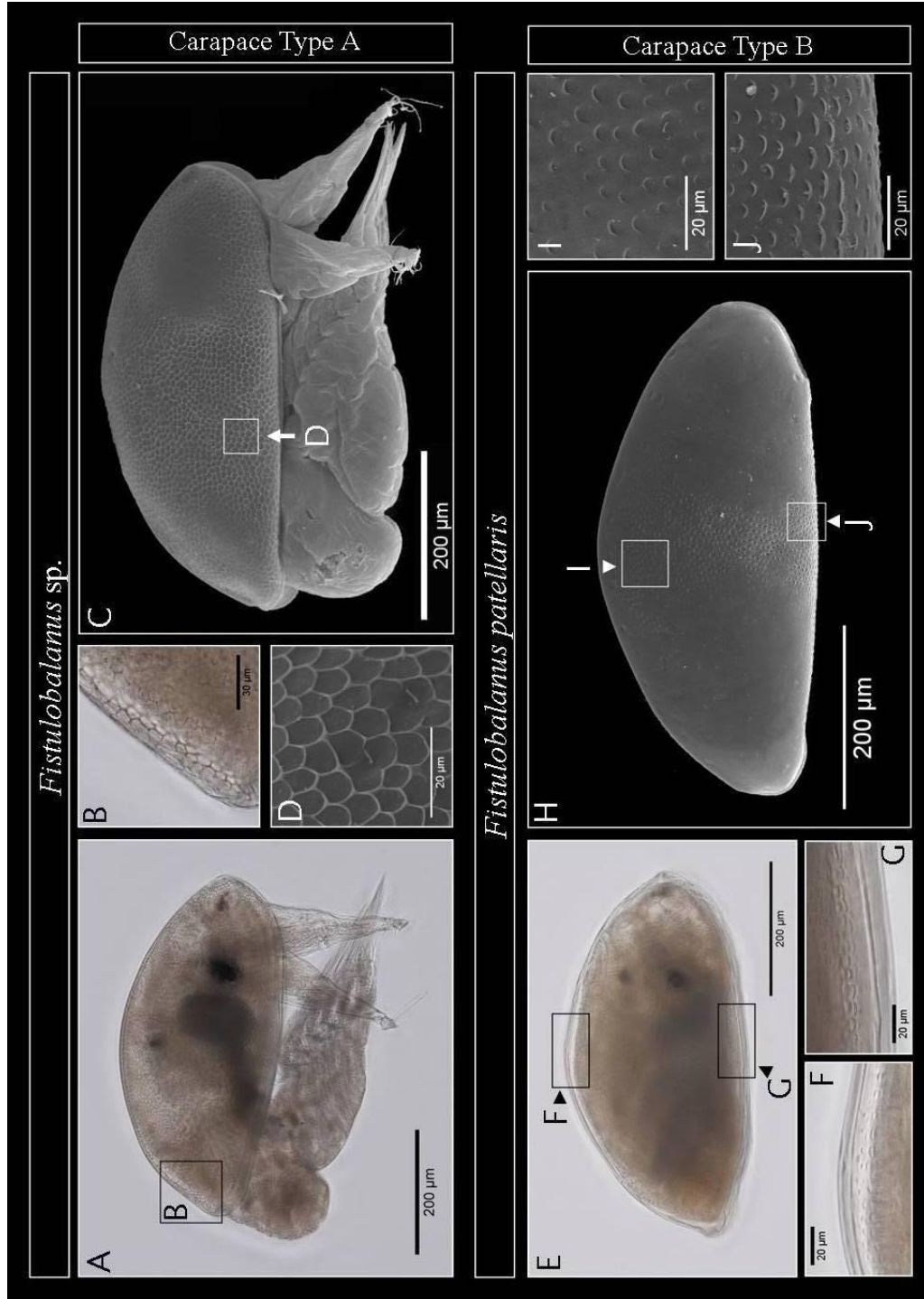
Species	Carapace length (µm)	Carapace height (µm)	Posterior Carapace Angle (°)	Length-to-height ratio	Remarks	Reference
<i>Amphibalanus amphitrite</i>	480.75±38.04	227.79±18.03	65.72±1.13	2.12±0.27	Field collected; mean± SD	This Study
	510	N/A	N/A	N/A	lab-reared; single measurement	Karande (1974)
	450±20	N/A	N/A	N/A	lab-reared; mean± 95% CI	Egan & Anderson (1986)
	550	250	N/A	N/A	lab-reared; single measurement	Glenner & Hoeg (1995)
	421-480	211-230	N/A	N/A	lab-reared; range	Anil <i>et al.</i> (2001)
<i>Amphibalanus reticulatus</i>	605±21.13	275.72±16.67	60.95±6.56	2.20±0.14	Field collected; mean± SD	This study
	547±26.3	250±25.5	N/A	N/A	lab-reared; mean±unspecified bar	Thiyagarajan <i>et al.</i> (1997)
	613±21	245±17	N/A	N/A	lab-reared; mean± SD	Lee <i>et al.</i> (1999)
<i>Amphibalanus variegatus</i>	636.46± 24.91	284.17± 17.41	65.16±6.79	2.25±0.14	Field collected; mean± SD	This study
	520±40	N/A	N/A	N/A	lab-reared; mean± 95% CI	Egan & Anderson (1986)
	615	275	N/A	N/A	lab-reared; single measurement	Karande (1974)
<i>Fistulobalanus sp.</i>	555.44±13.49	270.78±14.25	67.79±5.12	2.06±0.11	Field collected; mean± SD	This study
<i>Fistulobalanus patellaris</i>	554.31±19.45	281.19±14.41	71.69±6.56	1.97±0.09	Field collected; mean± SD	This study
<i>Euraphia withersi</i>	482.42±14.88	230.77±7.17	72.57±4.40	2.09±0.09	Field collected; mean± SD	This study
OTU 1	598.01±17.03	256.15±11.32	57.48±0.49	2.34±0.17	Field collected; mean± SD	This study
OTU 2	522.86±20.18	226.95±16.22	54.71±10.94	2.31±0.19	Field collected; mean± SD	This study

based on the presence of punctae on both the anterior and posterior ends of the carapace in type C, whereas punctae in type B are absent in both positions.

Type D is featured by ridges or folds at the posterior end of the carapace of *Euraphia withersi* (Fig 2.6J). These folds extend into the ventral aspect of the carapace (not shown). *Euraphia withersi* also has unique reddish pigmentation scattered around the ventral edge of carapace (Figure 2.6I, highlighted by arrows) and a dark rounded pigmentation spot posterior to the cyprid eye (Figure 2.6I, circled). The reddish pigmentation, however, faded after prolonged preservation in 95% ethanol. Four other species/ OTU (*Amphibalanus reticulatus*, *Amphibalanus amphitrite*, OTU 1 and OTU 2) do not have any carapace sculpturing and were named as type E (Figure 2.7). Classification of these taxa depends on their carapace size and shape, where *A. reticulatus* and OTU 1 are longer than *A. amphitrite* and OTU 2, while OTU 2 has a higher posterior carapace angle than *A. amphitrite*. Discrimination between *A. reticulatus* and OTU 1 is difficult.

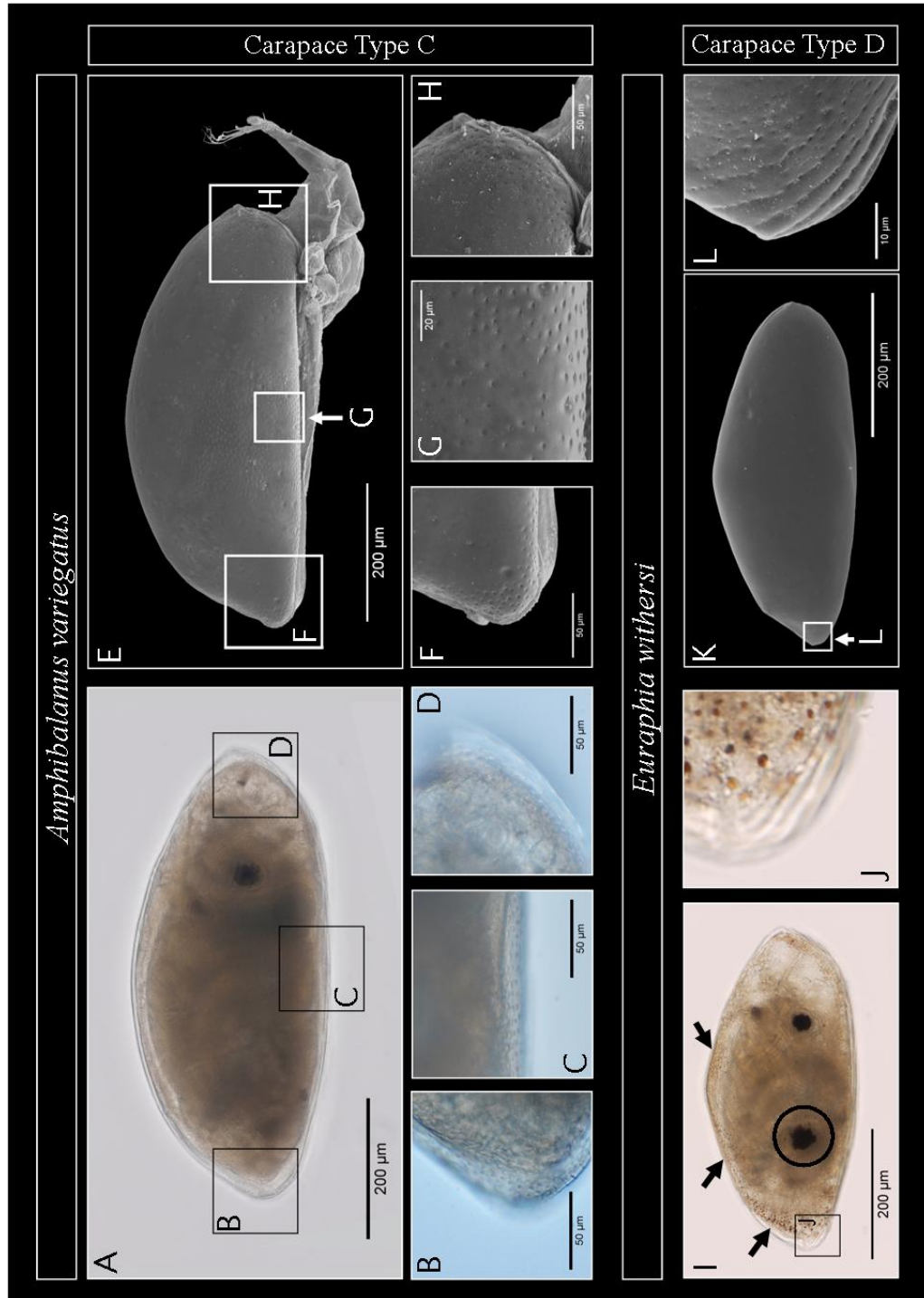
**Table 2.2:** Types of carapace sculpturing patterns

Type	Description	Species/ OTU
Type A	‘Honeycomb’ pattern of raised pentagonal and hexagonal units. Maximum feret diameter of the combs is $7.6 \pm 1.2 \mu\text{m}$ (mean $\pm$ SD, n=67)	<i>Fistulobalanus</i> sp.
Type B	Sculpturing spans through dorso-ventral axis, with punctae on the dorsal aspects and lunular pores on the ventral aspects. Maximum feret diameter of the punctae is $2.4 \pm 0.5 \mu\text{m}$ (mean $\pm$ SD, n=65), and $5 \pm 0.9 \mu\text{m}$ (mean $\pm$ SD, n=70) for the pores.	<i>Fistulobalanus patellaris</i>
Type C	Rounded punctae on ventral side, anterior and posterior ends. Diameter of the pits is $2.8 \pm 0.7 \mu\text{m}$ (mean $\pm$ SD, n=65)	<i>Amphibalanus variegatus</i>
Type D	3-4 distinct ridges or folds at posterior end	<i>Euraphia withersi</i>
Type E	No sculpturing of carapace	<i>Amphibalanus reticulatus</i> , <i>A. amphitrite</i> , OTU 1 and OTU 2

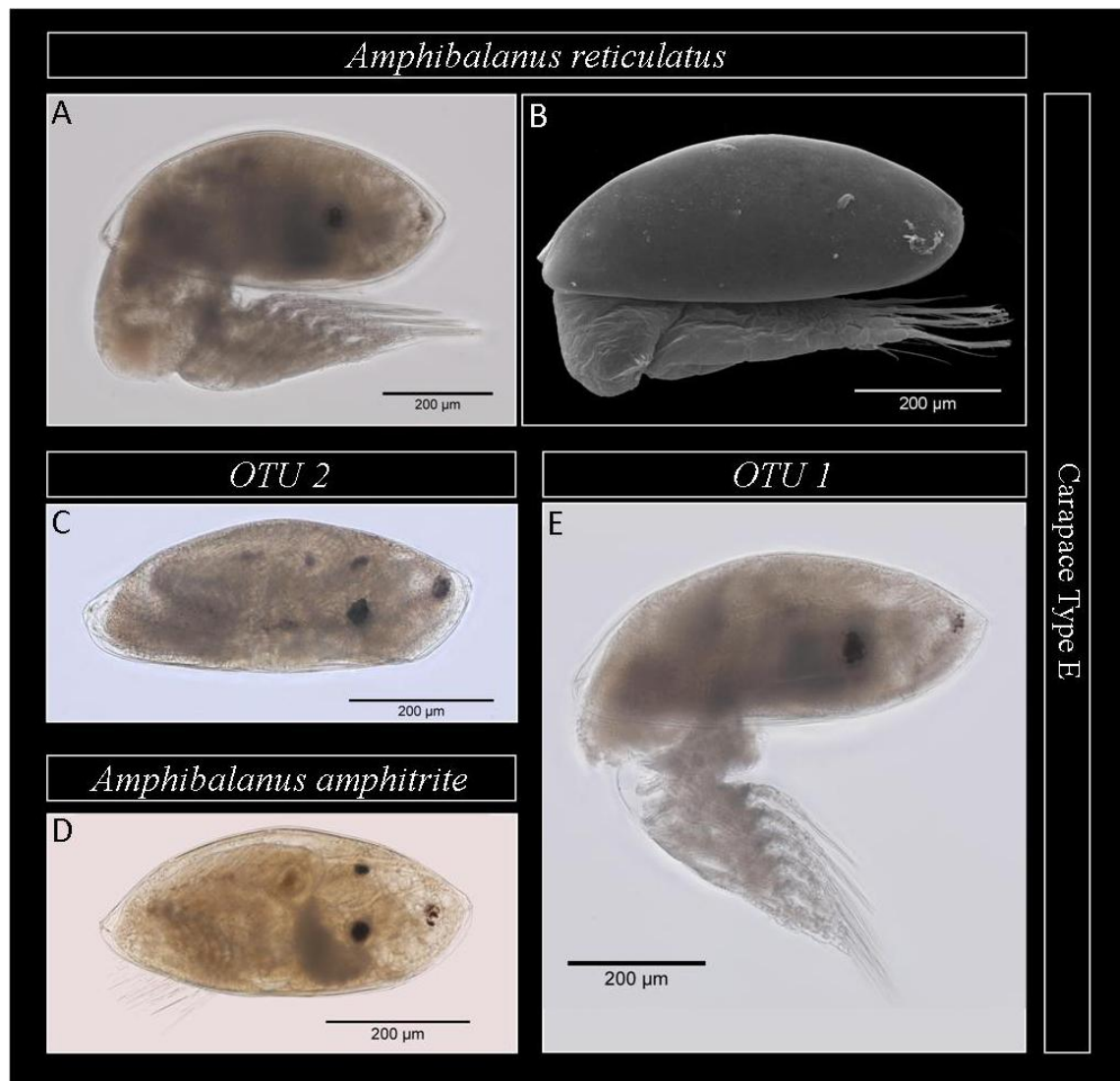


**Figure 2.5:** Light and scanning electron micrograph of cypriid larvae of (A-D) *Fistulobalanus* sp. and (E-J) *Fistulobalanus patellaris*. Details of specific carapace sculpturing patterns in each species are shown at higher magnification.





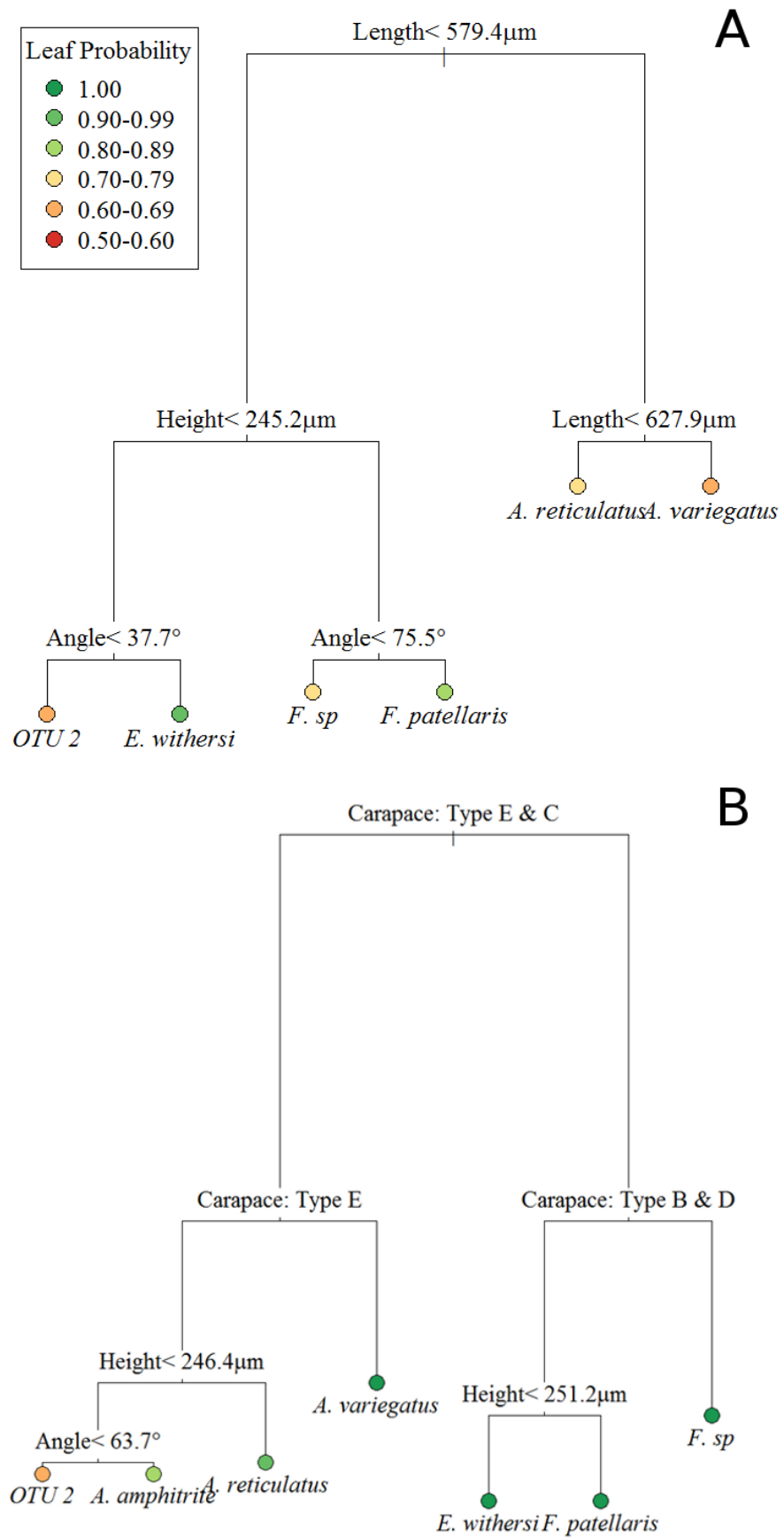
**Figure 2.6:** Light and scanning electron micrograph of cypris larvae of (A-H) *Amphibalanus variegatus* and (I-L) *Euraphia withersi*. *E. withersi* has reddish pigments around the carapace (I, arrows) and a dark rounded pigmentation spot (I, circled).



**Figure 2.7:** Light and scanning electron micrograph of cypris larvae of (A, B) *Amphibalanus reticulatus*, (C) OTU 2, (D) *Amphibalanus amphitrite*, and (E) OTU 1.

#### 2.3.4 Morphology-based classifier

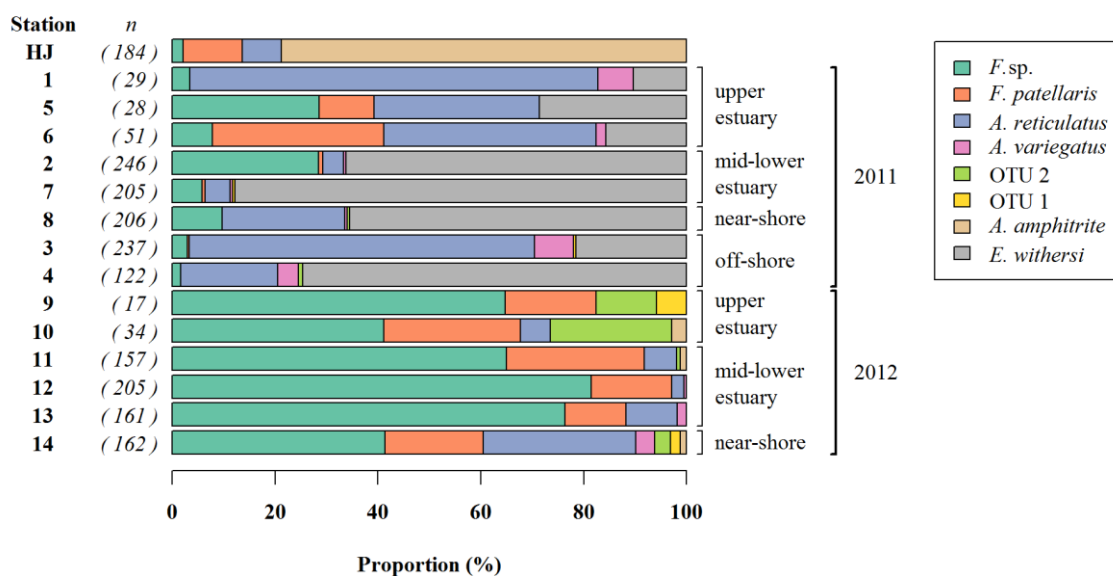
The performance of morphology-based tree classifier increased dramatically when the carapace sculpturing character was added. The estimated misclassification rate for the tree classifiers decreased from  $35.0 \pm 11.1\%$  ( $\pm$ SD) to  $5.7 \pm 5.0\%$  ( $\pm$ SD) respectively for datasets without and with carapace sculpturing characters (Figure 2.8). This decrease is mainly due to the increased accuracy of classification of species that have unique carapace sculpturing in the latter dataset. Recall was low for species/OTU present at low abundances in the training dataset (*A. amphitrite*, OTU 1, and OTU 2).



**Figure 2.8:** Classification tree model computed from the morphometric characters of (A) quantitative variables only (B) both quantitative and qualitative (carapace sculpturing) characters. A binary decision is made at each node, where ‘true’ for the node description lead to branch at left and ‘false’ to right. Probability of correct prediction (‘recall’) at each terminal node (‘leaf’) is also shown.

### 2.3.5 Testing the effectiveness of the classification key: assessing cyprid diversity in MMFR and Haji Dorani

The morphological classification model which was obtained in preceding steps (Figure 2.9B) was used to identify the cyprids collected at different stations in MMFR (Figure 2.9). A key was also written for the common cyprids in MMFR. A total of 1124 and 736 cyprids were classified for the 2011 and 2012 collections, respectively. Marked differences in species composition were observed between 2011 and 2012 collections. The 2011 (April) collection was dominated by *E. withersi* and *A. reticulatus* while the 2012 (June) collection was dominated by *Fistulobalanus* sp. and *F. patellaris*. All species were found in both years except *E. withersi* which was not found in the 2012 samples. The within-year variations in species composition among stations were smaller compared to annual variability. However some differences were observed between stations, in particular the composition between the upper estuary and the rest of the stations. For Haji Dorani site, the dominant group of cyprids belong to *A. amphitrute* (79%), which is expected. However, there were also other species of cyprids presented.



**Figure 2.9:** Composition of barnacle cyprid diversity at different stations and different year of collection at MMFR (station 1-14), and at Haji Dorani site (station HJ).

## KEY TO THE COMMON BARNACLE CYPRIDS IN MATANG MANGROVE FOREST RESERVE (MMFR)

1. Carapace sculpturing absent, i.e. smooth (carapace Type E) .....2  
 – Carapace sculpturing present .....3
2. Carapace length less than 550  $\mu\text{m}$ .....*Amphibalanus amphitrite*  
 – Carapace length more than 550  $\mu\text{m}$ .....*Amphibalanus reticulatus*
3. Carapace punctate at anterior, posterior and ventral aspects (carapace Type C) and large in size (carapace length more than 600  $\mu\text{m}$ ).....*Amphibalanus variegatus*  
 – Carapace not punctate with carapace length less than 600  $\mu\text{m}$  .....4
4. Carapace with honeycomb sculpturing pattern (carapace Type A)....*Fistulobalanus* sp.  
 – Carapace without honeycomb sculpturing pattern .....5
5. Carapace with dark rounded pigmentation spot posterior to cypris eye, ridged sculpturing on the posterior end (carapace Type D), height less than 250  $\mu\text{m}$ .....*Euraphia withersi*  
 – Carapace with rounded and lunular sculpturing at dorsal and ventral aspects, respectively (carapace Type B), height more than 250  $\mu\text{m}$ .....*Fistulobalanus patellaris*

## 2.4 DISCUSSION

### 2.4.1 Barnacle adult identification and DNA barcoding analysis

The adult barnacle species identified in MMFR belong to three genera, namely *Amphibalanus*, *Fistulobalanus* and *Euraphia*, which are commonly found in tropical and sub-tropical mangrove habitats (Rainbow *et al.*, 1989; Prabowo & Yamaguchi, 2005; Crona *et al.*, 2006; Marques-Silva *et al.*, 2006; Li & Chan, 2008). However, two of the clades (OTU1 and OTU2) derived from the cyprid data did not match any of the identified adult barnacle sequences by barcoding analysis. This suggests that the larvae may either be advected cyprids from offshore adult species which are not resident in MMFR, or the cyprids came from adults not sampled in the MMFR. The identity of these two unknown species awaits further detailed surveys of adult barnacles around MMFR. Non-matched results are common in barcoding analyses especially for areas that are not sufficiently surveyed. Barber & Boyce (2006) used COI fragments to study the diversity of coral reef stomatopods. They reported 22 distinct OTUs that could not

be matched with any adult stomatopod references. Chen *et al.* (2013) also reported 10 unidentified OTUs from wild collection of barnacle cyprids and suggested the possible invasion of cyprids from neighbouring regions. The presence of OTUs in the absence of their adults shows the apparent disconnectedness between the presence of larval and their adults, which could be due to a reasonably long larval phase (10-45 days, Lohse & Raimondi, 2007) and hence, the potential to be widely dispersed by ocean current. Cyprids in the absence of settlement cues may also not settle or survive on unsuitable substrates (Pawlik, 1992).

The 12S-DNA region has proven to be successful and reliable for barnacle identification in this study. The 12S-DNA fragments are shorter and relatively easier to amplify compared to COI fragments (unpublished data) and these are commonly used for species identification. Barnacle cyprids are usually small in size and their DNA can be easily degraded after a period of preservation. It is suggested that 12S-DNA fragments can be obtained from small cyprids or cyprids that have been preserved for a prolonged period of time. However, the 12S-DNA fragments have a smaller representation in online databases than COI fragments.

#### **2.4.2 Morphological analyses**

In the present study, the quantitative characters (carapace length, height, posterior angle and length-to-height ratio) have a low discriminating power. This problem is exacerbated in closely related species or genera within this study. Carapace length and carapace height are two common morphological measurements used for cyprids in previous reports, and have been suggested for use in species discrimination. For instance, Burrows *et al.* (1999) suggested using carapace length to differentiate the cyprids of *Chthamalus stellatus* from *Chthamalus montagui* in British waters, and this was later verified by molecular evidence using mtDNA RFLP profiles (Power *et al.*,

1999). Pineda *et al.* (2002) used carapace length and seasonal presence to select out the cyprids of *Semibalanus balanoides*. Nevertheless, the use of carapace length and height is only good enough to distinguish between a few species of cyprids which differ in size, and is of little use where many species are known to co-occur and similar in size, e.g. in the MMFR waters. Comparison of carapace length and carapace height of successfully identified species in this study (wild caught) to those obtained from previous reports (laboratory-reared) showed some discrepancy (Table 2.1). Discrepancy in carapace length and height was also found among the lab-reared cyprids from different studies of same species (Table 2.1). Thus, large within-species size variation may exist. Geographical origin or/and environmental conditions may be the cause(s) of size variation. O’Riordan *et al.* (2001) observed temporal and latitudinal variations in the length of cyprids collected from European localities. Desai *et al.* (2006) reported a significant effect of temperature and food concentration on the length of laboratory-reared barnacle cyprids. Thus, environmental and geographical factors may limit the usefulness of any cyprid identification key based only on morphometrics.

Carapace sculpturing is a useful character for discriminating the dominant species of barnacle cyprids found in MMFR. For the purpose of classifying large numbers of cyprids, carapace features that are observable under CM are preferable as diagnostic features. Although SEM provided enlarged and much clearer details of the carapace sculpturing, these are important only for the purpose of description but not necessary for applying the classification model. In fact, it is impractical to use SEM for the purpose of identifying cyprids in large numbers. Egan & Anderson (1986) did not include carapace sculpturing for their description of *Amphibalanus variegatus* due to the absence of SEM evidence. The honeycomb or Type A sculpturing that was found on unidentified *Fistulobalanus* sp. in the present study has also been previously reported for barnacle cyprids of *Chthamalus malayensis* (Yan & Chan, 2001), *Catomerus*

*polymerus* and *Chamaesipho tasmanica* (Egan & Anderson, 1989), and Cryptophialidae (Kolbasov & Høeg, 2007). Nevertheless, there could be some minor variations in the honeycomb sculpturing patterns of different species such as the size of the honeycomb unit, but the previous report did not describe its size and hence comparison is impossible. Lee *et al.* (1999) previously reported that the carapace of *Amphibalanus reticulatus* is covered with numerous small denticles but this was not supported by SEM evidence in the present study. Neither LM nor SEM in the present study showed any denticles. Instead, the species, whose identification was confirmed by molecular analysis has a smooth carapace. Thiyagarajan *et al.* (1997) also did not observe any denticles for *Amphibalanus reticulatus*. Such variation in the denticles on the carapace may also be due to the presence of cryptic species. Although the sculpturing pattern appears to be species-specific for the cyprids in Matang mangrove waters, the type of pattern shows no generic affinity. This supports the findings of Standing (1980), where he described carapace sculpturing in *Pollicipes polymerus*, *Balanus improvisus* and *Balanus glandula*, but none in *Chthamalus dalli*, *Balanus crenatus*, *Balanus nubilus* and *Semibalanus cariosus*. The function and evolutionary history of carapace sculpturing in cyprids is presently unknown.

#### **2.4.3 Morphology-based classification model**

The combination of quantitative characters with carapace sculpturing characters gave better classification accuracy. This suggests that a combination of both qualitative and quantitative characters in classification problems should be considered especially when few characters are available. The use of classification trees is suitable for combined characters, and a good alternative to LDA (Feldesman, 2002). The other advantage is variable selection. This is automatically performed by the classification tree algorithm, because the variables that are not useful in reducing the misclassification errors are not



used. This simplified the classification models. The classification tree based on the complete data with carapace sculpturing (Figure 2.8) did not use carapace length and length-to-height ratio as predictors, which is simpler than using all of the variables. It has to be noted that the selected variables may differ when a different statistical package is used to compute the classification tree. Classification trees have previously been used in the taxonomic identification of fish (Guisande *et al.*, 2010) and pollen grains (Lindbladh *et al.*, 2002.).

#### **2.4.4 Assessment of barnacle cyprid diversity at MMFR and Haji Dorani**

Field samples from MMFR waters showed that cyprid composition, dominated by four species, varied spatially (between stations) and temporally (between sampling years), indicating the dynamic nature of their supply. Cirripede nauplii were observed to be most abundant in the inshore waters of MMFR (<15 km off shore) compared to estuarine and offshore waters, being consistently found throughout the year but with peak abundance in May and October during the intermonsoon months (Chew, 2012). Thus, the difference in composition of cyprid samples in the present study is likely a result of temporal variability. The present field study is preliminary. Future studies with more exhaustive sampling over larger spatial and temporal scales are necessary to elucidate the supply-side ecology of barnacle larvae in the estuary.

For Haji Dorani, it was unexpected that the samples did not contain any cyprid of *E. withersi*, whose adults were found at the high intertidal zone. The presence of other species of barnacle cyprids (~21%) besides *A. amphitrite* was not unexpected, since the adults of these species had not been previously surveyed. Although Tan (2013) reported the presence of *Fistulobalanus patelliformis* (= *patellaris*) and *Amphibalanus thailandicus* on replanted mangrove seedlings it was not known whether they were translocated with the seedlings from elsewhere. More cyprids from Haji Dorani site

need to be DNA barcoded to verify the observations since not many sequences were obtained from this site. Modification of the sampling method for this site may be needed to obtain better quality of specimens for DNA amplification.

#### **2.4.5 Beyond carapace sculpturing characters**

Morphological characters besides those described in the current study may be used to discriminate species that do not have any sculpturing. Chen *et al.* (2013) showed that the antennular morphology provides higher inter-species variations than carapace morphology, which would appear very useful for species identification. However it is beset by the problem that not all preserved cyprids showed extended antennules. Kamiya *et al.* (2012) proposed a promising auto-fluorescence pattern approach to identify cyprids, but the method works only with fresh and unpreserved samples. At present, there is still no single approach that can provide identification of barnacle cyprids with high accuracy, high speed and low cost. The selection of the best approach will largely depend on the research question. The approach used in the current study aims to balance these three criteria. Since each approach has its strengths and limitations, future global or regional-scale cyprid identification keys are likely to use an integrated approach combining the usage of carapace sculpturing features, traditional morphometrics, geometric morphometrics and cyprid appendicular features.

#### **2.4.6 Conclusion**

The present study has successfully identified and described the barnacle cyprids from MMFR through a combination of molecular and morphological approaches. Six species of barnacle adults and cyprids had matched DNA sequences. These include *Fistulobalanus pattellaris*, *Fistulobalanus* sp., *Amphibalanus reticulatus*, *Amphibalanus variegatus*, *Amphibalanus amphitrite* and *Euraphia withersi*. Carapace sculpturing

pattern on the cyprids is the most important morphological discriminator. The constructed identification key is suitable to be used for identification of cyprids from either MMFR or Kg. Hj. Dorani, based on their morphology. Preliminary analysis of diversity of cyprids in MMFR shows a dynamic distribution. For Kg. Hj. Dorani's sample of cyprids, *A. amphitrite* was found to be the most dominant species.

## CHAPTER 3

### SUCCESSION OF MICROBIAL COMMUNITIES AND MORPHOLOGY OF BIOFILM IN RELATION TO THE SETTLEMENT OF BARNACLES

#### 3.1 INTRODUCTION

Bacteria constitute a large part in the microbial biofilm (Zobell & Allen, 1935; Faimali *et al.*, 2004). They are hypothesized as one of the important factors affecting the habitat selection of the exploring barnacle cypris larvae. There are many biofouling studies that aim to find out whether bacteria influence the larval settlement of barnacles. From the early studies using pure culture bacterial biofilm (e.g. Maki *et al.*, 1988; Maki *et al.*, 1990; O'Connor & Richardson, 1998; Maki *et al.*, 2000), to recent investigations with mixed populations of bacteria (e.g. Olivier *et al.*, 2000; Qian *et al.*, 2003; Lau *et al.*, 2005; Thiagarajan *et al.*, 2006), the cypris larvae showed differential response and considerable specificity and sensitivity to different species of bacteria or different microbial communities of biofilm. This led to the hypothesis that the barnacle cypris may have the capability to distinguish the different bacterial communities of biofilm (Qian *et al.*, 2003).

Microbial biofilm consist of not only of bacteria but other microbial organisms as well, including archaea and microbial eukaryotes. Microbial eukaryotes have received less attention in microbial ecology studies than prokaryotes, and their importance has often been underestimated (Moreira & López-García, 2002; Massana & Pedrós-Alió, 2008). Similarly, more emphasis has been put on the role of bacteria in microfouling studies and studies of interaction between microfouling and macrofouling. This is reflected by relatively low number of publications on microbial eukaryotes in biofouling studies (Dobretsov, 2009). Bacteria and microbial eukaryotes of biofilm are

often studied separately. When they were investigated in parallel, microbial eukaryotes were described in fewer details than bacteria.

Apart from microbial communities, the most prominent changes occurring during the succession of biofilm is its structural development. Whether the structural development of biofilm is accompanied by the succession of microbial communities has been of interest to biofilm ecologists (Martiny *et al.*, 2003). It has been observed that structural differences in biofilms correspond to differences in bacterial communities under different environments (Besemer *et al.*, 2007; Besemer *et al.*, 2009).

The hypothesis that barnacle larvae can distinguish biofilms of different bacterial community is compelling but little is known about the mechanism. It was suspected that the change in bacterial community during the biofilm succession may be associated with other changes such as the changes in biochemical profile, the associated cues is then utilized by the larvae as signals for selection of suitable settlement site (Hung *et al.*, 2007). Chung *et al.* (2010) tested this idea but found that the congruence between the biofilm bacterial community profile and chemical profile was less than expected. It is possible that the succession of biofilm bacterial community might interact with other biofilm attributes too, and the understanding of such interactions could open up more possible mechanisms to explain how barnacle larvae differentiate biofilms. Indeed, more exploration for possible mechanisms is still required despite years of investigations in biofilm- invertebrate settlement research (Hadfield, 2011).

### **3.11 Objectives**

This study aimed to

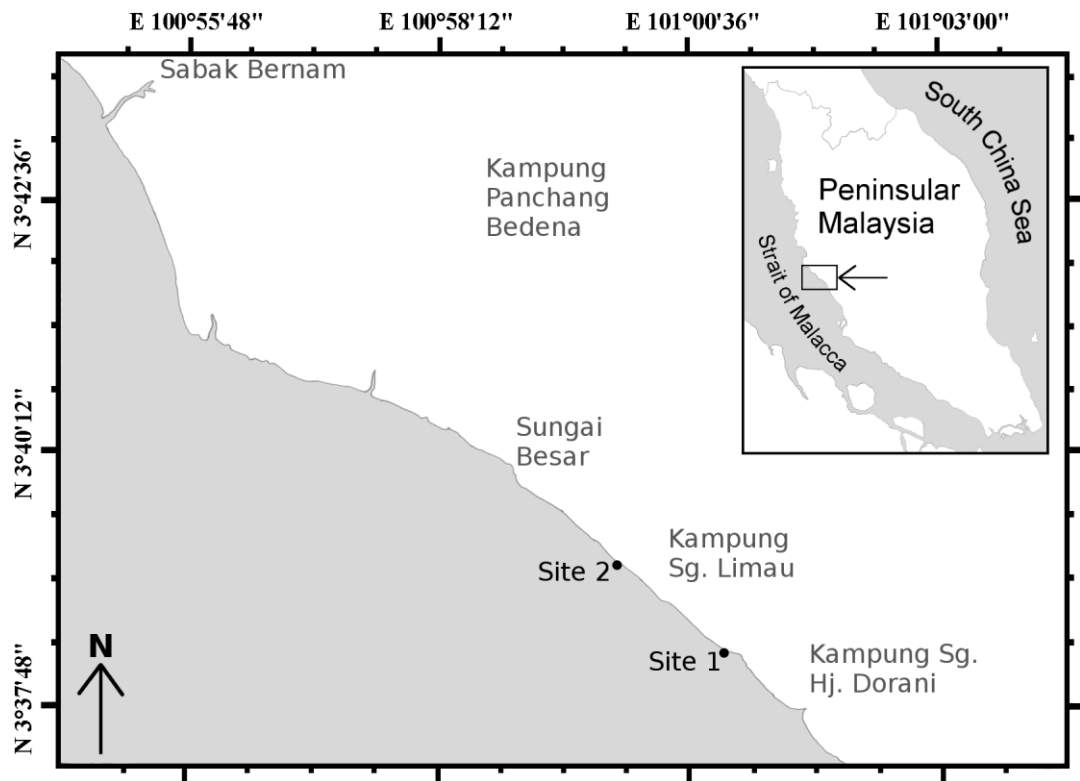
- (i) characterize the biofilm morphological structure and the successional changes of the biofilm communities with respect to the bacterial and microeukaryotic communities,

- (ii) determine the association between the biofilm morphological structure, bacteria and microeukaryotic communities, and
- (iii) evaluate the effect of biofilm age on the settlement of barnacles in relation to biofilm morphological structure, bacteria and microeukaryotic communities.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Study site**

All experiments in this study were conducted at the mangrove re-planting experimental site of the Forest Research Institute, Malaysia (FRIM) at Kampung Sungai Haji Dorani (Figure 3.1, Site 1). This site is an exposed intertidal mudflat where artificial wave breaker structures (geotubes) were built to protect replanted mangrove seedlings in the rehabilitated area (see Tamin *et al.*, 2011). Two barnacle species could be found at Site 1 and a nearby Site 2 (Figure 3.1): *Amphibalanus amphitrite* (= *Balanus amphitrite*, see note by Clare & Høeg (2008) for nomenclature discussion) and *Euraphia withersi*. *A. amphitrite* adults were the most dominant species observed at the experimental sites where the tidal height was ~200cm above C.D. Result of survey on the cyprid composition at this site from chapter 2 showed that 79% of cyprids caught belonged to *A. amphitrite*. For all settlement experiments, numbers of settled barnacle cyprids and metamorphosed juveniles were simply referred to as barnacles without distinction of development stage.



**Figure 3.1:** Study sites. Experiments in this chapter were conducted at Site 1(Kampung Sungai Haji Dorani) while experiments for chapter 4 were conducted at Site 2 (Kampung Sungai Limau).

### 3.2.2 Experimental design

#### 3.2.2.1 Preliminary study (microbial community structure of biofilms formed on mangrove seedlings after transplantation)

A preliminary study was conducted with the primary objective of testing a method for recovering microbial biofilms from mangrove seedlings for analysis by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR-DGGE). This study also examined the very short term effect of transplanting and tidal cycle on the microbial community of the biofilm. Twenty four mangrove seedlings from the nursery were transplanted to Site 1 at 1200hr on 26 May 2010. Subsequently, three mangrove seedlings were sampled each time at 4-hourly intervals over the next 24 hours, covering one (semi-diurnal) tidal cycle. Analysis methods were described in sections below (3.2.4- 3.2.5).

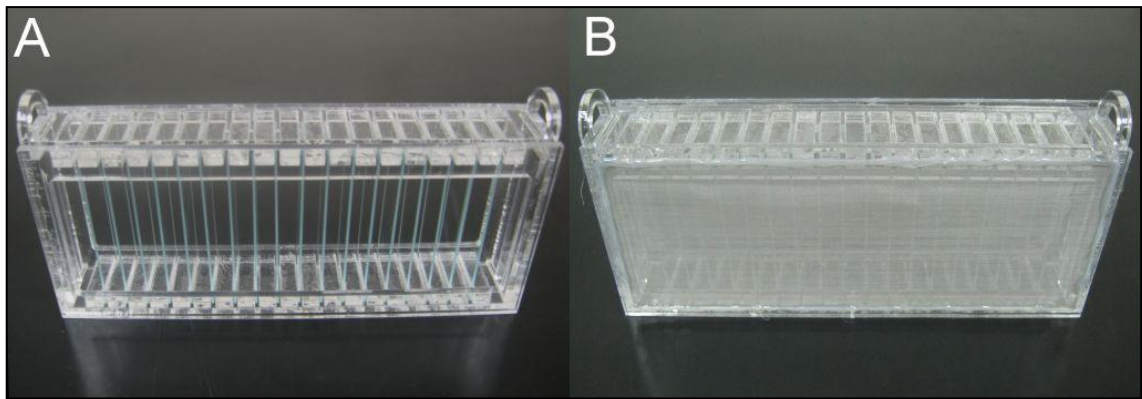
### **3.2.2.2 Effect of age of biofilm on the settlement of barnacles**

This experiment was carried out twice, the first time from 24 December 2010- 12 Jan 2011, and the second time from 20 April- 9 May 2012 (henceforth referred to as 2011 sampling and 2012 sampling, respectively). The second sampling was a repeat based on an improved experimental design. Glass slides were used as fouling substrates (barnacles in Site 1 could settle on the glass; Appendix B). They were encased in custom made acrylic holders that could hold 18 slides each. The holder design is similar to a commercially available periphyton meter (Appendix C). Each holder was considered as an experimental unit in this study. Wire mesh (150µm mesh size; Todd & Keough, 1994) made of stainless steel was used to wrap around the slide holders (sealed tightly with silicone) to exclude the entry of planktonic cyprids and other organisms, but allowed seawater and microorganisms to freely flow through it (Figure 3.2). Slide holders without the mesh cover were included as control. The effects of the wire mesh were examined and the results were included in Appendix D. Before deployment in the field, the slides were heat-sterilized while the holders were washed with bleach and copious amount of sterilized water.

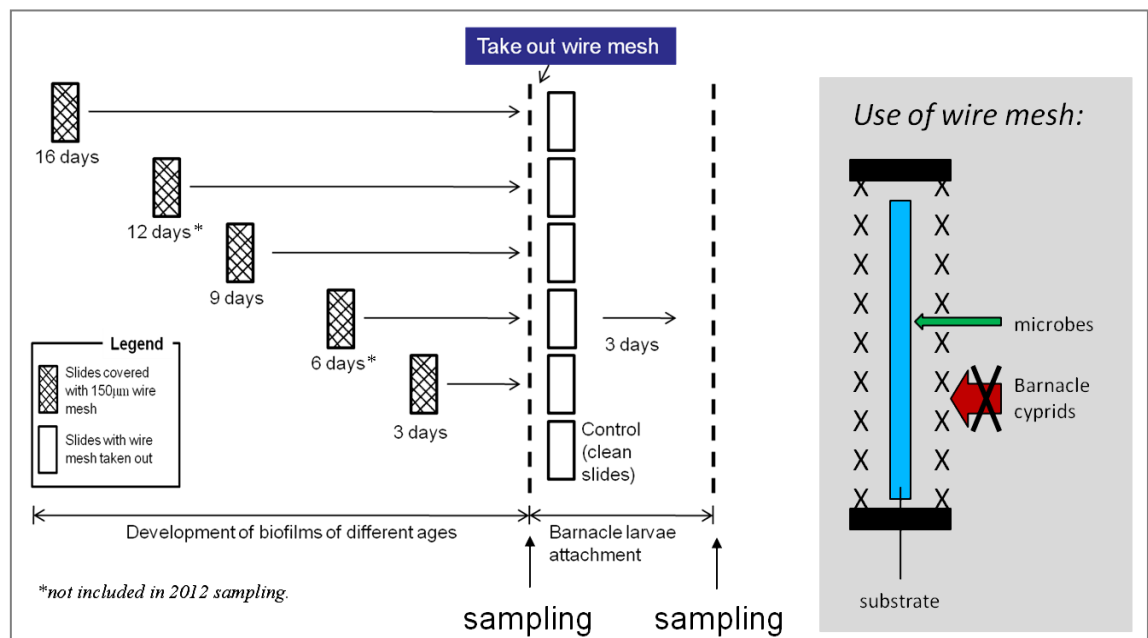
The slides were left at the study site for periods of 3, 6, 9, 12 and 16 days. This was to allow the growth of biofilms of different ages. After 16 days, the wire mesh was removed from the glass holders, and additional slide holders with new clean slides were added in as control. The treatment and control slides were left in the field for another 3 days. This was to give the cypris larvae a choice and sufficient time to settle on biofilms of different ages (Figure 3.3).

Completely randomised design (CRD) was used for the 2011 sampling, but as an improvement, randomised complete block design (RCBD) was used for the 2012 sampling. Frame structures constructed with PVC pipes were set up in the field to suspend the slide holders. The slide holders were suspended at ~ 30cm above ground or



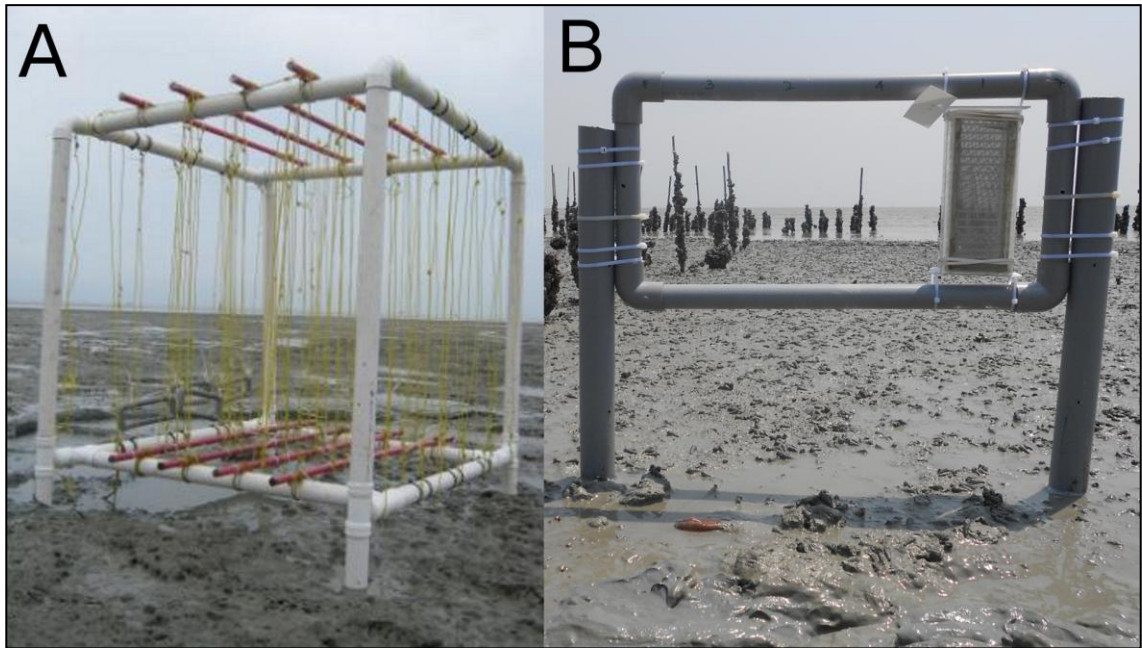


**Figure 3.2:** .Glass slides holder, (A) without wire mesh (B) with wire mesh to exclude invertebrate larvae and macro-organisms. Extra support rings were added to the holders in the final design (not shown).



**Figure 3.3:** .Experimental design to test the effect of biofilms age on the settlement of barnacles.

200cm above the Chart Datum. A different frame structure was used in 2012 because the first one (used in 2011) failed several times under harsh field conditions and it was also not suitable for RCBD (Figure 3.4). For the 2011 sampling, there were six levels of biofilm age treatment (including control) and each level had 3 replicates, but for the 2012 sampling, the number of levels was reduced to 4 (control, 3, 9, 16 days) based on the results from the 2011 sampling. Each age group had 9 replicates, which equals to 9 blocks, where each PVC frame structure (Figure 3.4B) was defined as a block. Blocking



**Figure 3.4:** Frame structures made of PVC tubes to suspend the glass slides holders on the mudflat, (A) frame used in 2011 sampling and (B) frame used in 2012 sampling.

was a means to address the potential confounding effects associated with position. The positions of the holders were either completely randomised (2011 sampling) or randomised within each block (2012 sampling).

In each experimental unit, 18 slides were divided randomly (with good interspersed) for different analyses (see Appendix E for arrangement of blocks and sampling scheme), with 6 slides for DGGE analysis, 6 for barnacle settlement analysis, 3 for confocal laser scanning microscopy (CLSM) analysis. For all analyses, the edges and areas of glass slides occluded by the holder ( $\sim 7.5\text{cm}^2$ ) were not used in subsequent analyses to avoid any possible effects from artifacts. Before subjected to detachment for analysis, biofilms from the occluded areas were removed by scrapping using heated blades. Extra slide holders without wire mesh were also deployed to assess the barnacle recruitment during the experimental period and to assess the effectiveness of exclusion by wire mesh (2011 sampling only). Samplings of slides for DGGE analysis and CLSM analysis were carried out on the day when the wire mesh was removed, while samplings of slides for assessment of barnacle settlement were carried out 3 days later. Slides for

DGGE analysis were stored at 4°C until further processing while the slides for CLSM analysis were fixed with 2% formaldehyde buffered with autoclaved filtered seawater (AFSW) and stored in the dark at 4°C. In the 2012 samplings, only 7 out of 9 blocks were sampled for biofilm analyses due to bad weather at the study site. However, all 9 blocks were used for the cyprid settlement assessment. The differences between 2011 and 2012 samplings were summarised in Appendix F. Other experimental conditions were held similar.

### **3.2.3 Recovery of microbial cells and DNA extraction**

After collection, the slides or the seedlings were rinsed with autoclaved filtered seawater (AFSW) to remove any loosely attached microbes and dirt. Biofilms were detached from the surfaces using ultrasonic bath, based on the methods modified from Hempel *et al.* (2008). The procedures were as follows: seedlings were cut aseptically into ~8cm lengths. The cut seedlings or glass slides were inserted into 50mL polypropylene tubes containing the washing buffer (0.1mM Tris HCl, 0.01mM sodium EDTA, 0.1% sodium pyrophosphate in AFSW). The tubes were then sonicated in an ultrasonic bath (Powersonic Model 603; Hwashin Technology, Korea) for 3 minutes followed by 3 minutes of vortex at full speed and 3 minutes of sonication again. The sonication method was chosen over the commonly-used scrapping method because the former could be applied to both plant and artificial substrates, and thus would facilitate better comparisons between the studies. Filtration was used to recover the detached microbial cells as earlier trials of using centrifugation resulted in unsatisfactory recovery. The washing buffer containing the microbial cells was filtered through a pre-sterilized 0.22µm membrane filter at moderate vacuum pressure (200mmHg). The filtration set was heat sterilized with flame between samples. The microbial cells retained on the membrane filters were then stored at -20°C until further processing.

The DNA extraction method was adapted from protocols used in soil microbiology. The commercially available UltraClean<sup>®</sup> Soil DNA Isolation Kit (MoBio Inc., Solana, CA) was used. The membrane filters were cut aseptically into small strips before DNA extraction to improve the efficiency of bead beating during extraction. The strips of membrane filters were inserted into the bead column and the extraction proceeded according to the protocols provided by the manufacturer. The extracted DNA was eluted in 50 µL buffer containing 10mM Tris-HCl (pH8) and stored at -20°C until further use. The DNA was electrophoresed on 1% (w/v) agarose gel and the concentration was compared to those of the 1kb DNA ladder (Vivantis, Malaysia).

#### **3.2.4 PCR amplification**

For bacterial assemblages, a nested PCR approach was used to amplify a region of the 16S rRNA gene from the DNA extracted from the biofilms. The primary PCR was performed using the universal bacterial primers 27F and 1492R (Lane, 1991) to amplify a region which corresponded to 1.5kb in length. The secondary PCR (nested) was performed using the primers set 341F and 907R with a 40bp GC clamp added to the forward primer (Muyzer *et al.*, 1997). This amplifies a fragment of about 560bp long. For micro-eukaryotic assemblages, a region of the 18S rRNA gene was amplified using the primers set Euk1A and Euk516r with a 40bp GC clamp added to the reverse primer (Díez *et al.* (2001). This also amplifies a fragment of about 560bp long. The GC clamp is a sequence of guanines (G) and cytosines (C) which act as a high melting domain and can prevent complete separation of two DNA strands and provide better separation of DGGE bands (Sheffield *et al.*, 1989). The primer oligonucleotide sequences are tabulated in Table 3.1.

**Table 3.1:** Primers used for the amplification of the 16S rRNA and 18S rRNA genes

Primers name	Sequences <sup>a</sup> (5'-3')	Reference
<i>Bacterial 16S rRNA gene</i>		
27F	AGA GTT TGA TCM TGG CTC AG	Lane (1991)
1492R	GGT TAC CTT GTT ACG ACT T	
341F-GC <sup>b</sup>	CC TAC GGG AGG CAG CAG	Muyzer <i>et al.</i> (1997)
907R	CCG TCA ATT CCT TTG AGT TT	
<i>Eukaryotic 18S rRNA gene</i>		
Euk1A	CTG GTT GAT CCT GCC AG	Díez <i>et al.</i> (2001)
Euk516r <sup>c</sup>	ACC AGA CTT GCC CTC C	

<sup>a</sup> Degeneracy is indicated by standard conventions: M can be A or C.

<sup>b</sup> A 40bp GC-clamp (CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C) was added to the 5' end of the primer.

<sup>c</sup> A 40bp GC-clamp (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G) was added to the 3' end of the primer

The PCR was performed using thermal cycler (Labnet International or Bio-Rad Laboratories). In all reactions, the final reaction mixture (50µL) contained 1x iTaqPlus PCR buffer, 0.4µM of each primer, 0.25mM dNTPs, 3.0mM of MgCl<sub>2</sub> and 1.5U iTaq<sup>TM</sup>Plus DNA polymerase (iNtRON Biotechnology, Korea). For 16S DNA amplification, 1µL of DNA and 1µL of primary PCR product (20× dilutions) were used as template for primary and secondary PCR, respectively. The PCR conditions for the primary PCR were as follows: initial denaturation at 94°C for 5min, 30 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and elongation at 72°C for 1.5min (increase 1s by each cycle), followed by 10min of final extension at 72°C. A touch-down PCR conditions were employed for the secondary PCR: 94°C for 5 min, 10 cycles of 94°C for 1min, 65°C for 1min with 1°C decreased every cycle until 55°C, 72°C for 3min, then 12 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1min, followed by final extension at 72°C for 30min. For 18S DNA amplification, 2 µL of DNA was used as template. In all amplifications, a negative control was included using deionized water as template. The PCR conditions were as follows: initial denaturation at 94°C for 5min, 35 cycles of denaturation at 94°C for 30s, annealing at 56°C for 45s and elongation at 72°C for 2min followed by 30min of final extension at 72°C. The PCR products were

electrophoresed on 1% or 1.5% (w/v) agarose gel, stained with ethidium bromide and photographed using a UV transilluminator (Syngene Bio Imaging, UK) to determine the presence of the expected size of 16S / 18S DNA fragments and to compare their concentrations to a 100bp DNA ladder (Bioatlas, Estonia).

### **3.2.5 Denaturing gradient gel electrophoresis (DGGE)**

DGGE was performed as described by Muyzer *et al.* (1997) using a DGGE-2001 system (C. B. S. Scientific, USA). Electrophoresis was performed on 0.75mm thick, 6% (w/v) polyacrylamide gels (37.5:1 of acrylamide: bis-acrylamide; Bio-world, Dublin, OH) with a denaturant gradient of 35%-60% / 35%-62% (for 16S DNA, range depending on samples), or 25%-45% / 25%-43% (for 18S DNA, range depending on samples), where 100% of denaturant is defined as 7M urea (GE Healthcare, Waukesha, WI) and 40% formamide (GE Healthcare, Waukesha, WI). The gels were casted with a gradient mixer after addition of 0.04% (final concentration) of ammonium persulfate (APS; Sigma-Aldrich, St. Louis, MO) and Tetramethylethylenediamine (TEMED; Sigma-Aldrich, St. Louis, MO), and were left to polymerize at room temperature for at least 2 hours. Top 1cm of the gel was filled with stacking gel (10 % polyacrylamide gel without denaturant).

The polymerized gels were pre-run at 100V, 60°C for 20min in 1× TAE (Tris-acetate-EDTA) buffer before 15-25µL of PCR products were loaded onto the gels and electrophoresed for another 16 hours. Volume of PCR products loaded was adjusted according to the estimation of their concentration from the agarose-gel electrophoresis. If more than 25µL was needed to achieve optimal concentration, double loading was performed. This would not affect the banding pattern as band positions were determined by denaturant concentration but not running time. The denaturant gradient, polyacrylamide concentrations, running time and voltage were all optimized by multiple

trials. Markers for DGGE were made from re-amplified excised bands from previous run and were included in each run for inter-gels comparisons of banding positions. After electrophoresis, the gels were stained with 1× SYBR<sup>®</sup> Gold nucleic acid gel stain (Molecular Probes, Eugene, OR; diluted in 1× TAE buffer) for 1 hour in the dark, rinsed and photographed under UV trans-illuminator. Digital images of the DGGE gels were then analysed with Quantity One 4.6.5 (BioRad, USA) or ImageJ. Although the gel images were enhanced in the presentation of results, all image analyses were performed on original images of the gels. Bands were determined from the intensity spectrum and their intensities were recorded for later analyses.

### **3.2.6 Confocal laser scanning microscopy (CLSM)**

Different dyes were used for the 2011 and 2012 samplings. For the 2011 samples, 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) was used to stain the biofilms (Koerdt *et al.*, 2010). For the 2012 samples, SYTO<sup>®</sup> 9 (Molecular Probe, Eugene, OR) and wheat germ agglutinin conjugated to tetramethylrhodamine isothiocyanate-dextran (WGA-TRITC; Sigma-Aldrich, St. Louis, MO) were used to stain the biofilms. Both DAPI and SYTO 9 are nucleic acids specific dyes, although DAPI can also bind to extracellular polymeric substances (EPS) and emit a different spectrum of wavelengths (Porter & Feig, 1980). WGA is a lectin from *Triticum vulgaris* that binds to the EPS components (specifically  $\beta$ -GlcNAc and sialic acid; Zippel & Neu, 2011) of the biofilms, while TRITC is a fluorescent label. The reason for the difference in the choice of dyes was to add a dye more specific for EPS (WGA-TRITC). Concentrations of the dyes used in staining were 5 $\mu$ g/mL DAPI, 10 $\mu$ M SYTO 9 and 10 $\mu$ g/mL WGA-TRITC. Biofilms were stained for 5 minutes, rinsed with AFSW, and examined under immersion oil with a Leica TCS SP5 CLSM system (Leica, Heidelberg, Germany) equipped with inverted objective lens. Observations were performed using a

63× oil immersion lens (1.4 numerical aperture) with type F immersion oil (Sigma-Aldrich, St. Louis, MO) as media. Ideally, biofilm should be observed directly in water using water lens, but the study was constrained by the non-availability of equipment. For each experimental unit, 3 slides were observed, and in each slide, 3 haphazardly selected fields (246  $\mu\text{m}$   $\times$  246  $\mu\text{m}$ ) were scanned in xyz mode, at a z-step size of 0.38 $\mu\text{m}$ .

For the 2011 samples, excitation wavelengths used were 405nm and 633nm, and the emission signals were collected with three channels: 440nm-470nm (DAPI signals), 630-635nm (reflection signals from 633nm beam), and 660-750nm (signals from Chlorophyll-*a* auto-fluorescence). For the 2012 samples, excitation wavelengths used were 405nm, 488nm and 543nm, and the emission signals were collected with three channels: 495-505nm (SYTO 9 signals), 570-600nm (TRITC-WGA signals), and 660-750nm (signals from Chlorophyll-*a* auto-fluorescence). CLSM images were analysed with ImageJ. For quantitative calculations, image stacks of all three channels were first combined into a single stack.

Four quantitative morphological descriptors were calculated, namely average thickness, biofilm volume, roughness coefficient and percentage cover. COMSTAT plug-in for ImageJ (version 2.0; Heydorn *et al.*, 2000) was used to calculate the first three descriptors, while the percentage cover was calculated from the projected maximum intensity image using a built-in tool. Image processing parameters such as thresholding value for calculation were strictly standardized across all images for each sampling date.



### 3.2.7 Statistical analyses

#### 3.2.7.1 Effect of biofilm age on barnacle settlement

To test the null hypothesis that biofilm age does not affect the settlement of barnacle, one-way analysis of variance (ANOVA) was performed. The ANOVA model used for 2011 sampling was

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

and an improved model was used for 2012 sampling,

$$Y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

where  $Y_{ij}$  is the barnacle numbers,  $\mu$  is the grand mean,  $\alpha_i$  is the Treatment effect,  $\beta_j$  is the Block effect,  $\varepsilon_{ij}$  is the error term, and  $i = 1, 2, \dots, 6$  (2011) or  $1, 2, \dots, 4$  (2012);  $j = 1, 2, 3$  (2011) or  $1, 2, \dots, 9$  (2012). Square-root transformation was performed on barnacle numbers to homogenize the variance. Fligner-Killeen test was used to test the homogeneity of variance. Tukey's 'Honestly Significant Difference' (HSD) test was used to compare the means of different treatment levels when the ANOVA was found to be significant.

#### 3.2.7.2 Changes of biofilm DGGE profiles and CLSM profiles

DGGE banding profiles were analysed using both qualitative and semi-quantitative approaches. Presence/absence of DGGE bands was used for qualitative analysis while the relative DGGE band intensities (proportion within each lane) were used for semi-quantitative analysis.

The semi-quantitative data were transformed using the Wisconsin double standardization method before dissimilarity matrices for the samples were calculated using Bray-Curtis distance. For CLSM data, different variables were first standardized or normalized to give zero mean and unit variance. Distance matrices for CLSM profiles were calculated based on Euclidean distance.

For all three types of profiles, non-metric multidimensional scaling (nMDS) ordination was used to display the relationships among the samples. For qualitative DGGE data, some samples had similar presence/absence profile which resulted in zero pairwise distance. To these data, a very small distance was added to these dataset to remove the zero distance as nMDS could not handle zero distance. Axis 1 and 2 of all nMDS plots were scaled to give an aspect ratio of 1 so that the visual distance was proportionately correct, and since the scales are relative and non-metric in nMDS, they were not shown in the plots.

To test the null hypotheses that biofilm age has no effect on the microbial DGGE and CLSM profiles, permutational MANOVA was performed. To show the associations among these three profiles, i.e. the successional concordances, Mantel's test and Procrustes analysis were performed. Both Mantel's test and Procrustes analysis calculate the correlation between two distance matrices, but Procrustes analysis additionally perform the calculation based on the ordination plots and display the results in graphical form. For graphical display, Procrustes superimposition method was used to superimpose one nMDS ordination onto another nMDS ordination. In brief, Procrustes superimposition achieves best matching between configurations (in this case, the nMDS ordination) through the minimization of sum of squares differences between configurations by means of translation, scaling, and rotation. Procrustes superimposition was performed for all three possible pair-wise combinations.

Procrustes correlation  $r$  was calculated to show the strength of the associations between pair-wise profiles. This  $r$  value was calculated from the Procrustes distances, and is also referred as  $m_{12}$  in literature (Peres-Neto & Jackson, 2001). To test the significance of the associations, a permutational based test was used, in which the observed value was tested against randomly re-sampled null distribution of the test statistic, based on 10000 permutations. Permutational test was also performed using

Mantel's test for both qualitative and semi-quantitative data, to check whether the conclusions were affected by the methods of analyses (Mantel's test vs. Procrustean test, and qualitative DGGE data vs. semi-quantitative DGGE data). Null hypothesis that the pair-wise profiles was not associated were rejected if  $p$ -value is smaller than the designated significance level  $\alpha$  ( $= 0.05$ , unless otherwise stated). Bonferroni's correction was used to adjust  $\alpha$  if multiple pairwise comparisons were involved. Since smallest possible  $p$ -value from 10000 permutations is 0.0001, if observed value fell outside of all values in permutated values, the  $p$ -value for these cases were stated as  $<0.0001$ .

### **3.2.7.3 Correlation between DGGE/ CLSM profiles and barnacle settlement**

The linear multiple regression model of barnacle numbers against the nMDS scores were used to check if there was any correlation between the DGGE/CLSM profiles and barnacle cyprid settlement. This was performed for 2012 sampling only because of a labeling problem with the 2011 samples. Barnacle numbers were square-root transformed to homogenize the variance. Although only 2 dimensions were used in the nMDS plots for visual display, regression analysis was also performed with the nMDS scores utilizing up to four dimensions (all multi-dimensional stress  $< 0.05$ ), to check whether the conclusions would be affected.

In addition to multiple correlation, multiple correlation coefficient  $R$  was calculated from the multiple regression models.  $R$  is actually the correlation between the actual value and the predicted value from the multiple regression model, but unlike the two-variable correlation, this multiple-variable correlation  $R$  has only strength but no direction. Analyses were performed for both the qualitative and semi-quantitative data of DGGE profiles.

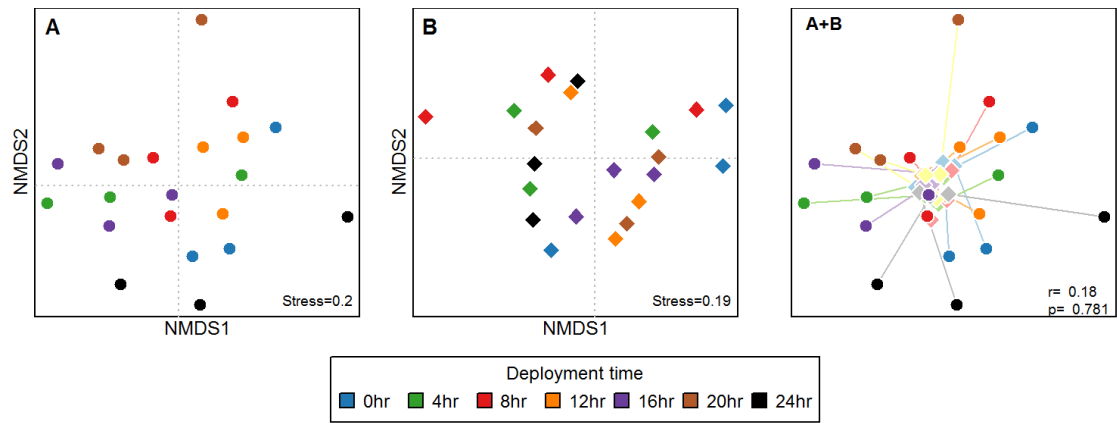
### 3.2.7.4 Statistical packages

All multivariate analyses were performed using the functions from ‘vegan’ package for R (Oksanen *et al.*, 2012), including ‘wiscosin’ for Wisconsin double standardization, ‘metaMDS’ for nMDS, ‘protest’ for Procrustes analysis, ‘mantel’ for Mantel’s test, and ‘adonis’ for permutational MANOVA.

## 3.3 RESULTS

### 3.3.1 Preliminary study: microbial community structure of biofilms formed on mangrove seedlings after transplantation

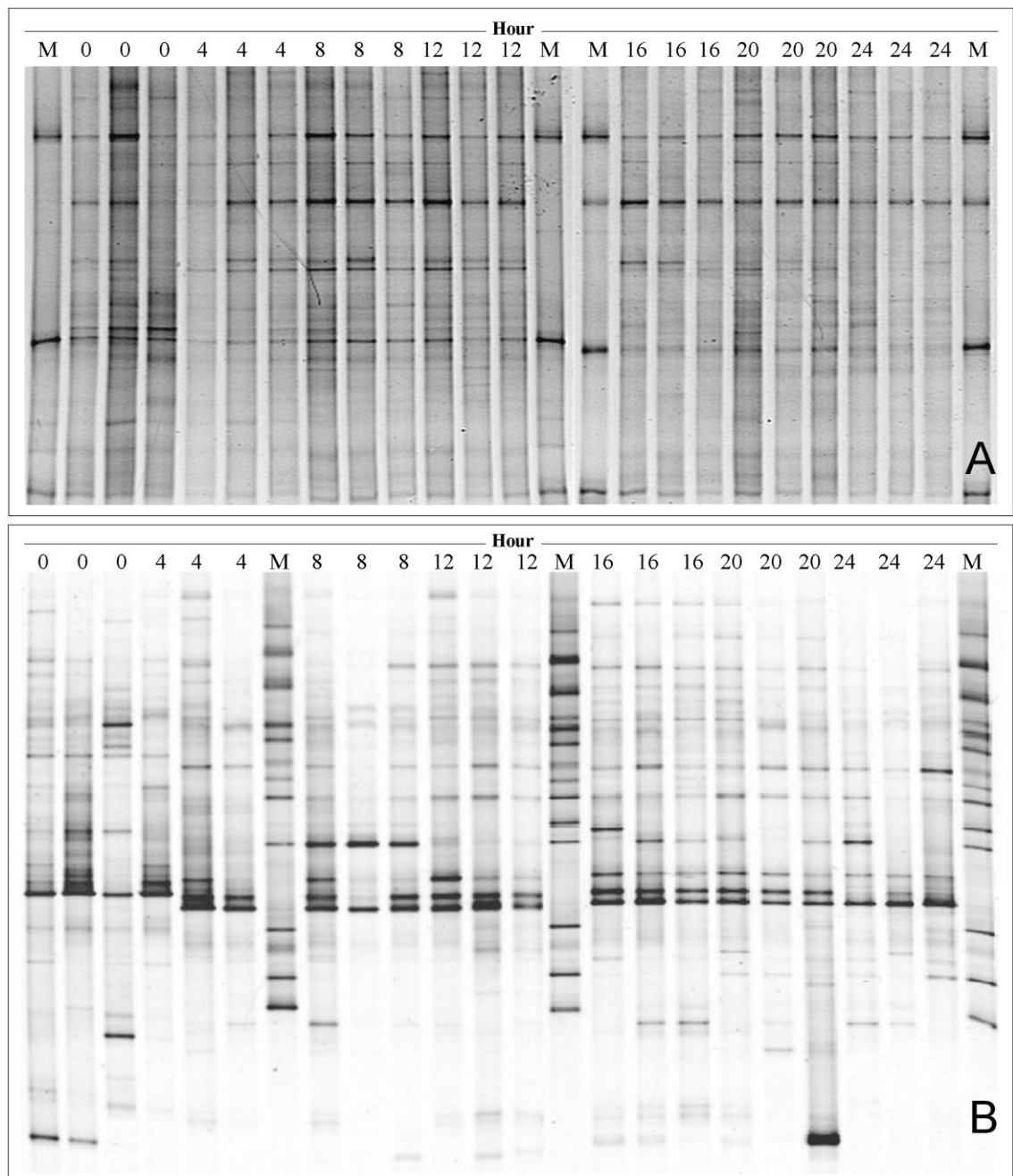
Microbial cells were successfully recovered from mangrove seedlings and PCR-DGGE analyses were successfully performed. No obvious clustering was observed from nMDS ordination of both the bacterial and micro-eukaryotes DGGE profiles (Figure 3.5). Looking at the DGGE gels (Figure 3.6), large within group variation was observed for microeukaryote DGGE gel, which was also reflected in nMDS plot. Permutational MANOVA showed that time had a weakly significant effect on the bacterial DGGE profile ( $F_{6, 14} = 1.5$ ;  $p = 0.02$ ;  $R^2 = 0.39$ ), whereas it had a non-significant effect on the microeukaryotic DGGE profile ( $F_{6, 14} = 0.8$ ;  $p > 0.05$ ). However, there was no association between the bacterial and microeukaryotic DGGE profiles ( $p > 0.05$ ), with failures to reject the null hypotheses from both Mantel’s test and Procrustean test, using either qualitative or semi-quantitative DGGE data. These results suggest that there were no or weak significant changes in microbial communities of mangrove biofilms in the very short timeframe (24 hours) after mangrove seedlings transplantation, and there was no evidence of successional concordance for this timeframe.



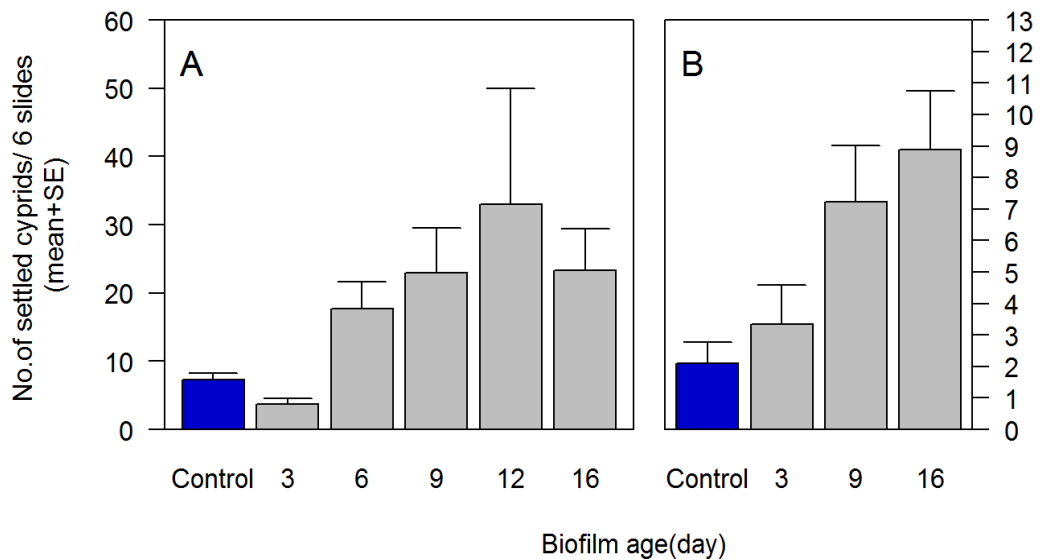
**Figure 3.5:** nMDS plots showing the relationships among the biofilms of different age (sampling time) on mangrove seedlings. (A) bacterial 16S DNA DGGE profile, (B) micro-eukaryotic 18S DNA DGGE profile, (A+B) Procrustes superimposition plots showing the pairwise concordance between profiles of bacterial DGGE and micro-eukaryotic DGGE.

### 3.3.2 Effect of biofilm age on barnacle settlement

Settlement of barnacle cyprids increased on glass slides with biofilms of more than 3 days of age (Figure 3.7), but the null hypothesis that biofilm age has no effect on the settlement of barnacle cyprids was rejected only for the 2012 sampling (Table 3.2). Blocking design greatly improved the statistical power in the 2012 sampling. Tukey HSD test showed that glass slides with biofilms of 9 day and 16 day of age had significantly higher number of cyprids settled on them (Table 3.3). Although the differences in number of barnacle cyprids observed were not very large compared to control (due possibly to the low larval supply in the field during the experimental period), the differences were highly significant ( $p < 0.001$ ) for the 2012 sampling. The results indicated that barnacle cyprids preferred to settle on older biofilms (9, 16 days) than on control. Higher larval supply was observed for the experimental period in 2011, as seen on the slides deployed in the field since the beginning of the experiment (Figure 3.8).



**Figure 3.6:** DGGE profiles of (A) bacterial 16S DNA, (B) micro-eukaryotic 18S DNA amplified from biofilms on mangrove seedlings at different times after transplantation. M = markers made from re-amplified excised bands from previous runs



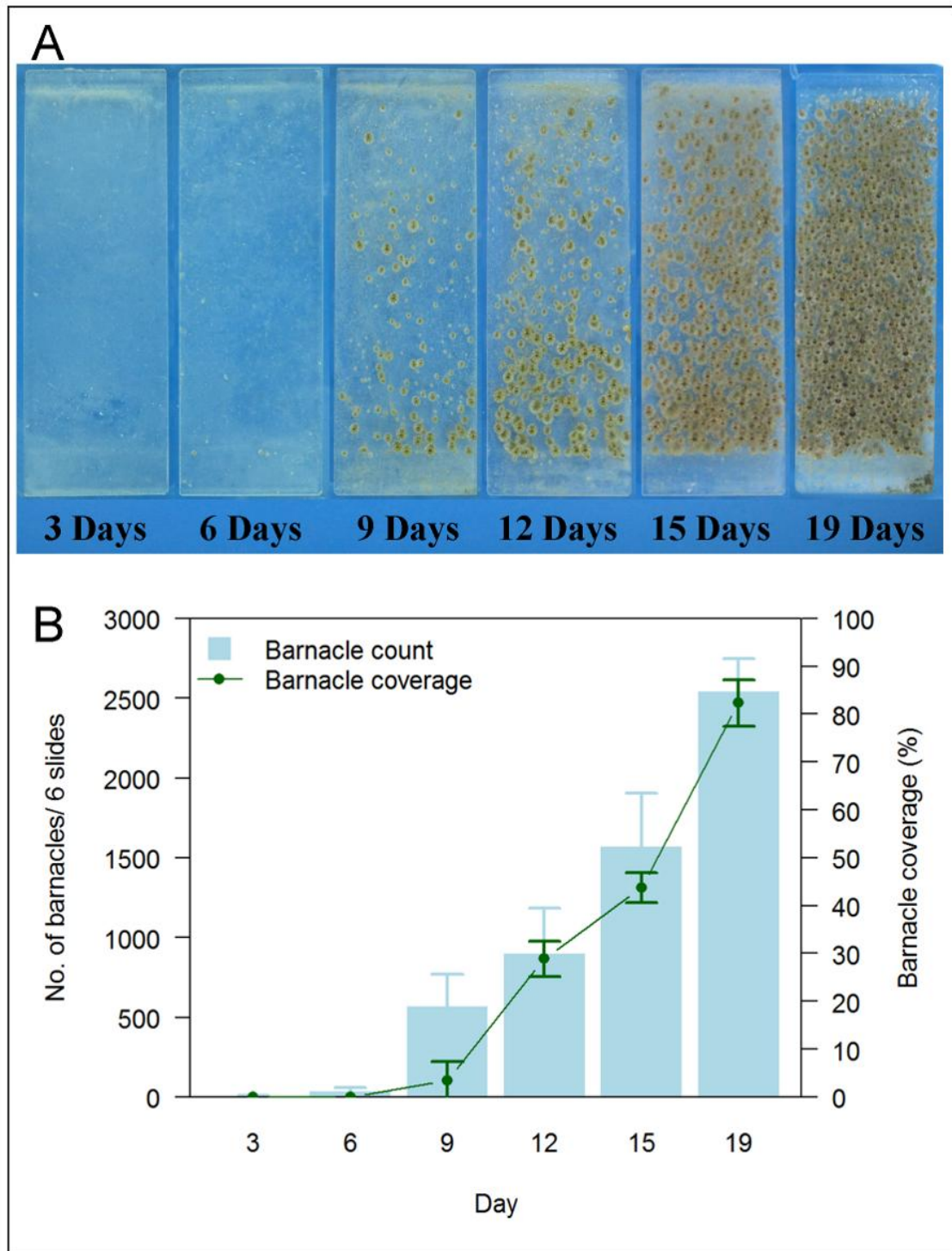
**Figure 3.7:** Number of settled cyprids on the glass slides with different ages of biofilms, 3 days after the wire mesh on the experimental units was removed. (A) 2011 sampling (B) 2012 sampling. Control = slides without biofilm at the time of mesh removal.

**Table 3.2:** ANOVA table of effect of biofilm age on the abundance of barnacle. Number of barnacle was square-root transformed. Biofilm age for 2011 (control, 3, 6, 9, 12, 16 days); 2012 (control, 3, 6, 9 days).

Source of variation	Df	Sum of Squares	Mean Squares	F value	Pr(> F)
<i>2011 sampling</i>					
Biofilm age	5	27.00	5.401	3.095	0.051
Residual	12	20.94	1.745		
<i>2012 sampling</i>					
Biofilm age	3	16.68	5.561	7.862	7.97×10 <sup>-4</sup>
Block	8	23.70	2.962		
Residual	24	16.97	0.707		

**Table 3.3:** Tukey's HSD test on barnacle numbers as influenced by biofilm age (control, 3, 9, 16 days) for 2012 sampling. Lower diagonal: *p*-value; Upper diagonal: 95% CI of back-transformed differences.

	Control	3	9	16
Control (mean=2.1)	-	0.71-1.81	0.06-5.89	0.25-7.22
3 (mean=3.3)	0.919	-	0.00-4.73	0.06-5.93
9 (mean=7.2)	0.013	0.054	-	0.69-1.83
16 (mean= 8.9)	0.003	0.012	0.912	-



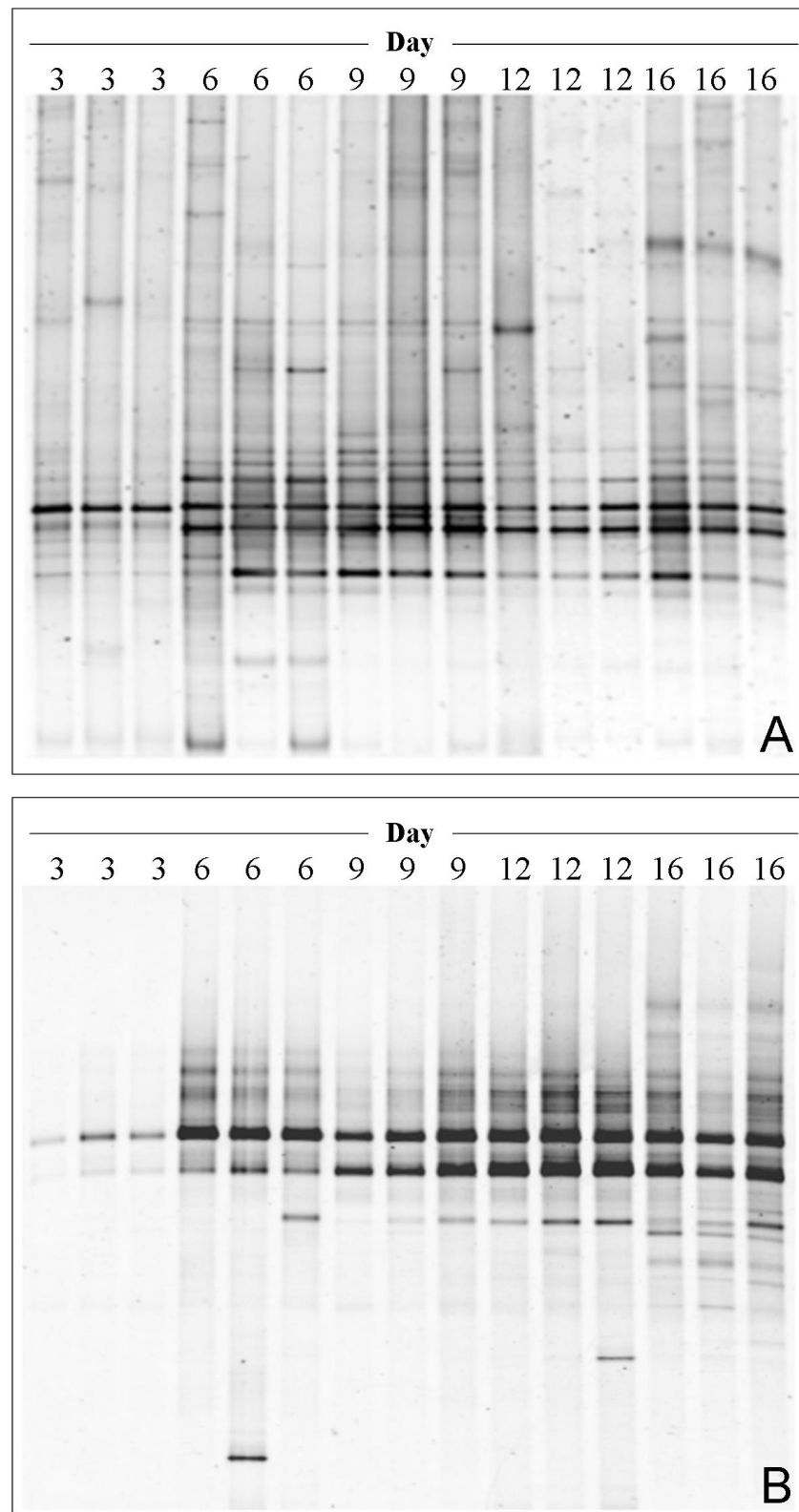
**Figure 3.8:** Barnacle fouling on glass slides during the experimental period of 2011 sampling. (A) representative photos of fouled slides for different lengths of deployment time. (B) Barnacle numbers and percentage cover at different days (mean  $\pm$  SD;  $n=3$ )



### 3.3.3 Changes of DGGE and CLSM profiles

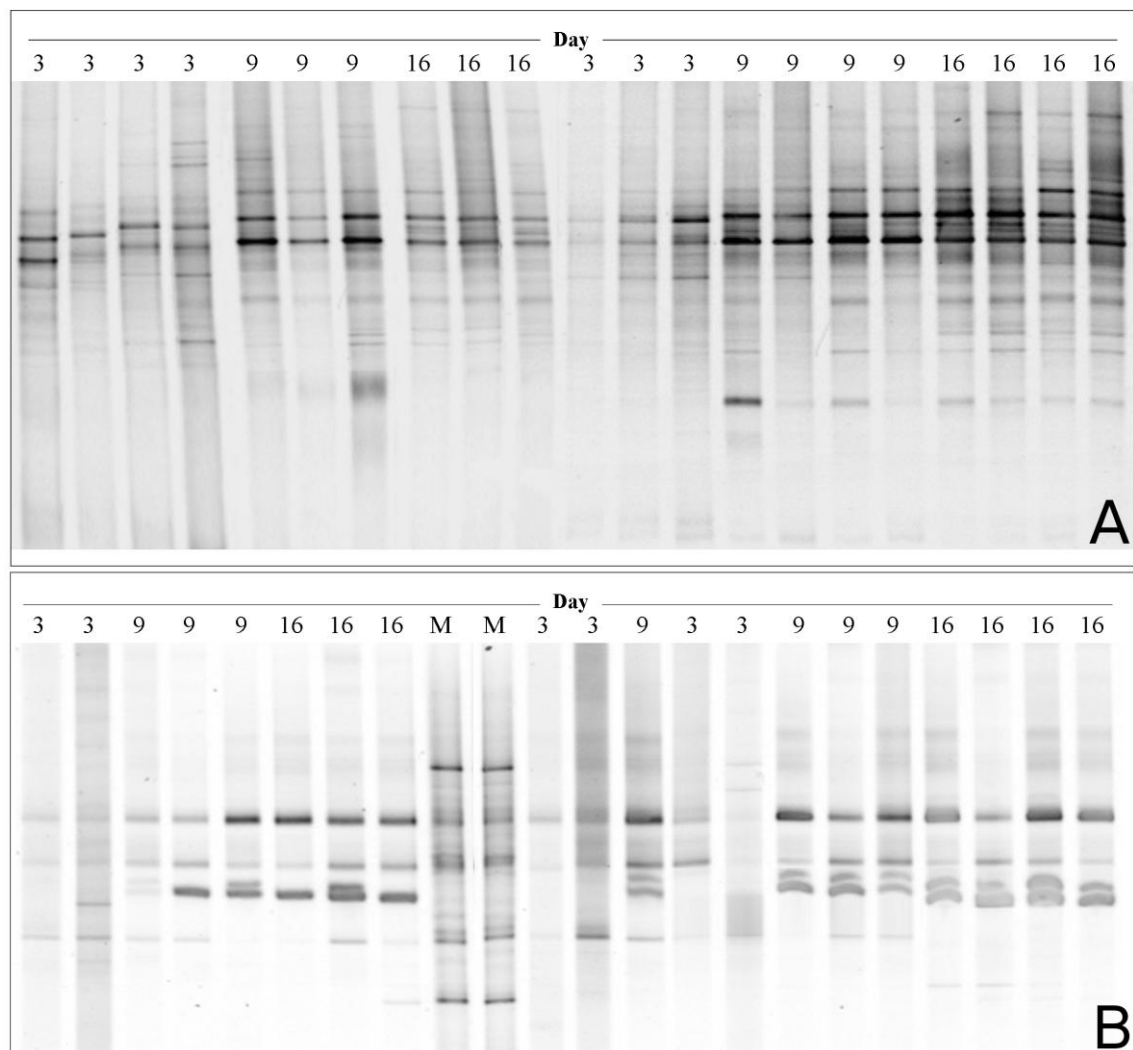
The DNA in all samples were successfully amplified for DGGE analysis, except the 3-day old sample of the 2012 sampling which failed to be amplified by the 18S rRNA gene primers. Thus the dataset had one less replicate in all subsequent analyses. Banding patterns of the DGGE profiles did not change a lot as the biofilms developed, but some differences between different ages could still be observed (Figure 3.9 & 3.10). Differences in band intensity between biofilm ages were observed for some of the bands, and semi-quantitative analysis of the DGGE banding patterns were carried out in subsequent analysis, to determine whether the qualitative and semi-quantitative analyses would arrive at the same conclusion. The DGGE gels from the 2011 samplings showed more bands than the 2012 samplings for both the 16S DNA and 18S DNA amplicons.

For CLSM analysis, biofilms from the 2011 samplings were observed to be thicker than the 2012 samplings, and autofluorescence signals were observed in biofilms of age 9 days or older (Figure 3.12). Results from quantitative analysis using COMSTAT confirmed this observation on thickness. Average thickness, biofilm volume and percentage cover increased with biofilm age while the roughness coefficient decreased with biofilm age (Figure 3.12). The relationships of the microbial community or biofilm morphology profiles with different biofilm ages were visualized using nMDS plots (Figure 3.13). Clear clustering could be observed from the nMDS plots for microbial DGGE profiles of both bacteria (Figure 3.13A) and microeukaryote origins (Figure 3.13B). This suggests that changes in microbial communities occurred as the biofilms developed. Three distinct clusters were found in nMDS plots of 2011 sampling, namely the 3-days, 6-9-12-days, and 16-days clusters. The same pattern was observed for nMDS plots of the CLSM profile (Figure 3.13C). When the sampling was repeated in 2012, observations of 2011 was taken into account, and the number of treatment levels of biofilm age were reduced to three (ie. 3, 6, 9). Similar patterns were observed

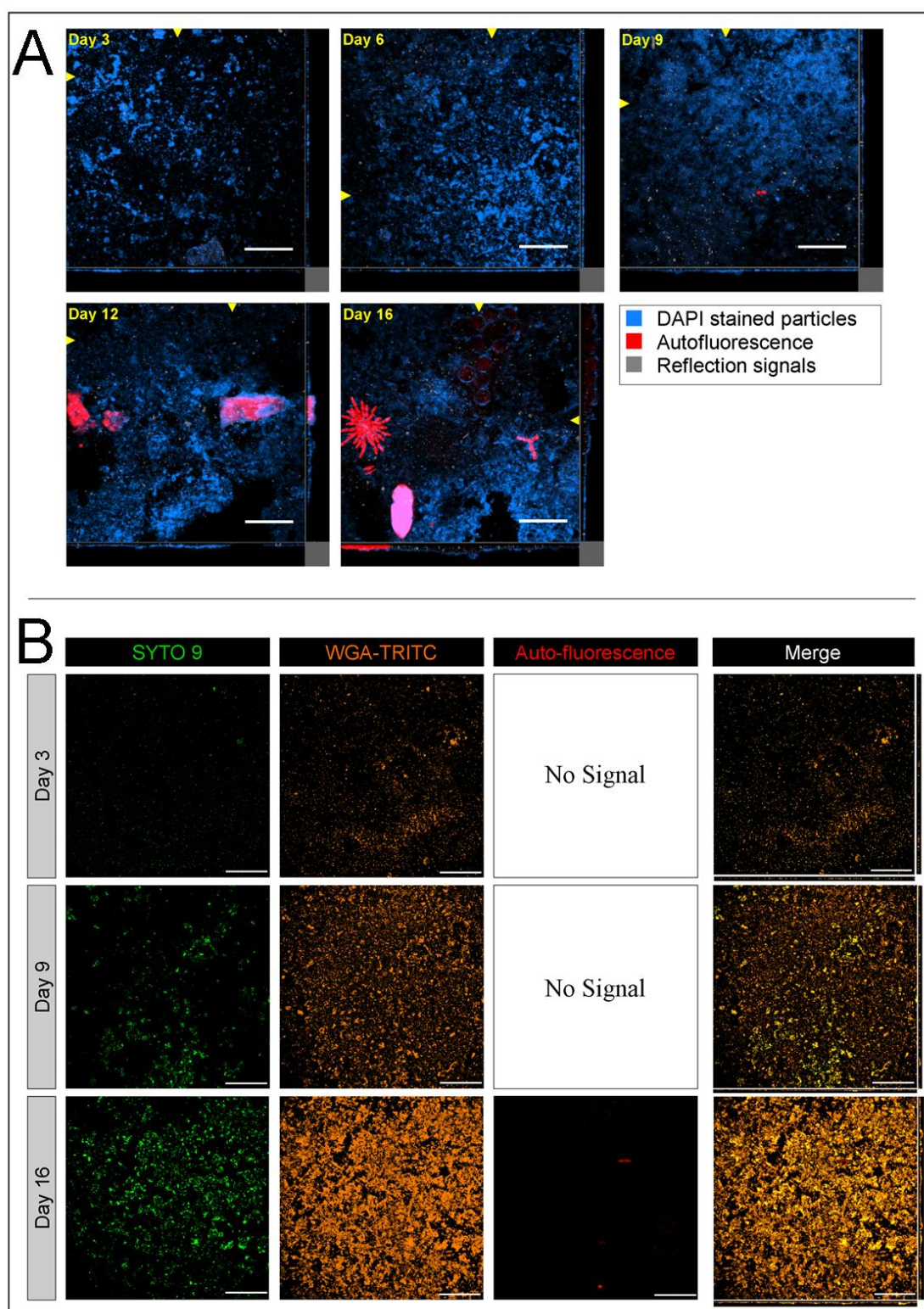


**Figure 3.9:** DGGE profiles of (A) bacterial 16S DNA (B) micro-eukaryotic 18S DNA amplified fragments, for 2011 sampling.

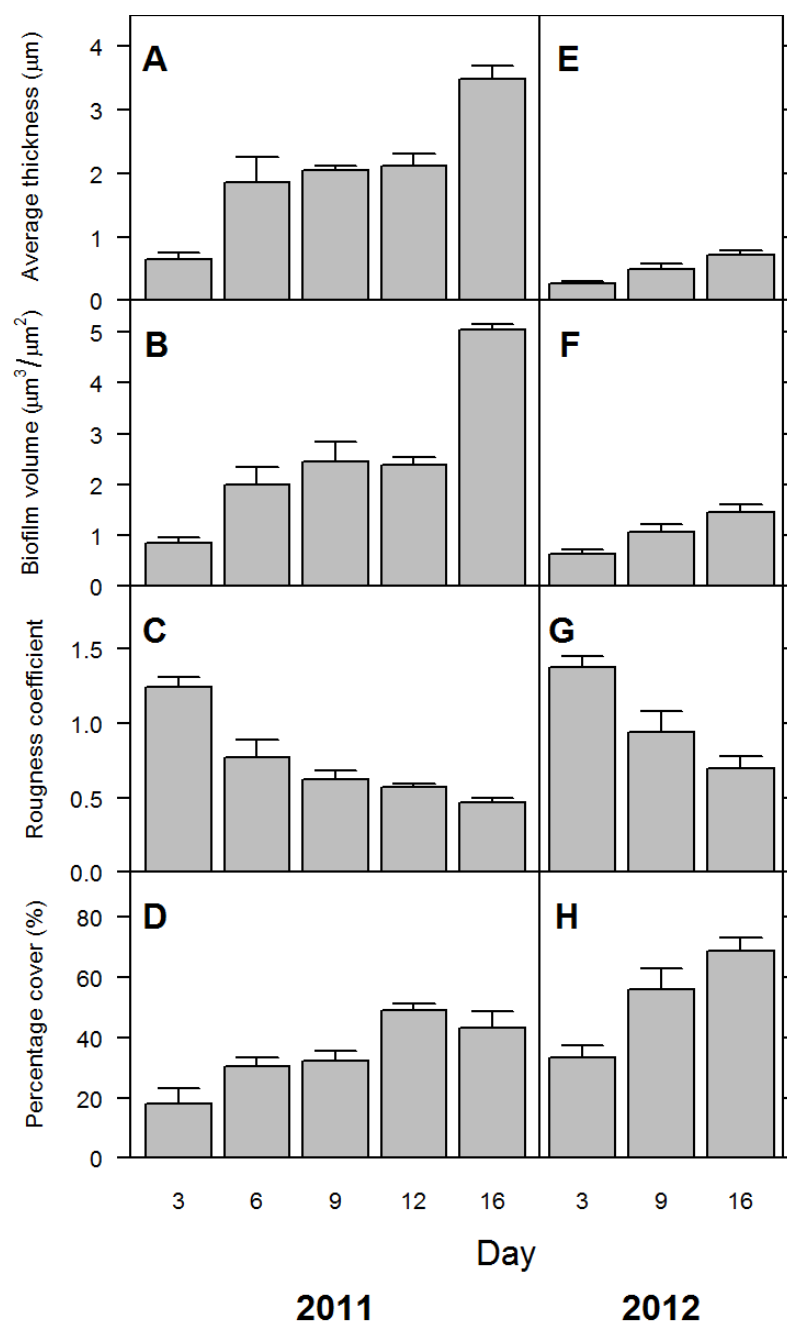
for 2012 sampling for microbial DGGE profiles, but less distinct clustering for CLSM profiles (Figure 3.14). Permutational MANOVA showed that biofilm age had significant effect on the microbial community profiles and morphology profile (Table 3.4).



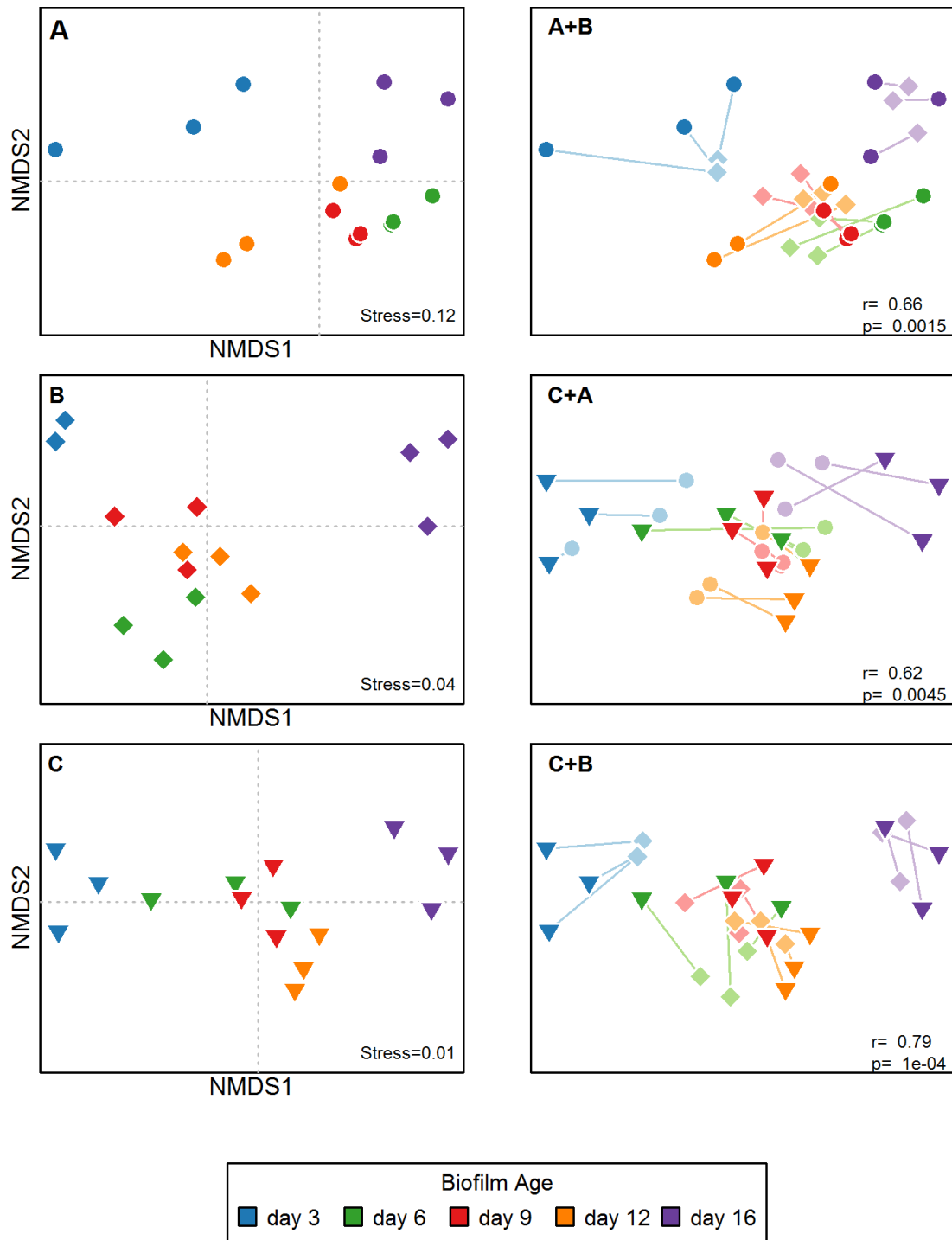
**Figure 3.10:** DGGE profiles of (A) bacterial 16S DNA (B) micro-eukaryotic 18S DNA amplified fragments, for 2012 sampling. M = markers.



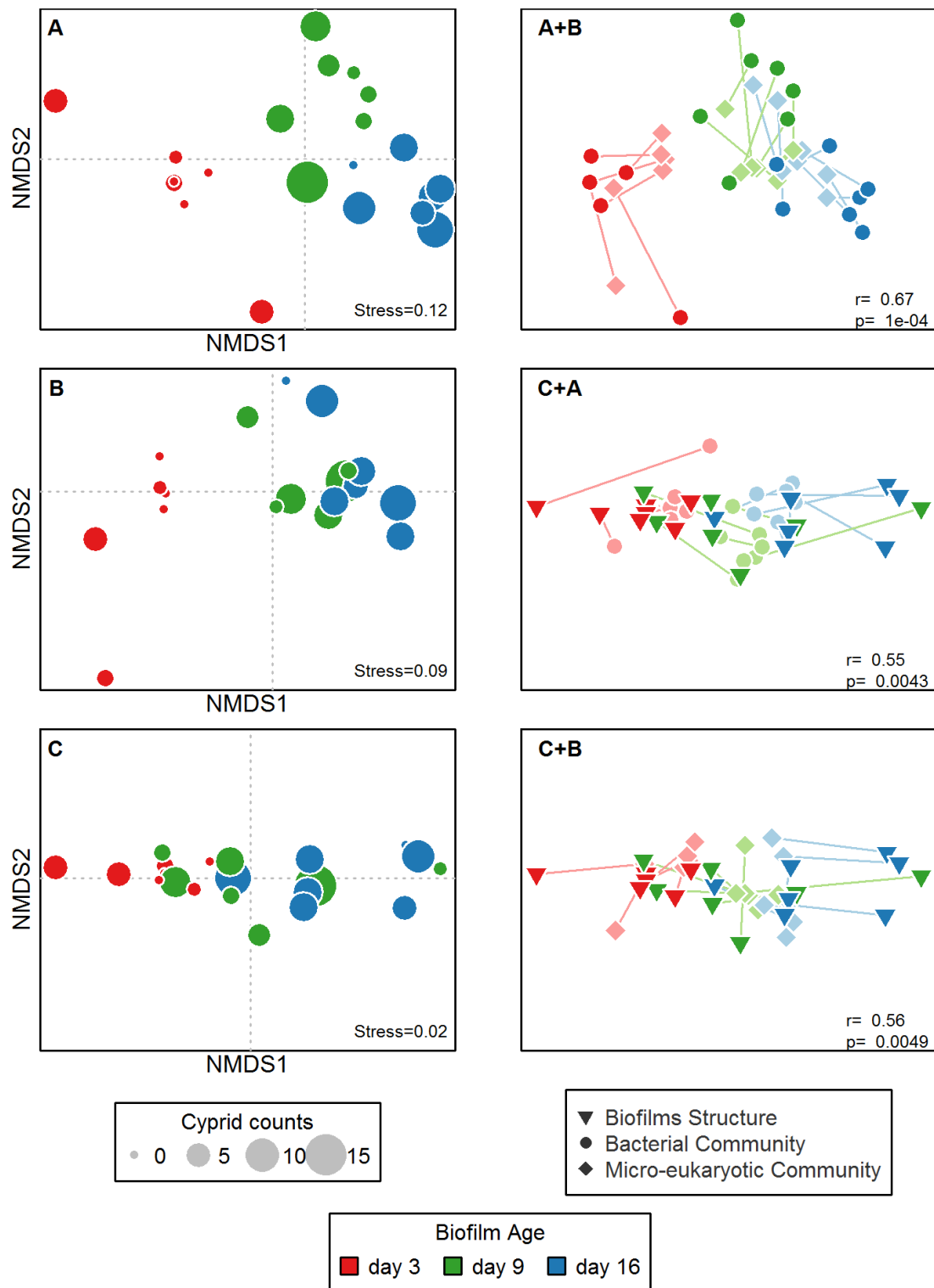
**Figure 3.11:** Maximum intensity projection of CLSM image stacks of biofilms of different ages in (A) 2011 sampling and (B) 2012 sampling. Scale bars are 50µm in length. *xz* and *yz* panels follow the same scale.



**Figure 3.12:** Calculated morphological parameters of biofilms of different ages (mean  $\pm$  SE). (A-D) for 2011 sampling and (E-H) for 2012 sampling.



**Figure 3.13:** nMDS plots showing the relationship between the biofilms of different ages for (A) bacterial DGGE profile, (B) micro-eukaryotic DGGE profile, and (C) morphological CLSM profile, for 2011 sampling (semi-quantitative data). Procrustes superimposition plots showing the pairwise concordance between profiles of (A+B) bacterial DGGE profile and micro-eukaryotic DGGE profile, (C+A) morphological CLSM profile and bacterial DGGE profile, and (C + B) morphological CLSM profile and micro-eukaryotic DGGE profile.



**Figure 3.14:** nMDS plots showing the relationship between the biofilms of different ages for (A) bacterial DGGE profile, (B) micro-eukaryotic DGGE profile, and (C) morphological CLSM profile, for 2012 sampling (semi-quantitative data). Procrustes superimposition plots showing the pairwise concordance between profiles of (A+B) bacterial DGGE profile and micro-eukaryotic DGGE profile, (C+A) morphological CLSM profile and bacterial DGGE profile, and (C + B) morphological CLSM profile and micro-eukaryotic DGGE profile. Numbers of barnacle cyprid (no./experimental unit) displayed as bubbles of different sizes (A to C).

**Table 3.4:** Results of permutational MANOVA model of the effect of biofilm age on microbial communities and biofilm morphology fitted using the distance matrix against biofilm age. Blocking effect was included in the model for 2012 sampling. Tests were performed for both the qualitative and semi-quantitative DGGE data.  $R^2$ = proportion of variation in distances explained by the biofilm age grouping. Diagnostic plots for constant variance are included in Appendix G for interpretation.

	Qualitative			Semi-quantitative		
	<i>F</i>	<i>p</i>	$R^2$	<i>F</i>	<i>p</i>	$R^2$
<i>2011 sampling</i>						
Bacterial community	40.51	<0.001	0.75	4.03	<0.001	0.62
Microeukaryotic community	7.63	<0.001	0.94	15.56	<0.001	0.86
Biofilm morphology	-	-	-	18.37	<0.001	0.88
<i>2012 sampling</i>						
Bacterial community	20.20	<0.001	0.65	11.30	<0.001	0.54
Microeukaryotic community	16.37	<0.001	0.72	11.06	<0.001	0.55
Biofilm morphology	-	-	-	10.91	<0.01	0.54

### 3.3.4 Successional concordance

Successional concordance is a measurement of agreement in compositional changes during succession. Successional concordance was evident in all three pairwise combinations, namely, bacteria community-microeukaryotic community, biofilm morphology-bacteria community, and biofilm morphology-microeukaryotic community. This indicates that during the early development of the biofilm, these components changed with similar pattern. These relationships were visualized using Procrustes superimposition plots (Figure 3.13 & 3.14). The null hypothesis that there is no correlation between any of the pairwise profiles was rejected using permutational tests. Permutational tests were also performed with the qualitative DGGE data. Most of the conclusions did not differ between the analyses using qualitative and semi-quantitative DGGE data (except one case, i.e. Procrustean test for biofilm morphology-bacteria community combination), although there were considerable differences in the strength of the correlations (Table 3.5). Another way to show that qualitative and semi-quantitative DGGE data did not differ from each other was by performing Procrustean analysis between qualitative and semi-quantitative DGGE data. This gave high



correlation coefficients in all cases (all >0.94, see Appendix H). Mantel's test results also showed the same conclusions except one case (biofilm morphology-microeukaryote combination). The null distribution of the test statistics resulting from the permutations using the qualitative and semi-quantitative DGGE data was also very similar to each other in terms of their means and variances, with only two cases where the means were different (Appendix I).

**Table 3.5:** Results of Mantel's test and Procrustean test for both qualitative and semi-quantitative data for all pair-wise comparisons of all three biofilm profiles (ns: not significant, \*:  $p < 0.0167$ , \*\*:  $p < 0.0033$ , \*\*\*:  $p < 0.0003$ ;  $\alpha$  adjusted).

	Mantel's Test				Procrustean Test			
	Qualitative		Semi-quantitative		Qualitative		Semi-quantitative	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
<i>2011 sampling</i>								
Bacteria-microeukaryote	0.46	0.0012 **	0.46	0.0010 **	0.77	0.0001 ***	0.66	0.0015 **
Morphology-bacteria	0.54	0.0002 ***	0.61	<0.0001 ***	0.69	0.0437 ns	0.62	0.0045 *
Morphology-microeukaryote	0.85	<0.0001 ***	0.72	<0.0001 ***	0.71	0.0001 ***	0.79	0.0001 ***
<i>2012 sampling</i>								
Bacteria-microeukaryote	0.57	<0.0001 ***	0.54	0.0001 ***	0.73	0.0001 ***	0.67	0.0001 ***
Morphology-bacteria	0.28	0.0065 *	0.26	0.0063 *	0.55	0.0035 *	0.55	0.0043 *
Morphology-microeukaryote	0.24	0.0168 ns	0.22	0.0349 ns	0.54	0.0096 *	0.56	0.0049 *

### 3.3.5 Correlation between barnacle settlement with DGGE and CLSM profile

Multiple regression fitting of the barnacle settlement numbers against the three biofilm profiles showed that there was a significant linear relationship between the barnacle settlement and the microeukaryotic community, but not for bacterial community and biofilm morphology (Table 3.6). Performing the multiple regression with higher

dimensions ( $k=3$  &  $4$ ) gave the same conclusions (data not shown), with higher  $R^2$  value (e.g.  $R^2=0.66$  for semi-quantitative data of microeukaryotic profile when  $k=4$ ). The results indicate that barnacle settlement was correlated with the composition of microbial eukaryotes of the biofilms. Although multiple correlation has no direction, it is evident from looking at the nMDS plot overlaid with barnacle numbers (Figure 13B) that the increase in number was positively correlated with the changes along the increase of biofilm age. Good agreement of conclusions was found for the results between qualitative and semi-quantitative DGGE data.

**Table 3.6:** Relationship between barnacle cyprid settlement numbers and all three biofilm profiles, assessed by multiple regression and multiple correlation (ns: not significant, \*:  $p<0.05$ , \*\*:  $p<0.01$ ).

	Qualitative			Semi-quantitative		
	Multiple Regression		Multiple correlation $R$	Multiple Regression		Multiple correlation $R$
	$R^2$	$p$		$R^2$	$p$	
Bacterial profile	0.16	0.220 ns	0.39	0.19	0.145 ns	0.44
Microeukaryotic profile	0.49	0.003 **	0.70	0.46	0.005 **	0.67
Morphological profile	-	-	-	0.07	0.501 ns	0.27

### 3.4 DISCUSSION

#### 3.4.1 Preliminary study: microbial community structure of biofilms formed on mangrove seedlings after transplantation

Very early succession of the biofilm on the mangrove seedlings after transplantation was examined, and a weakly significant result of bacterial community changes was detected. Biofilm formation has been shown to be a very quick process and large numbers of bacteria can be adsorbed onto an exposed substrate within 24 hours (Lee *et*

*al.*, 2008; Pohlen *et al.*, 2010). Effect of mangroves transplantation on its associated microbial diversity has attracted some interest recently. Gomes *et al.* (2010) showed that microbial diversity of mangrove rhizosphere differed among specimens from transplanted mangroves, natural mangroves and those in the nursery. However, future studies should consider extending the period of study of successional changes on the mangrove seedlings following transplantation.

#### **3.4.2 Effect of biofilm age on barnacle settlement**

The present study shows that barnacle cyprids prefer to settle on substrates with older biofilms over those without biofilm or with younger biofilms. Many studies have shown the effect of biofilm on barnacle settlement, including facilitative, neutral and inhibitive effects. Appendix J summarized the list of studies on biofilm-barnacle interaction. Besides barnacles, biofilm age had been shown to affect the settlement of other marine invertebrates as well, such as polychaetes (Shikuma & Hadfield, 2005), mussels (Bao *et al.*, 2007), bryozoans (Dobretsov & Qian, 2006), and ascidians (Wieczorek & Todd, 1997).

#### **3.4.3 Effect of biofilm age on microbial communities and morphology of biofilm**

Biofilm age has an effect on the bacterial community of the biofilm, this is in agreement with other studies of similar temporal scale (e.g. Qian *et al.*, 2003; Dobretsov & Qian, 2006; Hung *et al.*, 2008; Besemer *et al.*, 2009; Chung *et al.*, 2010) or of longer time (e.g. Jackson *et al.*, 2001; Martiny *et al.*, 2003) in different natural and artificial settings. Comparing similar studies of marine biofilm only, the differences between young and old biofilms is evident in most studies, although the number of days it took for a distinct bacterial composition to form may differ from study to study. Distinctively different young and old biofilms developed between 3-day and 6-day periods (e.g. Qian *et al.*,

2003; Hung *et al.*, 2008), or between 9-day and 16-day period (e.g. Chung *et al.*, 2010) in meshed cages. Different periods of immersion for the development of biofilm may be the reason; thus, an age measure standardized by immersion time may facilitate better comparisons between studies (Olivier *et al.*, 2000).

Changes of the microeukaryotic community followed a similar pattern as the bacterial community, mainly with a distinction between 3-days and 6-days, or between 12-days and 16-days. There is no study that offers a direct comparison to the present study for microeukaryotic community, but similar successional patterns have been observed in other environments. For instances, Sekar *et al.* (2004) found that the diatom community of the freshwater biofilm followed three distinct phases of 1-4 days, 5-7 days, and 10-15 days; Mihaljević & Pfeiffer (2012) found that colonization of periphyton algae formed three distinct phases of 1-9 days, 12-18 days and 21-42 days; Pfeiffer *et al.* (2013) similarly found distinct phases of 1-3 days, 6-15 days and 18-33 days. For studies using molecular fingerprinting method to study the succession of freshwater biofilm, Szabó *et al.* (2008) found no distinct clusters separated by biofilm age while Fechner *et al.* (2010) showed evidence of seasonal shift of micro-eukaryotic communities.

Changes in biofilm morphology are also affected by biofilm age. In the present study, thickness and volume were found to increase as biofilms aged, similar to findings in other reports in various environments (Neu & Lawrence 1997; Tsurumi & Fusetani, 1998; Mueller *et al.*, 2006). However, the decreasing roughness coefficient with biofilm age was different from the results of Mueller *et al.* (2006).

#### **3.4.4 Successional concordance**

Successional concordance of microbial communities of biofilms was shown in the present study. Concordance between communities from different taxonomic groups is

not an unfamiliar topic in ecological studies, where ecologists are interested in whether different taxonomic groups are distributed in similar ways across spatial and temporal scales, and whether they react similarly to environmental perturbations (e.g. Heino, 2002). There have been some interests in exploring all three domains of microbes (bacteria, archaea and eukaryotes) in the biofilm, for instances, studies of microbial communities' response to environmental gradients such as salinity (Casamayor *et al.*, 2002) and depth (Wilms *et al.*, 2006). Results of these studies showed that these three different components of microbial communities could have either similar or dissimilar changes along the gradients. More recently, study of freshwater biofilms using molecular fingerprinting methods showed that bacterial and microeukaryotic communities had different successional patterns (Szabó *et al.*, 2008; Fechner *et al.*, 2010) but showed similar spatial patterns (Dorigo *et al.*, 2009). However, none of the mentioned studies performed quantitative analysis to show the successional concordance.

Besides concordance between microbial communities, the present study also showed the concordance between biofilm morphology and microbial communities. It is not surprising that the change in the composition of microbial communities could change biofilm morphology, and this has also been observed in other studies (Besemer *et al.*, 2007; Besemer *et al.*, 2009). It is expected that the microeukaryotic community would be more strongly associated with the biofilm morphology than the bacterial community since the former is larger in size and has more diverse morphologies. The results of the 2011 sampling in the present study did show a stronger correlation between the microeukaryotic community and the biofilm morphology, however, the same result was not repeated in the 2012 sampling. This could be due to the lesser number of microbial eukaryotes attached to the substrate in 2012. As shown in the qualitative observation from the CLSM images, autofluorescence signals, mostly from

phototropic algae, were less in 2012 sampling. Besemer *et al.* (2009) also discussed that algae in biofilm could easily change the morphology of biofilm, and hence proposed that they are ecosystem engineers.

From the results of this study, successional concordance among the biofilm's microbial communities and structure is evident, within the timeframe investigated (3-16 days) in this study. The scale-dependent nature of the concordance of biodiversity patterns has been widely reported as reviewed by Reid (1998). The results in this study should be interpreted within the timeframe of 3 days to 2 weeks, and caution should be taken not to make any extrapolations beyond this period. As seen in the results of the preliminary study, successional concordance was not found between bacterial and microeukaryotic communities in the short time frame of 24 hours. The time frame used in this study has been widely used in other biofilm-barnacle interaction studies (e.g. Wieczorek *et al.* 1995; Olivier *et al.*, 2000; Qian *et al.*, 2003; Faimali *et al.*, 2004) and in studies of interaction of biofilm with other fouling organisms (e.g. Shikuma *et al.*, 2005; Chung *et al.*, 2010). Interpretations of concordance between diversity pattern and their associated profiles are scale-dependent too, for instances, Xu *et al.* (2007) showed that concordance between odour profiles and bacterial communities profiles did not show concordance with the full dataset, but when subsets of data sorted by families were used, concordance was evident in some of these subsets.

#### **3.4.5 Implications of successional concordance**

One of the important implications of concordance is that it shows that there are possible interactions among biofilm morphology, bacterial and microeukaryotic communities. As the earliest colonizers of the substrate, it has been long proposed that bacteria colonization may facilitate colonization of microbial eukaryotes (Zobell & Allen, 1935), by creating surface heterogeneity with the initial stochastic colonization of bacteria on a

newly submerged substrate. Indeed, bacteria and their EPS have been shown to be able to affect colonization of diatom, in both positive and negative ways (Gawne *et al.*, 1998). On the other hand, colonization of the microbial eukaryotes could also change the heterogeneity of micro-environment. As suggested by Besemer *et al.* (2009), microbial eukaryotes, which are relatively larger in size, could change the micro-scale hydrodynamics in the local environment of biofilm. This in turn will affect the attachment processes of bacteria and change the composition of the bacterial community. Microbial eukaryotes can alter the composition of bacterial communities, through grazing on bacteria (Parry, 2004).

It has been shown that diatom-associated bacteria differ between species of diatoms (Grossart *et al.*, 2005). Furthermore, it has been shown that EPS produced by diatom can drive changes of bacterial communities in the biofilm (Haynes *et al.*, 2007). There is also evidence that EPS produced by biofilm-related diatoms are utilized by bacteria (Taylor *et al.*, 2013). This interaction is likely to be bidirectional, as presence of bacteria has been shown to increase the EPS secretion by diatoms too (Bruckner *et al.*, 2008). There is much to be learnt about these interactions, and a consequence of the present study is the suggestion that future investigations on biofilm-barnacle interactions should look at the interaction dynamics and ecology of biofilm.

Quantitative analysis of concordance has become a useful tool but is currently under-explored. There are many interesting applications of the tool to examine the correlation between microbial community profiles and other profiles, such as diet profiles (Muegge *et al.*, 2011) and odor profiles (Xu, *et al.*, 2007), which might influence, or influenced by the microbial diversity. These are just some possible applications in microbial ecology, but perhaps the most relevant profile of great interest to biofouling studies is the chemical profile of biofilm. Although studies of the correlation between the microbial community profile and the chemistry profile of the

immediate environment of the microbes are not uncommon (e.g. Marschner *et al.*, 2003), the interest here in biofouling is whether the concordance between bacterial community profile and biochemical profile of biofilm could point to the selective settlement of macrofouling organisms on biofilm.

There were studies attempted to find concordance between bacterial community and chemical profile (Hung *et al.*, 2007; Chung *et al.*, 2010), but the application of quantitative analysis of concordance and inclusion of microbial eukaryotes are still lacking, which could be investigated in future. Since the biochemical profile is mainly affected by the microbial EPS composition of the biofilm, it is reasonable to state that inclusion of microbial eukaryotes in the investigation will help to give a clearer picture since EPS produced by different microbial communities is likely to be different. The biofilm structure where EPS make up the largest portion (Lawrence *et al.*, 1998; Zhang & Fang, 2001) may play an additional role in the larvae's perception of the biochemical signals that emanate from biofilm. For instance, Khandeparker *et al.* (2003) showed that lectins binding sites is important in settlement stimulating effect of biofilm, including the sites bound by WGA-TRITC, the sites used to study the EPS morphology in the present study.

Apart from biochemical profile, investigation of the biophysical profile might give important insights too. This will be an interesting direction in future, as physical properties of surface has been long suggested as important to barnacle settlement preference (Rittschof & Costlow, 1989).

#### **3.4.6 Relationship between barnacle settlement and microbial succession**

The present study found significant relationship between microeukaryotic succession profile and the number of barnacle settlement, but not for bacterial community, which



was quite unexpected. The conclusion came from only one set of experiment (2012 sampling), and more experiments are needed to check if the result is repeatable.

Biofilm bacterial community profile is previously reported to correspond to the settlement of barnacles (Qian *et al.*, 2003; Lau *et al.*, 2005; Thiagarajan *et al.*, 2006; Hung *et al.*, 2007; Hung *et al.*, 2008). However, conclusions from these studies were from direct qualitative comparison, and no information on the strength of the association was given due to the lack of quantitative analysis. For quantitative analysis, regression of larvae settlement numbers on nMDS scores of bacterial community pattern has recently been used to assess the relationship between bacterial community and larval settlement of oyster (Campbell *et al.*, 2011). Similar methods have been used in other studies of ecology where the community structure was used to predict the variables of interest (e.g. Hollingsworth *et al.*, 2008). Apart from the conventional multiple regression model used in the present study, other variants, such as the permutational-based or distance-based multiple regression models (Cuadras & Arenas, 1990) could be used in place. Perhaps these variants are better as some of the data in the present study did not fit very well with the current model, especially for qualitative DGGE data.

For correlation between biofilm structure and barnacle settlement, the closest previous study was that from Tsurumi & Fusetani (1998), in which the relationship between biofilm volume and barnacle settlement was investigated. Similar to the result of the present study, linear correlation was not found to be significant, but through repeated experiments, they have found that optimal settlement always corresponded to biofilms of certain range of volume ( $0.1-1 \mu\text{m}^3 \mu\text{m}^{-2}$ ).

The result of the present study suggests that succession of microbial eukaryotes may be important in affecting barnacle settlement. In the study by Chen *et al.* (2007), biofilms were developed using seawater filtered by sieves of different mesh sizes, and

*Fistulobalanus albicostatus* was found to preferentially settle on biofilmed substrate cultured with seawater filtered by all sizes in young biofilms. However, barnacle settlement was diminished on biofilms cultured in seawater filtered with larger mesh sizes in older biofilms. This suggests that the succession of microbial eukaryotes in the biofilm, which are of larger sizes than bacteria, is likely to influence the settlement of *F. albicostatus* in a negative way. Microbial eukaryotes that could be found in biofilms include diatoms, fungi and protozoans. Diatoms usually dominate the microeukaryote component of biofilms since they quickly colonize substrates as compared to fungi or protozoans (Faimali *et al.*, 2004).

The effect of microbial eukaryotes on settlement of barnacles or larvae of other marine invertebrates may be direct or indirect. The indirect effect could come from the interaction between bacterial and microeukaryotic communities. For instances, recently it has been shown that effect of bacterial biofilm on larval settlement of different species of marine invertebrate was affected by the co-cultivation of ciliates (Shimeta *et al.*, 2012). On the other hand, Jouuchi *et al.* (2007) showed that the effect of biofilm-associated diatom on the barnacle settlement was affected by co-cultivation of bacteria. This emphasizes the importance of understanding interactions among and within the microbial communities in biofilm-barnacle study.

The present study is basically an observational study despite all the experimental manipulations in the field. It can not provide strong evidence for a causal relationship. To elucidate the causal relationship between the microbial communities and barnacle settlement, strong experimental evidence will still be needed. For instance, an approach similar to that of Shimeta *et al.* (2012) can be used in future studies. But such experiments are often possible only by using laboratory-cultured biofilm and performing settlement assay in the laboratory, which may not represent the on-site

situation. With the many difficulties to overcome and the limitations of different approaches, it is best to test the hypothesis using multiple approaches.

#### **3.4.7 Problems of study**

Mangrove seedlings were initially planned to be used as the experimental substrate for biofilm development studies. Preliminary experiment (3.2.2.1) was carried out to test the methodology of PCR-DGGE using mangrove seedlings. However, it was difficult to experimentally manipulate the seedling's surface and caging the seedlings to prevent entry of barnacle cyprids. These problems discouraged the use of mangrove seedlings as substrate for subsequent biofilm and barnacle settlement experiments.

The major problem for settlement experiments was natural larval supply which was unpredictable. Besides the 2011 and 2012 sampling, there were several intervening experiments that between the 2011 and 2012 samplings, had all shown no settlement of cyprids due to the shortfall of larval supply. Thus the effect of biofilm age on cyprid settlement could not be verified. This represents one of the limitations of the field experiment, as larval supply cannot be manipulated. The same problem had also been encountered by other field study (e.g. Keough & Raimondi, 1995). On another note, although there could be seasonal or temporal effects between the 2011 and 2012 samplings, due to the NE monsoon and SW monsoon respectively, this was not the purpose of the experiments. Nevertheless, both sampling periods marked the period of heavy rainfall.

Another potential problem in the settlement experiment was the possible confounding effect of experimental manipulations. The interpretation of data from the present study considers the effect of wire mesh as the biofilms developed under the cover of wire mesh. The effect of wire mesh on water flow was checked using the clod card method, and was found to reduce the water flow significantly (see Appendix D).

Hydrodynamics could affect the microbial communities and morphology of biofilm (Besemer *et al.*, 2007), thus the result obtained in the present study might not represent that of the natural biofilm formed in the absence of meshed cage. Nonetheless, the focus of the present study required mesh treatment, and Keough & Raimondi (1995) reported that the mesh did not affect the conclusion of their biofilm-barnacle interaction experiment.

For data analysis, earlier studies had criticized the use of DGGE data in quantitative analysis as interpretations could be problematic due to multiple copies of rRNA genes and differential amplification of rRNA genes (Reysenbach *et al.*, 1992; Suzuki & Giovannoni, 1996). Beside these problems, there is possibility of presence of multi-cellular micro-eukaryotes, which would further complicate interpretation and hence, caution should be given to the semi-quantitative analysis of the micro-eukaryote DGGE profiles (van Hannen *et al.*, 1999). The present study used both qualitative and semi-quantitative data for statistical analysis of DGGE profiles (Xu *et al.*, 2007).

### **3.4.8 Conclusion**

The present study shows that biofilm age affects bacterial and microeukaryotic communities, and morphology of biofilms. It has also been shown that bacteria and microeukaryotic communities, and morphology of biofilms had changed following a similar pattern, i.e. they show a concordant succession. There is a positive effect of biofilm age on the settlement of barnacles. Furthermore, the numbers of barnacle settled on the biofilmed surface are correlated with the succession of the biofilm's microeukaryotic community.

## CHAPTER 4

### EFFECTS OF MANGROVE SNAILS (LITTORINIDS) ON DEVELOPMENT OF BIOFILM AND RECRUITMENT OF BARNACLES

#### 4.1 INTRODUCTION

Natural mangrove trees in adjacent areas of mangrove re-planting sites have been observed to have less biofouling problem than the re-planted mangrove seedlings and artificial substrates. This observation motivates the present interest to examine why the natural mangrove trees seem less prone to biofouling. Two possible reasons are suspected.

First, natural mature mangrove trees could have developed a defense mechanism against biofouling, which newly transplanted seedlings may not have developed yet once transplanted into a new and stressful environment. One possible defense mechanism includes the release of natural anti-fouling (AF) compounds. Natural AF compounds is a subject that has attracted a lot of interests in recent years, and many AF compounds have been discovered from various sources of organisms including plants (reviewed by Qian *et al.*, 2010). Indeed, in recent years there has been a growing interest in discovering potential AF compounds from mangrove plants and mangrove associated organisms (Chen *et al.*, 2008; Manilal *et al.*, 2009; Wang *et al.*, 2009; Prabhakaran *et al.*, 2012; Gopikrishnan *et al.*, 2013). Some mangrove extracts, even molecules, have already been identified as potential AF candidates.

Second, mature mangrove trees harbour gastropod predators and grazers that could exert a 'top-down' effect on biofouling organisms. Gastropod predators of barnacles have been known to have effects on barnacle populations on mangrove (Ellison & Farnsworth, 1992). Grazing pressure by gastropods has also been recognized

as an important factor controlling both the spatial and temporal distribution of barnacles in intertidal shores. While the effect of predators is direct (i.e. by ingestion of barnacles) and not related to biofilm, the effect of grazing (on the biofilm) on barnacle biofouling is indirect. Effects of herbivorous grazers such as littorinids and limpets on the recruitment of barnacle and/or microalgae have been extensively studied in the rocky shores (Denley & Underwood, 1979; Miller & Carefoot, 1989; Williams, 1994; Mak & Williams, 1999; Hutchinson & Williams, 2001; Chan & Williams, 2003; Holmes *et al.*, 2005; Hidalgo *et al.*, 2008). Littorinid gastropods (subfamily Littorininae) are commonly found in intertidal habitats including mangroves (Reid, 1986; Lee & Williams, 2002; Torres *et al.*, 2008; Printragoon *et al.*, 2008). These mangrove snails, also commonly known as periwinkles, are the few ‘true mangrove associates’ that use mangrove trees as their substrates (Reid & Williams, 2010). Despite the fact that both littorinids and barnacles are important ‘true’ members of the mangrove fauna diversity, their interactions have not been reported.

Preliminary survey showed that naturally grown mangrove trees (*Avicennia* sp.) at the shores of the present study sites had abundant littorinids inhabiting them (Figure 4.1), which was not observed on the replanted mangroves. It is hypothesized that littorinid grazing on the mangrove tree has a primary effect on hindering the recruitment of barnacles on natural mangrove trees. The hypothesis is based on the rationale that since barnacle settlement is linked to biofilm formation, any agent that disrupts biofilm development as for example by snails would discourage barnacle settlement. However, large numbers or heavy concentration of grazing snails may have a direct dislodgement effect on newly settled cypris larvae. Hence, this study also investigated the movements and behaviour of littorinids on the mangrove to provide further insights on their grazing activity.

#### 4.11 Objectives

- (i) To establish the littorinid grazing effect on recruitment of barnacles and development of biofilm and,
- (ii) To find out why littorinid snails did not colonize the newly replanted mangroves by studying the behaviour of these snails.



**Figure 4.1:** Abundant littorinids (*Littoraria scabra* and *Littoraria melanostoma*) on the natural mangrove trees (*Avicennia* sp.).

## 4.2 MATERIALS AND METHODS

### 4.2.1 Study site

All experiments in this study were conducted at Kampung Sungai Limau, Selangor, Malaysia (see Figure 3.1 in Chapter 3; Site 2). This site is an exposed intertidal mudflat with small patches of mangrove plants. *Avicennia* trees were found at the lower shore and *Rhizophora* trees on the higher shore. All experiments in this study were performed on the *Avicennia* plants or the snails collected from them.

#### **4.2.2 Preliminary study**

Prior to designing the actual experiment, a preliminary study was first carried out to survey the extent of mangrove tree differences in terms of trunk diameter. This was to ensure that the subsequent experiments could be designed with minimal effect of heterogeneity due to the size differences of the trees (and any associated variability). About 40 *Avicennia* trees were measured. The perimeter of each tree trunk at 0.9m height (they were not tall enough to be measured at breast height, the commonly used unit) was measured and converted into diameter. The number of littorinid snails inhabiting each tree was counted for some of these trees, when the tree was partially inundated by sea water to check on the relationship between tree diameter and number of littorinids. Shell length of each littorinid snail was measured too.

#### **4.2.3 Exclusion experiment I**

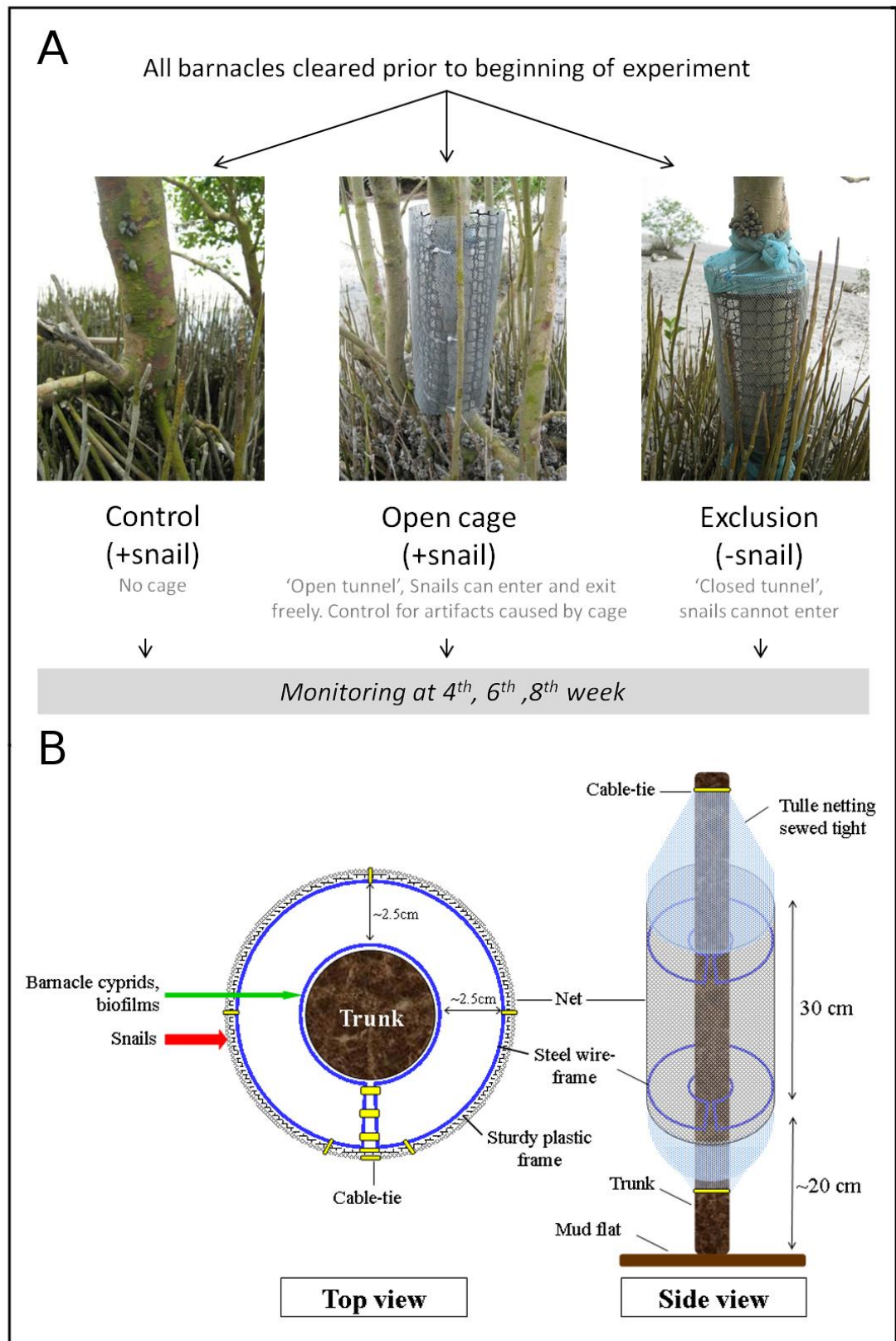
To conduct manipulative experiments on the mangrove snails, three levels of treatment were used: (i) Exclusion or close cage, the snails were excluded by using a custom-made cage of 30cm height, wrapped around the tree trunk and covered with fine nettings of 2mm x 3mm mesh size to prevent the entry of snails into the cage but not barnacle cyprids; (ii) Open cage, a procedural control using the same cage design described above, except that the top and bottom of the cage were not covered with nettings to allow the free passage of snails through it as well as unimpeded barnacle settlement; this treatment also replicated the possible confounding effect of reduced wave effect as in treatment (i); (iii) Control, no cage, snail movement, barnacle settlement and wave action were unimpeded (see Figure 4.2 for graphical explanations). All treatment trees were in the same monitoring site at a height of ~190cm above chart datum. Area of mangrove trunk under experimental manipulation was at the height of ~210cm above C.D.



Randomised complete block design was used in this experiment. Blocking was used to partition any possible confounding effects due to environmental heterogeneity. Three adjacent trees formed a block. All treatment levels were randomly assigned to each block. The vertical distance between the cage bottom and mud bottom was ~20cm, but it varied by  $\pm 5$ cm among blocks due to root obstructions. Nevertheless, this vertical distance was strictly standardized within each block. Out of the possible blocks along a transect of ~110m distance along the shore ( E 100°59'58.51" N 3°39'7.36" to E 100°59'55.59" N 3°39'9.22"), nine blocks were chosen to achieve minimal differences in tree perimeters (measured in preliminary study) among treatments ( $F_{2,8} = 0.061$ ,  $p = 0.94$ ; see Appendix K for the tree perimeter measurements and the assignments of treatment/ blocking levels).

All barnacles (< 30 individuals of both *A. amphitrite* and *E. withersi* per tree) were removed prior to the beginning of the experiment. The numbers of barnacle recruits were counted at 4<sup>th</sup>, 6<sup>th</sup> and 8<sup>th</sup> week of the experiment. As the numbers of recruits were very high starting from 6<sup>th</sup> week, subsamplings of barnacles instead of total enumeration of barnacles per tree were made. These were achieved by sampling the number of barnacles inside a 5cm  $\times$  5cm plastic quadrat square haphazardly placed on the tree trunk. A photo image of the quadrat with the barnacles in it was taken before all barnacles were counted. The number of barnacle recruits was standardized to individuals/ 50 cm<sup>2</sup>, instead of expressing as individual/ tree following the initial design where tree was the experimental unit.

Damage to cages/ breakage of nets was also checked at each sampling occasion, if the integrity of any cage was found to be compromised, the whole block where the unit belonged to was excluded from the experiment. This experiment was carried out from 3<sup>rd</sup> Sep 2011 (begin) to 22 Oct 2011 (8<sup>th</sup> week, end).



**Figure 4.2:** (A) Experimental design for Exclusion experiment I. (B) Design of the exclusion cage.

#### **4.2.4 Exclusion experiment II**

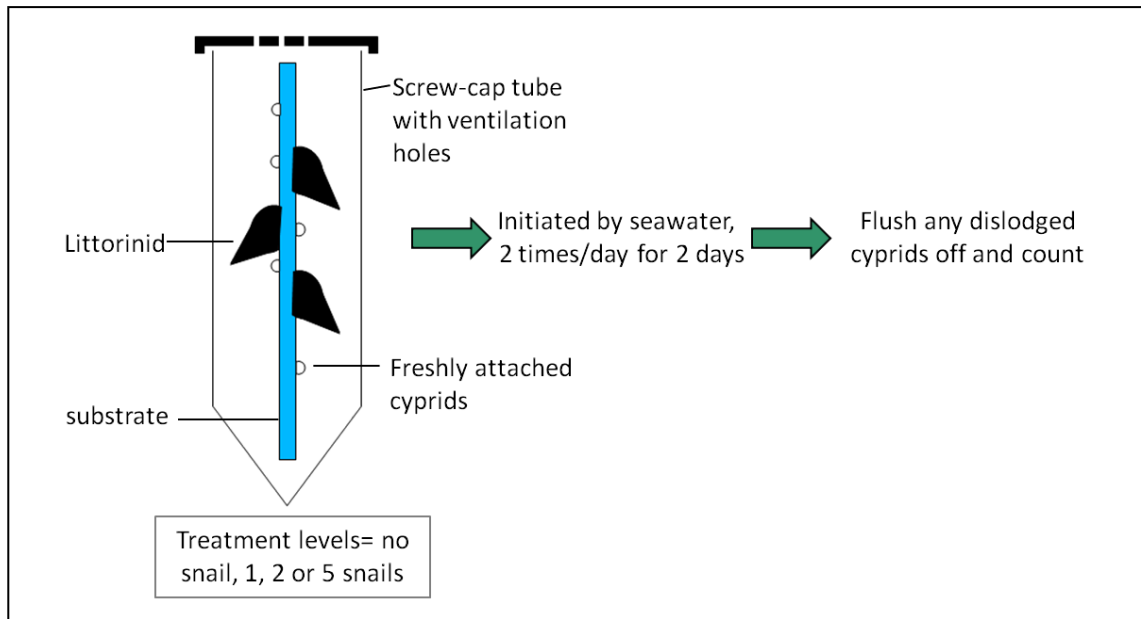
The second experiment investigated whether the same effect of snails on barnacles (in Experiment I) could be observed for biofilm. Using the same experimental setup of the first experiment, soft PVC plastic sheets (10cm in height) were wrapped around the tree trunk as the substrate for biofilm development and barnacle settlement. Sampling was carried out when barnacle cyprids were observed on the substrate, which was on the 11<sup>th</sup> day (date of experiment: 18 - 29 December 2011) from the beginning of the experiment.

Apart from cage and net damages, some snails were found trapped in the crevices between trunk and plastic sheet due to protruding surface of some trunks. If this happened, such units and the block they belonged to were excluded from the experiment. Plastic sheets were sampled at the end of the experiment, and all barnacle cyprids and newly metamorphosed juveniles attached on the plastic sheets were counted, excluding those at the edges to avoid edge effects. Alcian Blue was used to stain the EPS (extracellular polymeric substances) of the biofilms (Hiraki *et al.*, 2009).

The plastic sheets were stained with 0.2µm pre-filtered solution of 0.1% (w/v) Alcian Blue (pH adjusted to 2) for 5 minutes, rinsed, and photographed. Negative control of plastic sheet without deployment in the field was included in the analysis. The percentage cover by biofilm was then determined by using image analysis software, ImageJ. Photographing conditions and image processing parameters such as thresholding value were strictly standardized among images (see Appendix L for original images and images overlaid with thresholded selection).

#### **4.2.5 Bulldozing experiment**

To examine the 'direct' effect from littorinids on the barnacle cyprids by dislodging the cyprids when the snails move (the 'bulldozing' effect), a laboratory experiment was carried out. Littorinids were collected from the study site (site 2). Glass microscope



**Figure 4.3:** Experimental design for bulldozing experiment.

slides were deployed in the field fixed with custom made casings and stands, and retrieved when freshly attached cyprids were observed. The experimental unit in this experiment was the glass microscope slide. Number of cyprids on each slide was not manipulated, thus each slide had different numbers (median=5, min=3, max=9). Mean number of cyprids in each treatments before the experiment did not significantly differ from each other (ANOVA;  $F_{3,32} = 0.235$ ,  $p = 0.87$ ). To manipulate the effect of littorinids, four levels of treatment were used: Control (no snails), one littorinid, two littorinids, and five littorinids, using a completely randomised design. Littorinids were added into screw-cap tubes (with ventilation hole) containing one slide in each of them, and left for 2 days. Seawater was added to initiate the movement of the littorinids, two times a day. This experiment was performed from 9 to 11 July 2011. At the end of the experiment, the slides were flushed with seawater to remove any possible dislodged cyprids, and the remaining attached cyprids were counted. See Figure 4.3 for graphical explanations.

#### 4.2.6 Snail behaviour

This study was performed on 21 Feb 2012 at the study site (site 2). Observation of the mangrove snails behavior was done on two chosen neighbouring *Avicennia* mangrove trees of ~5m apart, and henceforth referred to as Tree A and Tree B. Poles with measuring tape (accuracy  $\pm 0.5\text{cm}$ ) were set up as reference gauge for measurements of tide levels and vertical levels of snails above ground. Heights above ground were later expressed as heights above the Chart Datum (C.D.), calculated based on the tidal levels above CD at the nearest secondary port, Bagan Datuk (Tide table, 2012, National Hydrographic Centre).

Two digital cameras (Canon Powershot G12 & S95 models) were fixed on custom made stands, and photos were taken at intervals of approx. 6 min (median; min=3min, max=16min) intervals over flood and ebb tides of daytime. Although the fixed cameras provided only partial view of a tree trunk from a single direction, the captured photos had the advantage of easier comparison and more objective error of measurements compared to holding the cameras and taking the pictures from different angles. Thus, this photographic setup for studying the snail behavior was decided as a better choice given also the limitations of the number of cameras available for the study.

Measurements of snail vertical heights were done based on the photos including the vertical gauge. Snail orientations were also measured for a subset of photos (Tree B, at larger time intervals) to investigate the collective behavior exhibited by the snails. Orientations were measured from the shell apex to the tip of the aperture (shell axis). The orientations measured were not necessary the exact direction which the snails were heading but this is good enough to measure the uniformity of the orientations, which was of interest in this study. However, since the tree trunk surface was not flat, snails at the edge of the trunk or those whose orientation cannot be determined confidently were

not included in the measurement. A total of  $48.3 \pm 0.11\%$  (mean  $\pm$  SD) of observations were removed.

In addition to field observations, a simple laboratory experiment to observe the littorinid snails collected from the field was carried out. Ninety snails were randomly put on a glass Petri dish and their behavior was captured on a video camera (Canon S95 model). Frames from the video were then analysed using ImageJ, taking digital measurements of their positions and directions. Snails that moved to the edge of the petri dish, or those which could not be measured confidently were excluded from the analysis. A total of  $8.5 \pm 6.2\%$  (mean  $\pm$  SD) of observations were removed. Only a very short period (200s) was analysed as there was not much change observed afterwards.

#### 4.2.7 Statistical analyses

##### 4.2.7.1 Exclusion experiment I

Repeated-measures ANOVA was used to test the null hypothesis that the treatments (exclusion) have no effect on the recruitment of barnacles. Subjects that were repeatedly measured over time in this experiment were the mangrove trees, which were the substrates that were monitored. The model used was,

$$Y_{ijkl} = \mu + \alpha_i + \gamma_{j(i)} + \tau_k + (\alpha\tau)_{ik} + (\gamma\tau)_{j(i)k} + \beta_l + \varepsilon_{ijkl}$$

where  $Y_{ijkl}$  is the Barnacle counts,  $\mu$  is the grand mean,  $\alpha_i$  is the Treatment effect,  $\gamma_{j(i)}$  is the Subject effect (tree) nested within Treatment levels,  $\tau_k$  is the Time effect (week),  $(\alpha\tau)_{ik}$  is the interaction between Treatment and Time,  $(\gamma\tau)_{j(i)k}$  is the interaction between Subject (nested within Treatment) and Time,  $\beta_l$  is the Block effect,  $\varepsilon_{ijkl}$  = error term, and  $i = 1, 2, 3; j = 1, 2, \dots, 18; k = 1, 2, 3; l = 1, 2, \dots, 6$ .

The model did not meet the assumption of homogeneity of variance (checked with Fligner–Killeen test), and since common transformations such as square-root and logarithmic transformations were tried and proven ineffective, Box-Cox transformation

(Box & Cox, 1964) was used. Violation of assumptions of ANOVA model is common in ecological experiments, and application of Box-Cox transformation has been suggested as one of the methods to improve the fulfilment of required assumptions when other common transformations fail (Peltier *et al.*, 1998). Because inverse power was used for the transformation, the sign for the response variable was reversed and a constant of 1 was added in the final ANOVA model to bring the values back to positive (Osborne, 2002).

#### 4.2.7.2 Exclusion experiment II

Multivariate analysis of variance (MANOVA) was first performed to test the null hypothesis that the treatments (exclusion) do not have any effect on the recruitment process. The model used was,

$$(Y_{1ij} \ Y_{2ij})^T = \mu + \alpha_i + \beta_j + \varepsilon_{ij}$$

where  $Y_{1ij}$  is the biofilm cover,  $Y_{2ij}$  is the barnacle settlement numbers,  $T$  indicate transposed vector,  $\mu$  is the grand mean,  $\alpha_i$  is the treatment effect,  $\beta_j$  is the blocking effect,  $\varepsilon_{ij}$  is the error term, and  $i = 1, 2, 3$ ;  $j = 1, 2, \dots, 5$ .

Follow-up one-way ANOVA models were then used to show the treatment effects on each response variables, i.e. biofilm cover and barnacle numbers. Arcsine transformation and square-root transformation were performed on biofilm cover and barnacle recruits, respectively, to satisfy the assumption of homogeneity of variance across treatments. Finally, Spearman's rank correlation test was performed to check the correlation between biofilm cover and barnacle recruits.

#### 4.2.7.2 Bulldozing experiment

The number of cyprids successfully detached (= before – after) and number of cyprids that failed to be detached (= before – success) at the end of experiment were fitted

against treatment levels using the logistic model, where binomial distribution was used for errors, and logit specified as link function. Effect of differences in number of attached cyprids on each slide, which was not manipulated experimentally in this study, was adjusted by the logistic model by putting weights on them (Crawley, 2007). Due to overdispersion of the data, a quasi-binomial distribution was used to replace the binomial distribution as an adjustment to the problem (Crawley, 2007). Analysis of deviance (Crawley, 2007) was then carried out to test the null hypothesis that grazing pressure does not affect the detachment of attached cyprids (testing the constructed logistic model against null model).

#### **4.2.7.4 Snail behaviour**

The recorded tide levels were ‘smoothed’ by local regression (LOESS) fitting. This was because rigorous wave correction measures were not taken in the study, so smoothing was used as a method to remove the noise of the data caused by waves. Fitted values of smoothed tide levels were used in subsequent analyses. To show the correlation between tide levels and snail vertical levels, Kendall’s *tau-b* rank correlation test was performed. Kendall’s *tau-b* method was chosen over Spearman’s test here because of the presence of ties (identical values) in the tide levels (Kendall, 1945). Distances between the snail vertical levels and the tide levels during flood and ebb tides were compared using histograms. For easier visualization of comparisons, outliers (defined as  $< 1^{\text{st}} \text{ quartile} - 1.5 \text{ inter-quartile range}$  or  $> 3^{\text{rd}} \text{ quartile} + 1.5 \text{ inter-quartile range}$ ) were removed in the histograms. Permutational Kolmogorov-Smirnov test (permutational test was used to avoid the problem caused by ties) was used to test the null hypothesis that the distribution of the distance between snail and tide levels do not differ between flood and ebb tides, using the original data without removal of outliers.



To analyse the distribution of the snails' directions, sample mean resultant length  $R$  was used as a measure for concentration towards any direction, and Rayleigh's test of uniformity was performed to test the significance of  $R$ , i.e. testing the null hypothesis that the snail directions were randomly distributed (Jammalamadaka & SenGupta, 2001). When there is a high concentration,  $R$  will approach 1, while uniformly distributed directions will give a value close to 0. For laboratory observation, aggregation index  $R$  for spatial point pattern (Clark & Evans, 1954) was used as an indication of ordering/ aggregation, and a significance test was performed to test the null hypothesis that the snails exhibited complete spatial randomness (CSR). A value of  $R > 1$  suggests ordering,  $R = 1$  suggests random pattern and  $R < 1$  suggests aggregation. Bonferroni adjustment was used to adjust the significance level of both Rayleigh's test and CSR test.

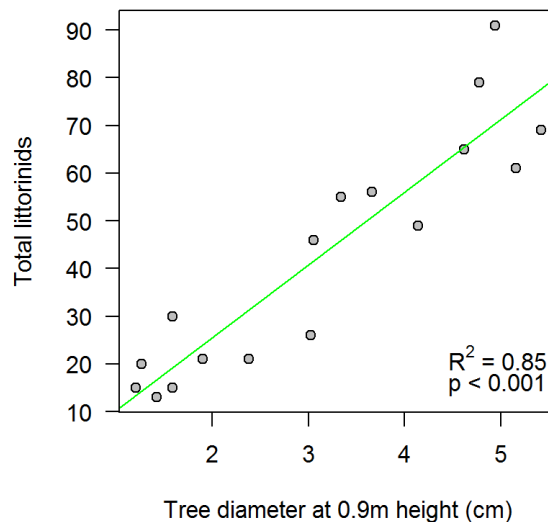
#### **4.2.7.5 Statistical packages**

For Box-Cox transformation, the power ( $\lambda$ ) required for the transformation was estimated using function from 'car' package for R (Fox & Weisberg, 2011). Other packages used include 'spatstat' for spatial statistics (Baddeley & Turner, 2005), i.e. to calculate the aggregation index and plotting spatial points pattern, and 'circular' for circular statistics (Agostinelli & Lund, 2011), i.e. to calculate mean resultant length and plotting rose diagrams. Permutational Kolmogorov-Smirnov test was performed following the method developed in the book by Rizzo (2008). Significance ( $\alpha$ ) level of all statistical tests was set at 0.05 unless otherwise stated.

### 4.3 RESULTS

#### 4.3.1 Preliminary study: relationship between tree diameter and littorinids number

Significant linear relationship ( $p < 0.001$ ,  $n = 17$ ) was found between the mangrove tree diameter and the total number of littorinids that inhabited them, with a coefficient of determination ( $R^2$ ) of 0.85 (Figure 4.4). The mean shell length of *Littoraria scabra* at the study site was measured to be  $0.94 \pm 0.19$  cm (mean  $\pm$  SD;  $n = 50$ ).

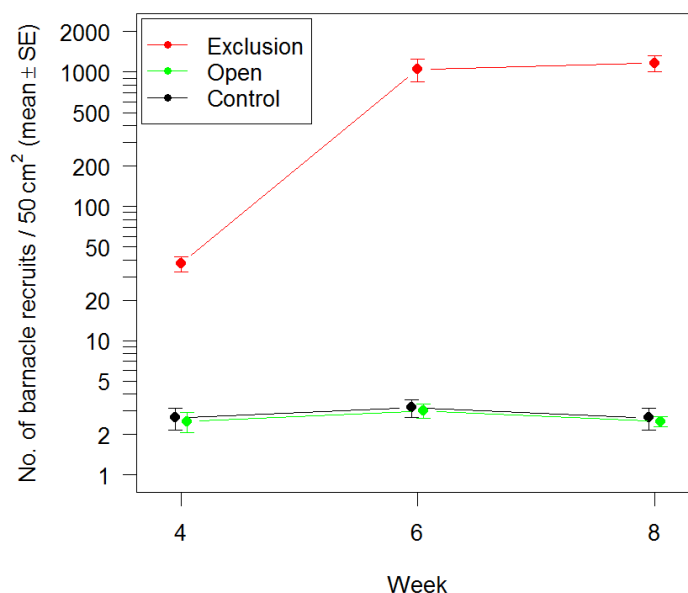


**Figure 4.4:** Relationship between diameter of *Avicennia* sp. and number of littorinids found on them.

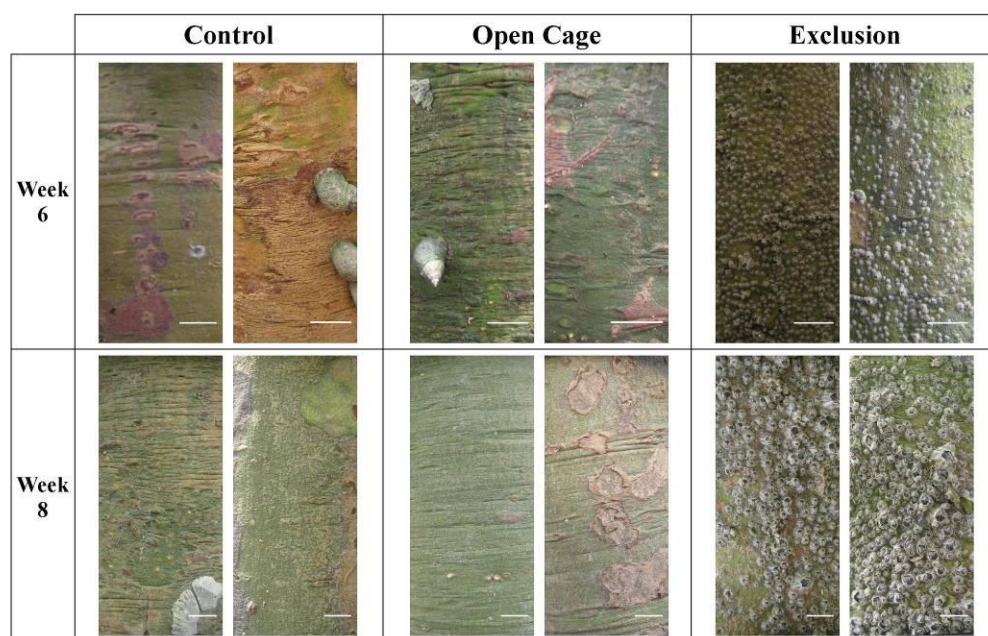
#### 4.3.2 Exclusion experiment I: effect of snail exclusion on barnacle recruitment

Mean number of barnacle recruits per 50 cm<sup>2</sup> for the (snail) exclusion treatment reached  $38 \pm 5$  at 4<sup>th</sup> week, further increased to  $1048 \pm 204$  at 6<sup>th</sup> week, and then stopped increasing at 8<sup>th</sup> week. At the same time, the number of barnacle recruits for both the control and the open cage remained much lower throughout the experiment ( $< 5$ ; see Figure 4.5, 4.6). The effect of the open cage was found to be not significant, as the number of barnacle recruits did not significantly differ from each other between the control and the open cage treatments ( $p > 0.05$ ). Repeated measures ANOVA indicates

that the exclusion of the littorinids had a great effect on the recruitment of the barnacle, and the recruitment of the barnacle changed with time (see Table 4.1). The barnacle juveniles were identified as *Amphibalanus amphitrite* based on their morphology.



**Figure 4.5:** Effects of snail exclusion on the recruitment of barnacles (mean ± SE) over 8 weeks (Logarithmic scale is used for y-axis for better comparisons).



**Figure 4.6:** Representative photographs of the mangrove tree trunk surfaces subjected to cage (open and exclusion) and no-cage (control) treatments taken during Week 6 and 8 (scale bar denotes 0.5cm).

**Table 4.1:** Repeated measures ANOVA table of effect of treatments on the recruitment of barnacles over three sampling times. Number of barnacles was transformed using  $-1/(\text{Barnacle}^{0.35}) + 1$  ( $\lambda$  was determined to be  $-0.35$  using Box-Cox method).

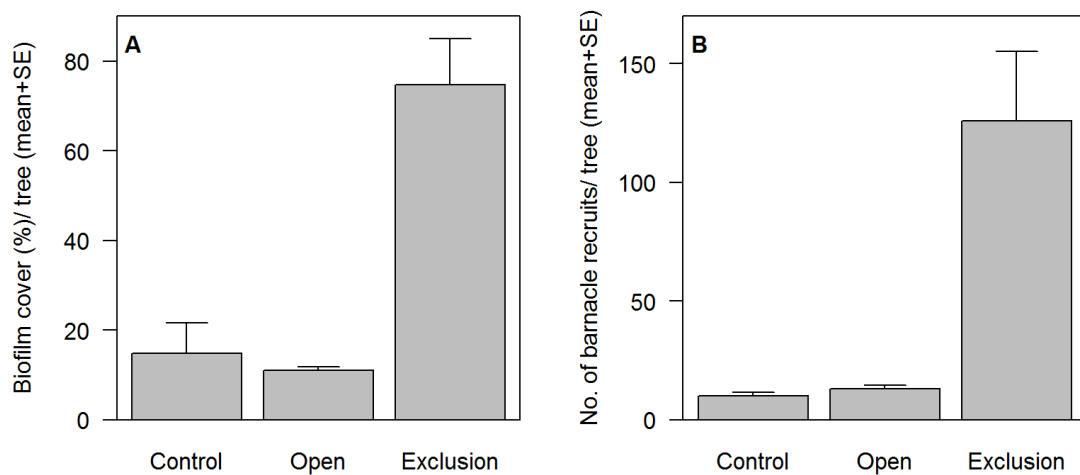
Source of variation	Df	Sum of Squares	Mean Squares	F value	Pr(> F)
<i>Between subjects</i>	17				
Treatment	2	3.895	1.948	168.363	$2 \times 10^{-8}$
Block	5	0.094	0.018		
Residual <sup>1</sup>	10	0.116	0.116		
<i>Within subjects</i>	36				
Week	2	0.111	0.055	14.052	$4.94 \times 10^{-5}$
Treatment:Week	4	0.069	0.017	4.403	0.006
Residual <sup>2</sup>	30	0.118	0.004		

<sup>1</sup> This residual is actually effect of trees nested within treatments

<sup>2</sup> This residual is actually effect of trees within treatments by weeks

### 4.3.3 Exclusion experiment II: effect of snail exclusion on biofilm growth and barnacle settlement

Two response variables were measured in this experiment, and MANOVA showed that the treatments had significant effects on them (Table 4.2). This was confirmed by follow-up ANOVA (Table 4.3). Both the biofilm coverage and the number of barnacle settlement were significantly higher in the exclusion treatment than the control and open cage treatment (Figure 4.7). This showed that littorinids reduced the development of biofilms, and confirmed the effect of littorinids on the barnacle in Exclusion Experiment I, on artificial substrate. A check on the correlation between biofilm coverage and barnacle settlement number showed a significant correlation (Spearman's correlation  $r = 0.59$ ,  $p = 0.02$ ). Qualitative examinations of the positions of the settled barnacle cyrpid/ juveniles on the substrate also showed a relatively higher affinity towards the biofilmed areas (Appendix M).



**Figure 4.7:** Effect of snail exclusion on the (A) biofilm development and (B) barnacle recruitment after deployment for 11 days in the field.

**Table 4.2:** MANOVA table of effect of treatments on the recruitment processes

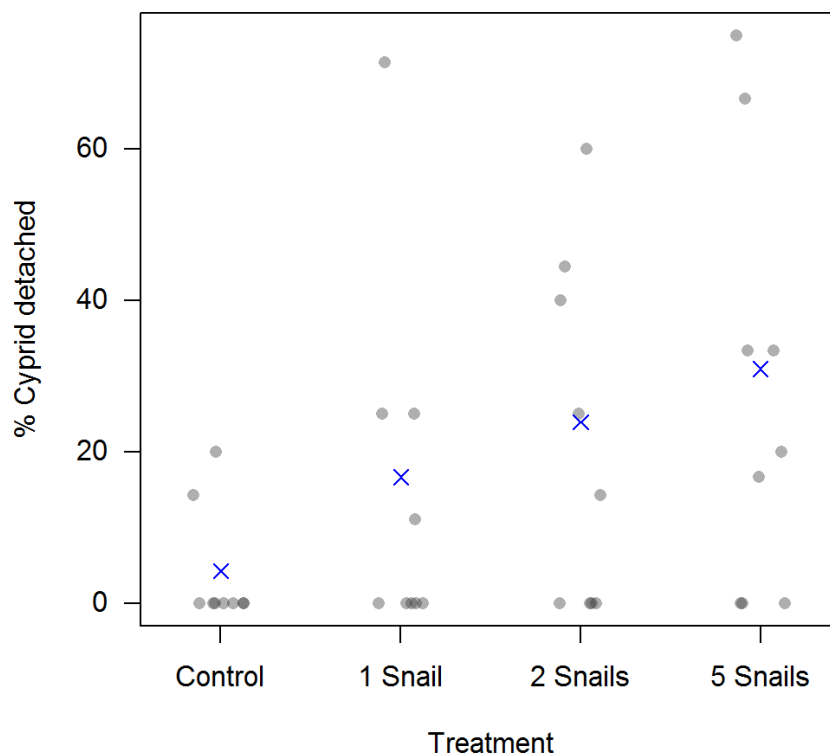
Source of variation	Df	Wilks	Approx. F.	Num Df	Den Df	Pr(> F)
Treatment	2	0.027	17.717	2	8	$2.28 \times 10^{-5}$
Block	4	0.455	0.845	4	8	
Residuals	8					

**Table 4.3:** ANOVA table of effect of treatments on the individual variables

Source of variation	Df	Sum of Squares	Mean Squares	F value	Pr(> F)
<i>Biofilm Development</i>					
Treatment	2	1.998	0.999	15.611	$1.73 \times 10^{-3}$
Block	4	0.098	0.024		
Residual	8	0.512	0.064		
<i>Barnacle Recruits</i>					
Treatment	2	190.295	95.147	31.733	$1.57 \times 10^{-4}$
Block	4	11.386	2.846		
Residual	8	23.987	2.998		

#### 4.3.4 Snail bulldozing effect on settled barnacles

The mean proportion of cyprids dislodged by littorinids increased as the number of the snails increased (Figure 4.8) but this difference was not statistically significant (Table 4.4). The null hypothesis that the bulldozing effect by snails on the dislodgement of the cyprids is random could not be rejected.



**Figure 4.8:** Percentage of cyprids successfully detached at the end of Bulldozing Experiment. ( $n=9$  for each treatment; cross (×) is the mean for each treatment).

**Table 4.4:** Analysis of Deviance table of the effect of the treatments on the detachment of cyprids.

Source of variation	Df	Deviance	Residual Df	Residual Deviance	F	Pr(> F)
Treatment	3	16.027	32	68.679	2.661	0.065
Null			35	84.706		

#### 4.3.5 Snail behaviour

It was observed that the littorinids at their resting position did not actively move until they encountered the incoming tide water. Once the snails encountered the rising tide water, they actively migrated upwards to avoid immersion. This response was also observed in snails resting at higher positions, usually at crevices or branches, which only actively moved up just before the water level reached them. The snails appeared to maintain a distance above the water level (median= 17cm for both trees observed). The snails' upward movement stopped at high slack water, and they started moving downward as the tide level began to fall during ebb.

The tide level appeared not to be the sole factor dictating their resting position as some snails broke off from the group as they were moving down, and rested earlier at higher positions while the majority moved in rhythm with the tide, to the bottom (see Figure 4.9 and 4.10).

During this return journey, they also maintained a distance above the falling tide water (median= 14cm and 12cm for Tree A and B, respectively). Permutational Kolmogorov-Smirnov test showed that the distribution of the vertical distances between snails and water level differed significantly between flood and ebb tide ( $p < 0.001$  for both case, see Figure 4.11). Overall, strong correlation between the snail levels and tide levels was found (For tree A, Kendall's  $\tau_B = 0.78$ ,  $p < 0.001$ ; For tree B,  $\tau_B = 0.78$ ,  $p < 0.001$ ). It has to be noted that, because each tree trunk only had one camera directed on it with fixed view, not all the snails would appear in each photo frame. Therefore, the number of snails recorded at each moment of time varied (Figure 4.9, 4.10). The snail number also appeared very low if they hid inside the root crevices, e.g. at time 1940 for Tree B.

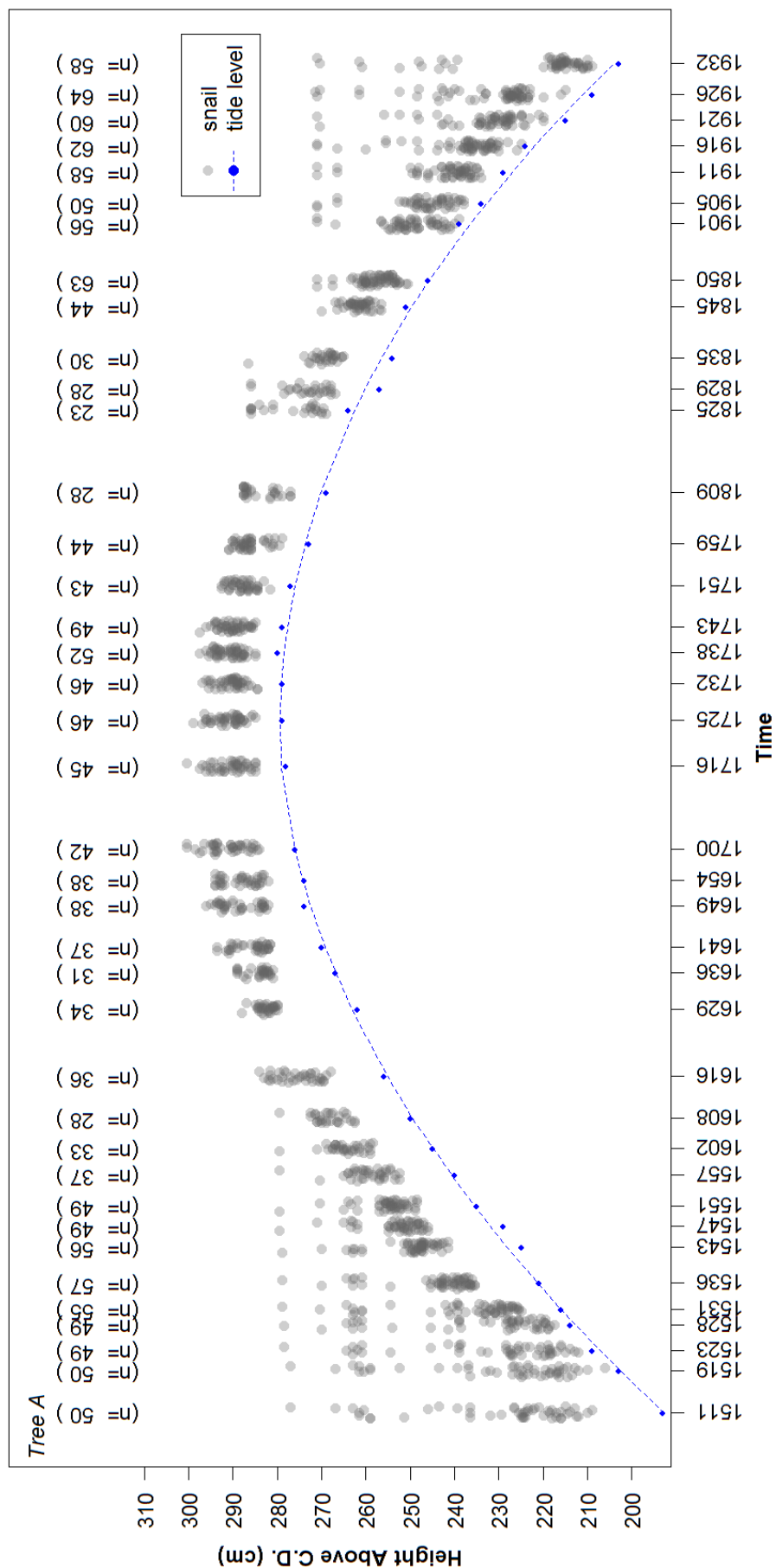
Besides vertical migration, it was also observed that the snails avoided the trunk surface that faced the strongest wave action by moving laterally around the trunk so as

to seek shelter behind it on the leeside of the wave direction (Figure 4.12). However, this tendency was not quantified in the present study.

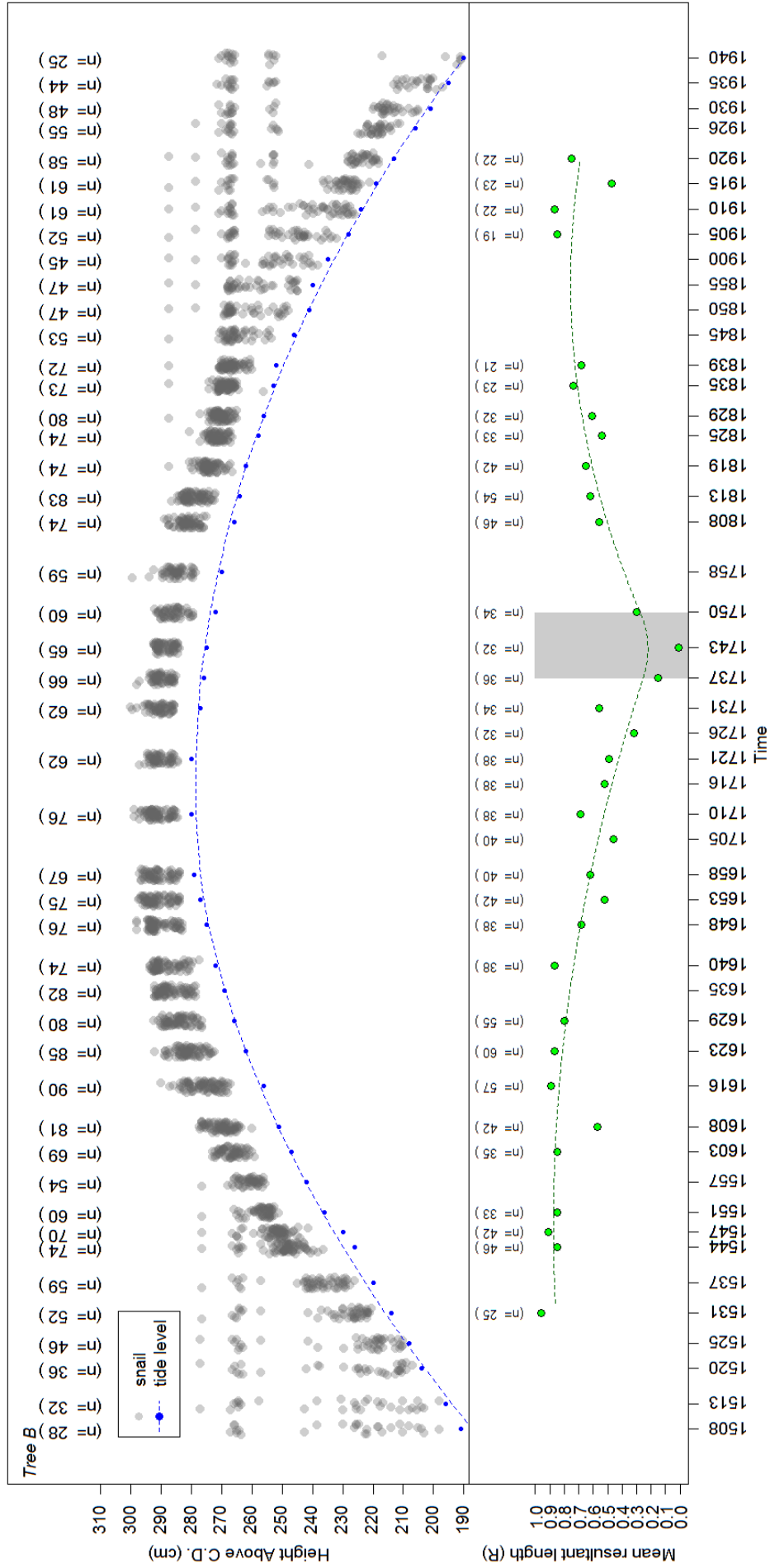
Mean resultant length ( $R$ ) was used to quantitatively show the directional uniformity of the snails during vertical migration, and the result showed a high value of  $R$  (0.46 to 0.96) during the observation period, except for four time points around high slack water ( $R = 0.01$  to  $0.32$ ) (see Figure 4.10). These low  $R$  values at slack water indicated the short period when the change of direction from upward to downward direction occurred. Decreasing trend was observed for  $R$  before high slack water, while increasing trend was observed after it. Overall, there was a significant negative correlation for  $R$  values with the water levels (Kendall's  $\tau_B = -0.44$ ,  $p < 0.001$ ).

In the laboratory study, the randomly scattered snails moved and form aggregations on the petri dish, breaking the random spatial pattern after a short time (80s) from initiation of the experiment (Figure 4.13). The aggregation index was less than 1, and significance test showed that it did not happen by chance (significance level  $\alpha$ , after Bonferroni correction = 0.006; see Figure 4.13). In contrast to the field observation, the mean resultant length was low throughout the laboratory observation, showing no significant trend of unified direction (all  $p > 0.006$ ).

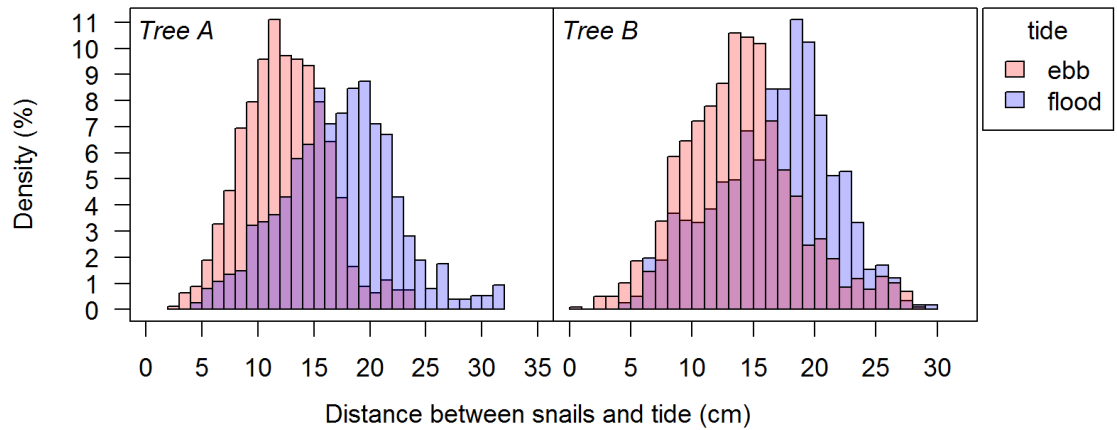




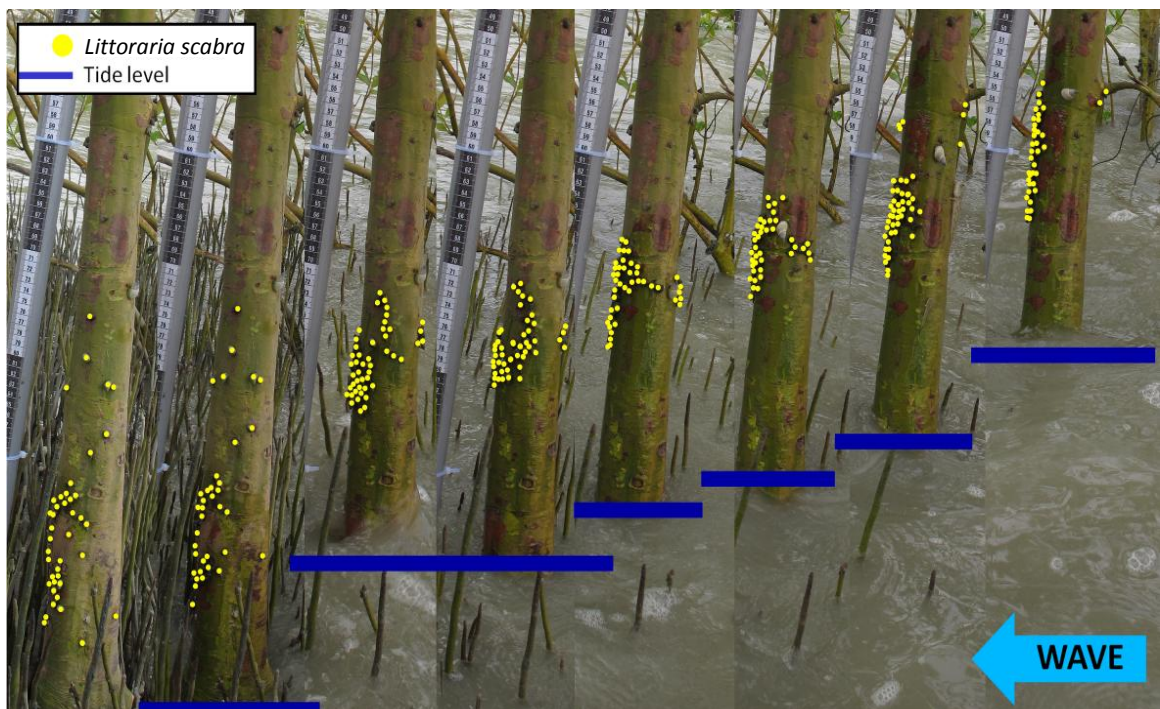
**Figure 4.9:** Vertical migration pattern of mangrove snails in relation to tide levels during flood and ebb tides, measurements for Tree A (For better visualization, data points on each time point was made to jitter randomly around each time axis, and the color was made partially transparent, where densely clumped points appear darker in colour; C.D.= Chart Datum).



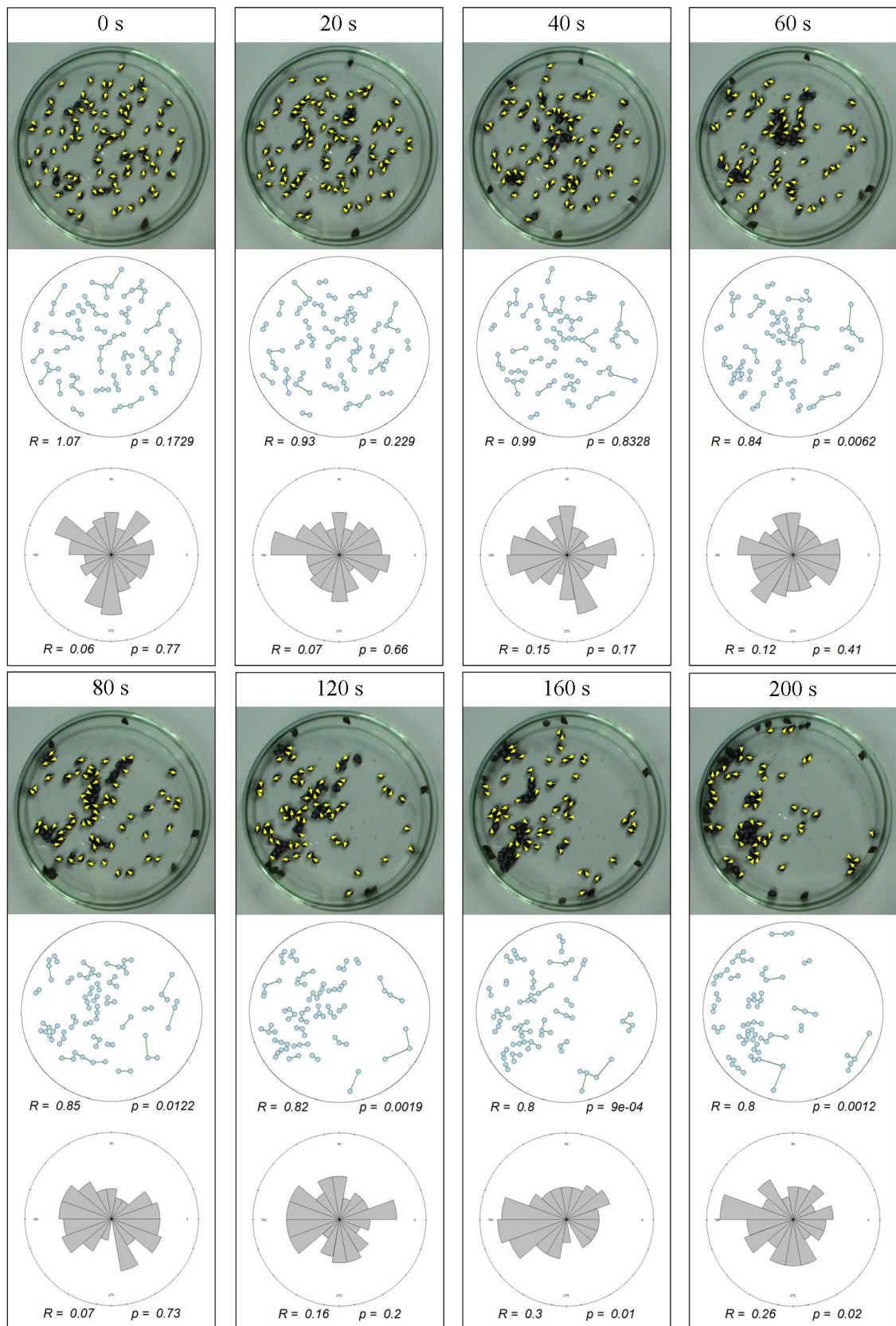
**Figure 4.10:** Upper panel, same as Figure 10, for Tree B. Lower panel, mean resultant length (R), broken line showing trends fitted with second degree piecewise polynomial regression with knots designated at time 1737 and 1750 (shaded), believed to be the period when changing of direction occurred.



**Figure 4.11:** Histogram of frequency of distance between vertical levels of snails and tide during flood tide and ebb tide. (purple region indicates overlapped histograms of both flood and ebb tides)



**Figure 4.12:** Stitched time-series photographs of Tree B, arranged sequentially (left to right), to show both the vertical (upward) and horizontal (lateral) migration of *L. scabra* during flood tide. Lateral movements involved snails moving around the trunk towards the leeward side of the wave direction. Snails are marked yellow for easier visualization.



**Figure 4.13:** Laboratory observation of the littorinid aggregation behaviour. At each time interval, photo in the first row is the original frame overlaid with directional marks. In second row, snail positions are shown with lines connecting each snail's nearest neighbour.  $R$  here refers to the aggregation index. In third row, snails' directions are summarized in a rose diagram.  $R$  here refers to mean resultant length.  $p$ -values ( $<0.006$ ) indicate rejection of null hypothesis that distribution (spatial/ directional) is random ( $\alpha = 0.006$ ).



## 4.4 DISCUSSION

### 4.4.1 Preliminary study and design issues associated with mangroves

The experimental design used in the snail exclusion experiments was actually not a strict RCBD design, but a hybrid approach between randomised block design and systemic design (Hurlbert, 1984), with the goal of minimizing the pre-manipulation variability in tree diameters among treatments by means of ‘restricted randomisation’, i.e. selecting combination of randomised blocks that gave the acceptable spatial and internal property (tree diameter) interspersed. However, this thoughtful design can lose its designed effectiveness too if some of the blocks are excluded later in the experiment, which happened in the snail exclusion experiments in this study when the compromised cages had to be excluded in the analysis. Difference in tree diameters was thought to affect the density of littorinids and other associated variations such as small scale hydrodynamics, and thus interspersed among treatments was desired. Rittschof *et al.* (2007) showed that when cylinders of different diameters were used as fouling substrates, different flow profiles were obtained and that affected the subsequent settlement of *Amphibalanus amphitrite*. The preliminary study found a strong linear relationship between the trunk diameter and number of littorinids. This could be one of the reasons why very few littorinids were found on replanted mangrove saplings which usually have small stem diameters (<1cm). This observation is different from the observation made at an East African mangrove area, where Torres *et al.* (2008) found no correlation between the diameter at breast height of mangrove trees and the abundance of littorinids in the seaward zone. This discrepancy is likely due to the different survey methods used.

#### **4.4.2 Effect of littorinids on barnacle settlement and biofilm development**

##### **4.4.2.1 Grazing effect on barnacles**

The results of Exclusion experiment I showed evidence on the negative effect of mangrove littorinid grazers on the recruitment of barnacles on mangrove trees, consistent with the previous findings from rocky shores (Denley & Underwood, 1979; Williams, 1994; Hutchinson & Williams, 2001; Chan & Williams, 2003; Holmes *et al.*, 2005; Hidalgo *et al.*, 2008). The present finding appears to be the first empirical evidence of snail grazing effect on mangrove barnacles. Previous works conducted using exclusion experiments had studied snail predation effect on barnacles that colonized mangrove roots in Belize (Ellison & Farnsworth, 1992) and Panama (Starczak *et al.*, 2011). In the Belize study, caged mangrove roots which kept away gastropod predators (*Melongena melongena*) were found to have increased barnacle numbers, but had no effect on algae. In the Panama study, species of predators were not named and thus it is not clear whether the effect was caused by the predators alone. The cage design in the present study was able to keep grazers and other animals from entering the experimental substrates. Mudskippers, crabs and snakes were sometimes seen resting on the mangrove trees during flood tide. Gastropod predators (*Thais* sp.) were also found in the study area of the current study, but were never found on the substrate of the experimental units during all monitoring surveys. Therefore, predation is a less likely cause of low barnacle abundance as compared to grazing by the abundant littorinids. In summary, results from Exclusion experiment I suggest that grazing pressure is an important factor structuring the vertical distribution of barnacles on mangrove trees.

#### 4.4.2.2 Grazing effect on biofilm

Mangrove littorinids graze on biofilms. Similar findings have been previously reported for rocky shores. The method used to quantify biofilms in the present study however differs from the commonly used methods. Chlorophyll-*a* has been the most frequently measured response variable for indicating biofilm development in studies at rocky shores. The exclusion of grazers mostly results in higher Chlorophyll-*a* concentration (Williams, 1994; Mak & Williams, 1999; Hidalgo *et al.*, 2008; Macusi, 2012; but see Hutchinson *et al.*, 2006; Skov *et al.*, 2010). Observation using SEM is also commonly used to study biofilm on rocky shore. Prior to Exclusion experiment II, the preliminary study was carried out using mangrove bark directly as substrate, but there was difficulty in manipulation as well as analytical interference from the bark. Thus, staining techniques employing Alcian Blue and other dyes, and PVC sheets were used instead. The use of artificial substrate is one of the limitations of the study.

The other concern is the scale of the measurement. In the study by Macusi (2012), the effect of grazers (littorinids and limpets) exclusion on biofilm, measured by the level of Chlorophyll-*a* and percentage cover of biofilm as observed in SEM, were found to give different conclusions, i.e. weakly significant effect for Chlorophyll-*a* but not significant effect for percentage cover using SEM. But the same difference measured as visual percentage cover of the plots was more distinct. Similar scale effect on patchiness was also observed by Hutchinson *et al.* (2006). This highlights the problems of differential sampling scales used, and the scales used in SEM (usually less than 1 cm<sup>2</sup>) and Chlorophyll-*a* (usually rock chips of about 2-4 cm<sup>2</sup>) may not be sufficient to detect differences in the biofilm cover affected by macrobiota. Measurement scale used in an experiment should be relevant to the size of the organisms being studied, and its behavioural attributes (Raffaelli *et al.*, 1994). In the present study, the research question was whether the mangrove littorinids ('independent

variable') affect the barnacles and biofilm ('response variables'), and the measured scale between them should match, i.e. both measured at the scale of whole experimental units. This problem also presented in the experiment of previous chapter, where the analysed area of biofilm morphology is much less than those sampled for microbial communities analysis and barnacle counts. This mismatching appears to be a common problem in manipulative experiments in ecology where the response variable is measured at a scale much smaller than the scale at which the independent variable operates (Raffaelli, 2006). The present study used Alcian Blue to stain biofilm of the whole experimental unit. This was an attempt to avoid this mismatch problem.

Staining the biofilms using Alcian Blue also had its problem. Alcian Blue, which stains mucopolysaccharides/ mucoproteins, was found to stain also the mucous trail left by the littorinids. Laboratory test using clean petri dish and staining the trails left by littorinids confirmed that the dye could stain the mucus trails as well (data not shown). However, due to the large mean differences of Alcian Blue stained area between the treatments, even with the inclusion of the mucous trails, the conclusion made in this study is not affected. Nonetheless, appropriate biofilm assessment method remains a challenge in future studies. Furthermore, it is also not clear whether the mucus trails left by *L. scabra* have any effect on the barnacle and biofilm settlement. Holmes (2002) showed that the mucus trails of limpet (*Patella vulgata*), but not littorinid (*Littorina littorea*), can positively affect the settlement of cyprids of *Semibalanus balanoides*. There are also reports on the positive effect of littorinid (Davies & Beckwith, 1999) and limpet (Connor, 1986) mucus trail on the growth of microalgae.



#### 4.4.2.3 Direct and indirect effects of grazing

Although the barnacle settlement and biofilm development were measured in Exclusion experiment II and they were shown to be correlated, it is not clear how grazing could affect barnacle settlement. Grazing can have a direct effect on cyprids as the snail moves about (through ‘bulldozing’) and/or indirect effect (through removal of biofilms). The laboratory ‘bulldozing’ experiment however did not show high removal of cyprids (<40 %) unlike in the snail exclusion experiments. However, this lab bulldozing experiment was not replicated due to the difficulty of obtaining freshly attached cyprids. Also, the single trial experiment was not carefully designed and the results should be taken with caution.

It is not clear whether *L. scabra* had ingested the cyprids. Ingestion of cyprids by limpet grazers has however been reported (Miller & Carefoot, 1989; Chan & Williams, 2003). Different conclusions have been given in other studies aiming to resolve the direct and indirect effects. Anderson (1999) concluded that the effect of grazers (*Bembicium auratum* and *Austrocochlea porcata*) on *Balanus variegatus* and *B. amphitrite* was largely due to the indirect effects of grazing on algae, but with little evidence of indirect effect for *Hexaminus* sp. or *Elminius covertus*. Buschbaum (2000) showed that the indirect effect of grazing on algae was negative in early settlement of *Semibalanus balanoides* but positive in the long term. But Holmes *et al.* (2005) concluded that the effect of grazing on *S. balanoides* was not mediated through indirect effect.

Besides changes in total biofilm coverage/ biomass, another possible mechanism of the indirect effect of grazing is through the change in biofilm composition, which in turn, changes the positive effect of biofilm on barnacle settlement. As shown in the results from the previous chapter, changes in micro-eukaryotic composition but not the bacterial composition, was associated with barnacle settlement preference. Barranguet

*et al.* (2005) and Lawrence *et al.* (2002) both found reduced microalgal and diatom biomass in grazed biofilm but not for bacterial biomass. Furthermore, grazing was shown to be able to change the microalgal composition of biofilm (Lowe & Hunter, 1988), possibly due to differential grazing preference/ efficiency towards different micro-eukaryotic species. Investigating the indirect effect due to differential grazing efficiency on the eukaryotic/ bacterial components of biofilm will make a good future study.

#### **4.4.2.4 Grazing effect versus inherent mangrove antifouling effect**

Results of Exclusion experiment I suggest that the low number of barnacles naturally present on the mangrove trees at the study site is unlikely due to the anti- (macro) fouling capability of mangrove trees. However, lack of baseline observation and the use of PVC substrate in Exclusion experiment II prevent us from drawing any conclusion about the antifouling capability of the mangrove against the development of biofilm. But even if there is any antifouling effect from the mangrove trees (macro- and/or micro-fouling), it appears not effective against the settlement of barnacles which occurred in large numbers in the snail exclusion experiments. Therefore, under natural settings, less fouling on mangrove trees could be explained by the effect of grazers.

#### **4.4.2.5 Other factors**

Physical limitations (e.g. temperature) have been suggested as the major factor limiting the vertical limit of recruitment success of barnacles at the higher shores, while biological factors maybe important at lower shores (Williams, 1994). It is noted that the shore level occupied by natural mangrove trees described in this study (site 2; about ~210cm above C. D.) was slightly higher than site 1 (~200cm above C.D.) described in Chapter 3. However, the result showed that barnacles can recruit to higher levels e.g. at

Site 2 where littorinids were excluded by net cages. Compared to exposed rocky shores, temperatures on the mangrove trunk under the leafy canopy are not expected to be higher. For tropical rocky shores, Mak & Williams (1999) found that even at the high shore, the experimental exclusion of littorinids increased biofilm development even at high temperature. It has also been shown that physical factors affect biofilm and barnacle differently. The exclusion of grazers affects biofilms at both higher and lower shores, whereas this had effect on barnacles only at the lower shore (Mak & Williams, 1999; Hidalgo *et al.*, 2008). The other important factor is the density of the grazers. As discussed by Holmes *et al.* (2005), the density of limpets can change its effects on the recruitment of barnacles. Buschbaum (2000) also showed that the density of *Littorina littorea* could be the key factor to the recruitment of *Semibalanus balanoides* on intertidal mussel beds, giving either positive or negative effects.

#### **4.4.3 Littorinid behaviour**

##### **4.4.3.1 Vertical migration**

Observation of vertical migration of littorinids in rhythm with the tidal cycle is consistent with the observations of previous studies in mangroves (Nielsen, 1976; Yipp, 1985; Reid, 1986; Ohgaki, 1992; Alfaro, 2007). This behavior was also observed for other types of grazing snails such as turbinids (Alfaro, 2006) and limpets (Gray & Williams, 2010). Among these studies, the work of Alfaro (2007) who studied the behaviour of *L. scabra* on *Rhizophora* sp. in Fiji was the closest to the current work. Given that *Rhizophora* sp. and *Avicennia* sp. have very different root structures, it is reasonable to say that vertical migration pattern by *L. scabra* may be different between the two substrates, especially at the lower part of the tree where the snails could be scattered around the roots of *Rhizophora* which was described by Alfaro (2007) as ‘tortuous paths’. In the study by Ohgaki (1992), it was reported that the distribution of

littorinids on different mangroves was different and furthermore, vertical migration behaviour exhibited by different species of littorinids such as *L. intermedia* and *L. pallescens* were observed to be specialized. However, a side-by-side comparison of *Rhizophora* and *Avicennia* could not be performed since the two species occupies different levels of the shore; the former on the upper shore experiences lesser and much shorter duration of tidal inundation. In the present study, the littorinids on the *Avicennia* trees were observed to be quite restricted to the trunk and branches of a single tree but sometimes were seen to move to the nearest pneumatophores too. Observations indicate that the snail movement appears to be initiated by the splashing of the tide water. This is consistent with observations made in other studies on littorinids and limpets (Gray & Williams, 2010). Little and Stirling (1984) suggested that the physical disturbances, seawater or freshwater can initiate *L. scabra* movement but not humidity nor tidal immersion. Alfaro (2008) suggested that humidity is the activation factor. These discrepancies in the response of *L. scabra* could be due to the snail's adaptations to different environments.

The present study shows that littorinid vertical distributions are very similar before and after the flooding by tides. The behavior of returning to the resting position has been described as homing behavior, an adaptation developed to avoid stress (Chelazzi, 1990). Although there is evidence for exogenous factor (tide cycle) regulating snail movement, it is not clear whether *L. scabra* has endogenous circadian rhythm that regulates its movement, as both types of behavior have been shown to be present in limpets (Gray & Williams, 2010). Both the snail's moving speed and its distance to the water level differ between flood (faster and farther) and ebb tides. This is consistent with the observation by Alfaro (2007), but the magnitude of the difference observed in the present study (distance: <5cm, speed: 0.1cm min<sup>-1</sup>) is not as large as in Alfaro (distance: >50cm, speed: 0.4cm min<sup>-1</sup>). Alfaro (2007) attributed this difference to

avoidance during flood tide and feeding behaviour of the snails during ebb tide. This difference may also be caused by the difference in the strength of the wave action between tides, which was observed to be stronger during flood tide. However, more observations and quantitative data are needed to support this.

The energetically expensive excursion of the littorinids to avoid immersion during flood tide appears to be compensated by active feeding during ebb tide. In an important study combining both the migration pattern and diet of *L. scabra*, comparison of gut contents of *L. scabra* between the flood and ebb tides showed significantly increased number of food particles in ebb tide, indicating that the snails graze on their way down (Alfaro, 2008). Further analysis of snail's fatty acid profiles showed significantly elevated signals of microalgae and bacteria in ebb tide compared to flood tide, suggesting the ingestion and assimilation of biofilm. The study also indicates the importance of biofilm as food source for *L. scabra* because although it is a generalist feeder which consumes various types of food (plant tissue, zooplankton, phytoplankton, and algae), bacteria and microalgae are better assimilated than the rest.

#### **4.4.3.2 Implications of vertical migration pattern**

One of the major differences between the natural mangrove trees and the replanted seedlings is that the seedlings are fully inundated during high tide, and any littorinids on them would be subjected to stress due to immersion. This may be one of the reasons why littorinids have problems to be recruited on the re-planted mangrove seedlings. Beside the avoidance of physical stress, the other benefit of the vertical migration includes avoidance of predation by aquatic predators (Catesby & McKillup, 1998), such as puffer fish (Duncan & Szelistowski, 1998). Biofilm grazing by littorinids happens at every tidal cycle immediately after the biofilm is replenished by the high tide. Thus, grazing provides a constant perturbation to the development of biofilm. As shown in

previous chapter, significant positive effect of the biofilm on the settlement of barnacle is restricted to biofilms of older age, and thus barnacle colonization is constrained by biofilm perturbation due to grazing pressure. An implication on snail studies, however, not related to biofouling, is the common practice of surveying snail abundance and diversity during low tide (e.g. Torres *et al.*, 2008). Counting during low tide has been shown to underestimate both the abundance and diversity of molluscan grazers (Hutchinson & Williams, 2003)

#### **4.4.3.3 Aggregation behaviour**

Although the aggregation behaviours of littorinids have been widely described (Rojas *et al.*, 2013), more studies focused on snail aggregations at resting positions rather than on the moving snails. The observed aggregative moving behaviour is often referred as ‘swarming’ behaviour. Aggregation was also observed in the study by Alfaro (2007), quantified as the nearest neighbour distance. One of the differences between the present study and that of Alfaro (2007) is that the density of *L. scabra* is higher in the present study and a stronger collective movement was observed. Although nearest neighbour distance was not quantified in the field observation, it was very obvious that the distance between contiguous snails was much closer. While laboratory observation showed spontaneous aggregation behaviour in the absence of any physical tidal cues, swarming behaviour (i.e. moving together in a self-organized group towards a polarized direction) was not apparent. This indicates the importance of water movement on the moving snails. While the vertical migration pattern was influenced by the tidal height to avoid immersion, the horizontal movement is likely elicited to avoid a direct wave hit. Underwood (1972) showed that water movement both initiated and influenced the rate of activity of four species of littorinids, i.e. the greater the water movement, the greater the activity.

#### **4.4.3.4 Implication of aggregation pattern**

Benefits of aggregation in both homing and swarming behaviours of littorinids are similar, that is, to reduce physical stresses such as desiccation or dislodgement by waves (Stafford, 2002). Biotic benefit such as increased foraging efficiency has received relatively less attention, albeit being one of the major incentives for swarming behaviour (Parrish & Hamner, 1997). Future studies should include the study of the effect of swarming behaviour on the feeding efficiency, since it is apparent that this effect, if present, will also mean better removal of micro- and macro-fouling communities on the mangroves. In fact, it is suspected that the low removal efficiency of cyprids by ‘bulldozing’ effect in the laboratory experiment is due to the lack of the swarming behavior observed in the field. Thus, cyprid encountered by littorinids is more random in the lab experiment, whereas in the field, swarming behaviour likely increases the probability of encounters. In addition, the wave avoiding behaviour of the littorinids, i.e. by moving to the leeside of the trunk, also coincides with the settlement preference (to avoid strong currents) of both barnacles (Qian *et al.*, 2000) and biofilms (Battin *et al.*, 2003). The wave action affects all three trophic levels, and in this case, it helps to regulate biofouling.

Both barnacles and biofilms preferentially settle or grow in crevices. Similarly, littorinids also like to aggregate at the crevices (Stafford *et al.*, 2007), thereby increasing their chances of encountering both barnacles and biofilms. However, there are also reports that showed crevices, such as those provided by the adult barnacles, also offer refuges to freshly settled barnacles (Miller & Carefoot, 1989).

#### **4.4.4 Using littorinids to control biofouling on re-planted mangroves?**

The present study suggests that littorinids may control barnacle biofouling. Future studies could be carried out to confirm this by translocating *L. scabra* from nearby

natural mangroves onto the re-planted trees. Alternatively, the same purpose may be achieved by studying methods that could encourage the natural recruitment of littorinids onto the re-planted trees. Most mangrove littorinids disperse by releasing planktonic egg capsules into the sea (Reid, 1986), thus, natural recruitment supplied from other areas is possible. Mangrove trees are important habitat for littorinids, thus successful recruitment of these snails can be viewed as part of the mangrove rehabilitation endeavours, or as an indicator of the rehabilitation progress. Mangrove rehabilitation without the recovery of faunal diversity will be incomplete. However, it is important to recognize that the factors that may encourage the recruitment of littorinids, could encourage the recruitment of barnacles as well. As shown in a long term study in a temperate rocky shore, physical influences such as temperature and wave exposure could affect three trophic levels (snails, algae, barnacles), whether in similar ways or in contrasting fashions, and change the magnitude of the interaction effects among them as well (Thompson *et al.*, 2004). Therefore, solving the problem of biofouling of replanted mangrove will require not only the good understanding of these interactions but also the physical factors that could affect them.

#### **4.4.5 Conclusion**

Exclusion of littorinid affects the recruitment of barnacles negatively, suggesting grazing pressure as the underlying factor regulating barnacles' recruitment onto the naturally grown mangrove trees. Similarly, biofilm cover is affected negatively by the grazing pressure. There is a significant relationship between biofilm cover and the number of settled barnacles. However, the relative importance of 'direct' (bulldozing) and 'indirect' (biofilm) effects from the grazers is not clear. Littorinids on naturally grown mangrove avoid tide submersion by moving in rhythm with tide cycle, which they cannot avoid on the newly replanted mangrove as the seedlings are too short and



fully submerged during high tide. Mangrove littorinids, when present in high number, also show strong collective movement that may enhance their effects on biofilm or barnacle settlement.

## CHAPTER 5

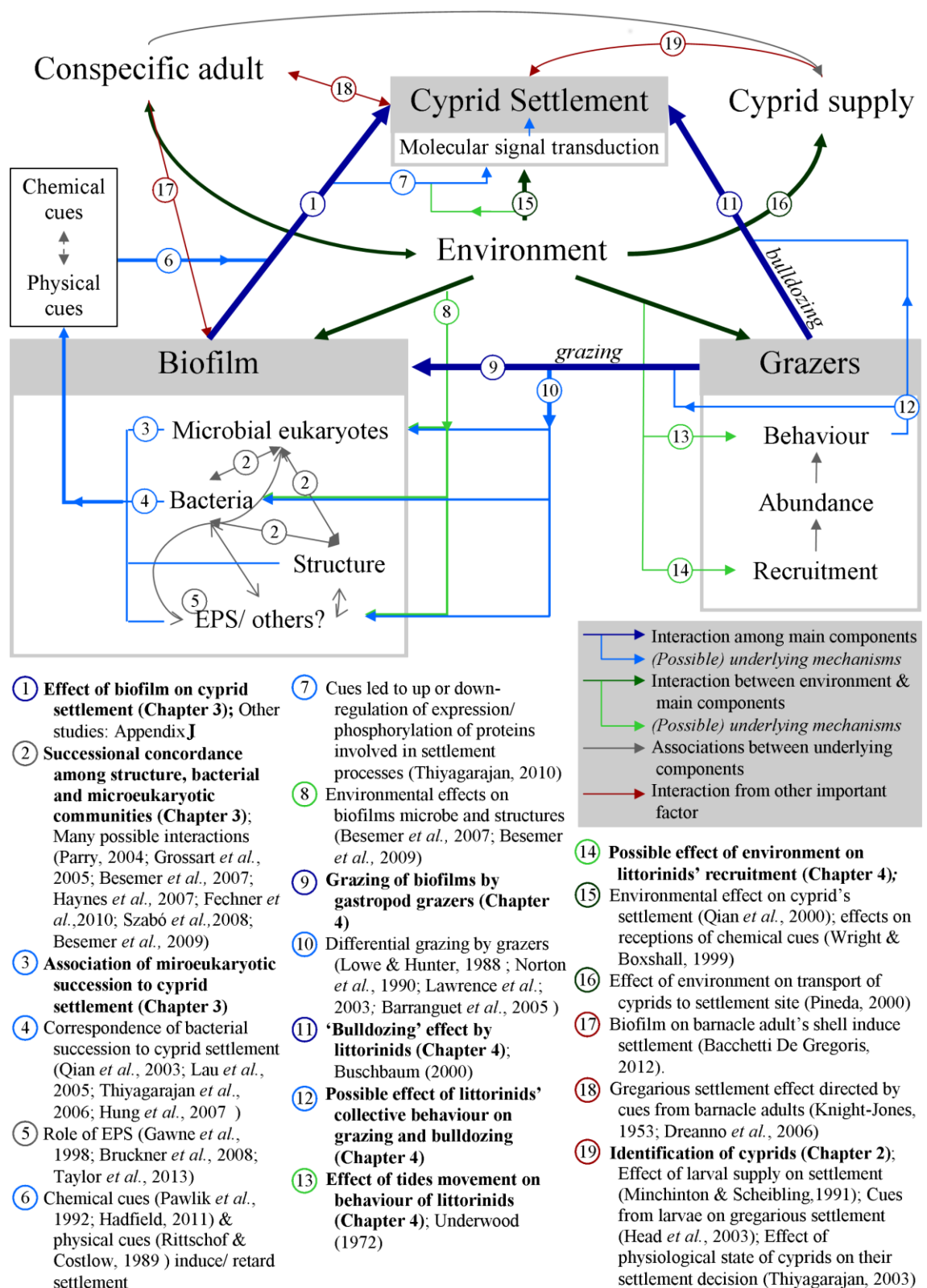
### GENERAL DISCUSSION AND CONCLUSION

#### 5.1 Contributions of present study and future directions in biofouling studies

Many factors affect the settlement of barnacles. Figure 5.1 summarizes the web of interactions between some of these factors, and illustrates where and what the current thesis has contributed to elucidating the complex problem of biofouling. . These include:

- (i) Barnacle cyprid could be identified using a combined morphological and molecular approach (Chapter 2)
- (ii) Biofilm has a positive effect on cyprid settlement (Chapter 3)
- (iii) Successional concordance among structure, bacterial and microeukaryotic communities is evident (Chapter 3)
- (iv) Cyprid settlement is associated with the succession of micro-eukaryotes (Chapter 3)
- (v) Gastropod grazing controls barnacle settlement (Chapter 4)
- (vi) Gastropod grazing controls biofilm growth (Chapter 4)
- (vii) Collective movement behaviour exhibited by littorinids may remove biofilm and cyprids (Chapter 4)
- (viii) This aggregation behaviour of littorinids is mediated by tides (Chapter 4)
- (ix) Tidal height may influence the recruitment of littorinids (Chapter 4)

The understanding on some of these interactions is still at the beginning stage, and more detailed studies are needed. Some of these interactions are important to enhance our understanding of biofouling and the role of natural grazers, and to use them as biological controls of mangrove biofouling.



**Figure 5.1:** Summary of interactions related to the settlement of barnacle cyprids, with contributions of the current study (bolded). Interactions recommended for more detailed studies to advance the knowledge in biofouling/ barnacle ecology are: 2, 3, 5, 7, 8, 10, 12, 13, 14, and 15. Some of these are important to be understood in order to use grazers to control mangrove biofouling (10, 12, 13, and 14).

Of particular interest pertaining to grazers in future studies are:

- (i) Differential grazing preference by littorinids on different components of biofilms (bacteria, microeukaryotes, and EPS)
- (ii) Effect of littorinid behaviour on on biofilm and barnacles
- (iii) Effect of environmental factors on behavior and recruitment of littorinids

It has to be noted that discrepancies among studies are not uncommon in some aspects that are better studied, such as the studies on biofilm-barnacle interaction. The current knowledge on barnacle settlement, as summarized here, is obtained from findings in a wide range of studies that can be divided using different criteria. Some examples are laboratory vs. field study, subtidal vs. intertidal environment, man-made structures vs. natural environment, rocky shores vs. mangroves, (natural vs. re-planted mangrove, biological substrate vs. artificial substrate, freshwater vs. marine (for biofilm and grazer studies). Different approaches of study had been used in the different studies. Perhaps the development of a more standardized approach is as important as conducting more detailed studies, so that the results from the different types of studies can be compared more easily. The use of biological substrate, such as the mangrove surface, gives more challenges as the living surface and its responses (interactions) add to the complexity. Plant-microbe-grazer interactions can be very complex, such as microbe farming by littorinids in the salt marsh (Silliman & Newell, 2003).

This thesis has come up with several methodological approaches in the study of biofouling although they remain to be further verified in future studies. It is also hoped that these approaches could have wider applications in biofouling studies, both for barnacle and other macrofouling organisms. For instance, in chapter 2, the method of using a morphology-based classification model to facilitate classification of large numbers of cyprid larvae could be applied to not only more species of barnacles, but also to larvae of other fouling organisms. The developed identification tool using the

suggested approach could be used to facilitate study of biofouling in places where multiple species of barnacles/ fouling organisms are present, as well as to study biofilm and grazing effects. In chapter 3, the combination of a concordance study of different biofilm components and relating them to barnacle settlement, could be useful in the study of interaction between biofilm and barnacle or any other macrofouling organism. This could be used either for exploratory study in the field like the present study, or for experimental study in the laboratory. One particularly recommended application of the approach in future is the study of biofilm succession on barnacle shells, as for example, the study by Bacchetti De Gregoris *et al.* (2012) has shown that the gregarious settlement of *A. amphitrite* could be due to the positive effects of bacterial biofilm on barnacle shells, and it will be interesting to examine interaction between cyprids and microbial eukaryotes or biofilm structure. And last, in chapter 4, observations on the snail behaviour have offered insights into the possible explanations of differential grazing pressures.

This thesis has contributed new perspectives on the study of the three main regulating factors of macrofouling: larval supply, biofilm, and grazers. However, the main challenge is to integrate the different approaches in order to study these factors together or to partition off the other effects while concentrating on one of them, especially in field studies where all factors have to be taken into consideration.

## **5.2 Biofouling control in mangrove rehabilitation site**

In chapter 3, it has been shown that microeukaryotic composition was correlated with the settlement of barnacles. This means that it is possible to manipulate the effect of the biofilm on barnacle settlement by altering the composition of the microbial community, especially since the accumulating evidences have shown that the composition of microbial communities could affect the settlement of barnacle. However, it is necessary

to first clarify the complexity of the many interactions that are associated with the composition of the microbial community before biofouling control measures could be developed. Methods such as the use of biocidal agents (Mary *et al.*, 1993) to change the microbial compositions may not be very suitable on the use of mangrove seedlings. The use of chemicals will also raise concerns over their effects on the natural environment. Other measures, such as the altering the nursery conditions before transplanting the mangrove seedlings, could be more suitable (Gomes *et al.*, 2010). One possible direction includes exploring whether nursery conditions could help to establish suitable biofilms that deter barnacle settlement.

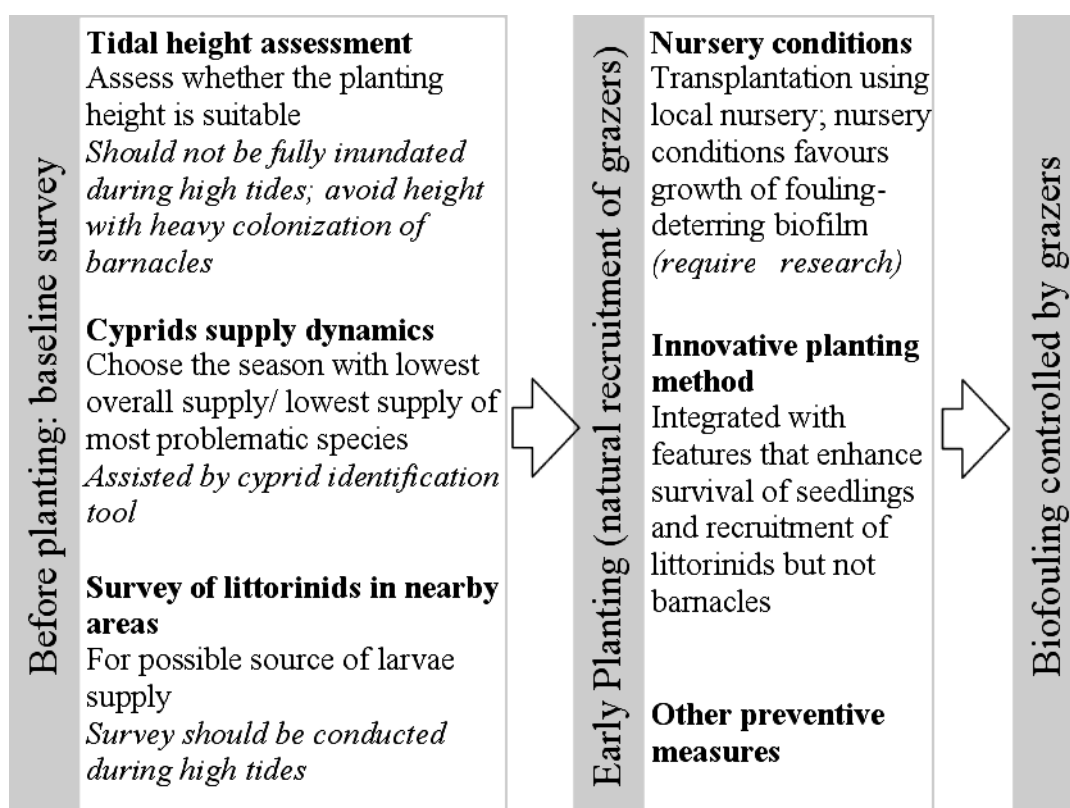
The findings in chapter 4 show that littorinid grazing could be a very efficient and natural solution to the problem at both microfouling and macrofouling levels. Furthermore, the disruption of microbial succession by littorinids, could be a possible way to alter the microbial composition and reduce the positive effect of biofilm on barnacle settlement. Although tests of translocating littorinids have not been carried out, some of the current findings could be directly incorporated into the design of mangrove re-planting efforts to curb biofouling. For examples, planting taller seedlings (Tamin *et al.*, 2011), or planting seedlings on the higher shore (Tan, 2013), have been shown to increase the plant survival and/ or reduce barnacle infestation. In fact, the natural mangrove trees at site 2 (chapter 4) were on higher ground than the replanted mangroves at site 1 (chapter 3). The rehabilitation site should not be at a tidal height that is too low down the shore (Tan, 2013). Besides, negative effects of biofouling on saplings or taller seedlings are less than that on younger seedlings (Li *et al.*, 2009). Planting taller seedlings can enhance the recruitment of littorinids too, since the plants will not be fully submerged. Modifications or the use of innovative planting methods developed in the replanting project (Raja Barizan *et al.*, 2008) to create refugia space at the base of supporting devices could encourage recruitment and increase the survival of

the littorinids. Another challenge in the use of mollusc grazers to control barnacles is to recruit grazers before the barnacles recruitment happens. The grazing effect seems effective only before (by removing the biofilm) or just after cyprid settlement (by bulldozing effect); it would not be effective once the cyprid metamorphoses into the adult. Therefore, the adoption of other anti-fouling methods at the early stage, such as those suggested in Tan (2013), may be important before biological control using littorinids could be used. A two-stage strategy is proposed for controlling of biofouling in mangrove rehabilitation projects (Figure 5.2).

### **5.3 Limitations of present study**

- (i) There was low representation of *A. amphitrite* cyprid specimens used in the classification model; thus, the range of morphological variations of this important species might not be well represented (Chapter 2).
- (ii) The cyprid identification tool developed is useful for wild-caught planktonic cyprids. However, the tool's suitability for newly settled/metamorphosing cyprids has not been tested (Chapter 2).
- (iii) The findings from some of the experiments conducted using artificial substrate cannot be similarly generalized for mangrove seedlings; caution is warranted (Chapter 3 & 4).
- (iv) Testing of cyprid settlement preference on biofilms took three days due to low larval supply during the experimental period. This could affect the interpretation of results (Chapter 3).
- (v) Experiments were conducted in one rehabilitation site only. Hence, generalization of present results to other rehabilitation sites should be done with caution (Chapter 3 & 4).

- (vi) Although large number of observations was made in the littorinid behavioural study, few repeats were carried out due to logistic constraints (Chapter 4).



**Figure 5.2:** A two-stage strategy aiming at using natural recruits of littorinids to control biofouling on re-planted mangrove.

## 5.4 Conclusion

Despite the limitations and difficulties of the field studies in the mangrove rehabilitation site, this thesis has achieved some important contributions and provided answers to the primary objectives set out in chapter one. A tool for identification of barnacle cyprids has been developed, which is useful for both natural mangrove habitat (MMFR) and mangrove rehabilitation site (Kg. Sg. Hj. Dorani). A link between the biofilm succession and barnacle settlement on artificial substrate has been established, and the present study further provides several information highlights on the microeukaryotic community which has been previously unexplored. Based on the experiments using mangrove trunk directly or using artificial substrate, there is empirical evidence of snail



grazing effect on barnacle recruitment on mangrove, as well as a link between grazing and the development of biofilm. This work provided possible avenues to the mangrove fouling problem at both the microfouling and macrofouling level. Further studies could focus on the factors regulating the recruitment of littorinids at the rehabilitation site and the testing of the proposed two-stage integrated control approach comprising of a physical prevention method at the early stage and biological control at the later stage.

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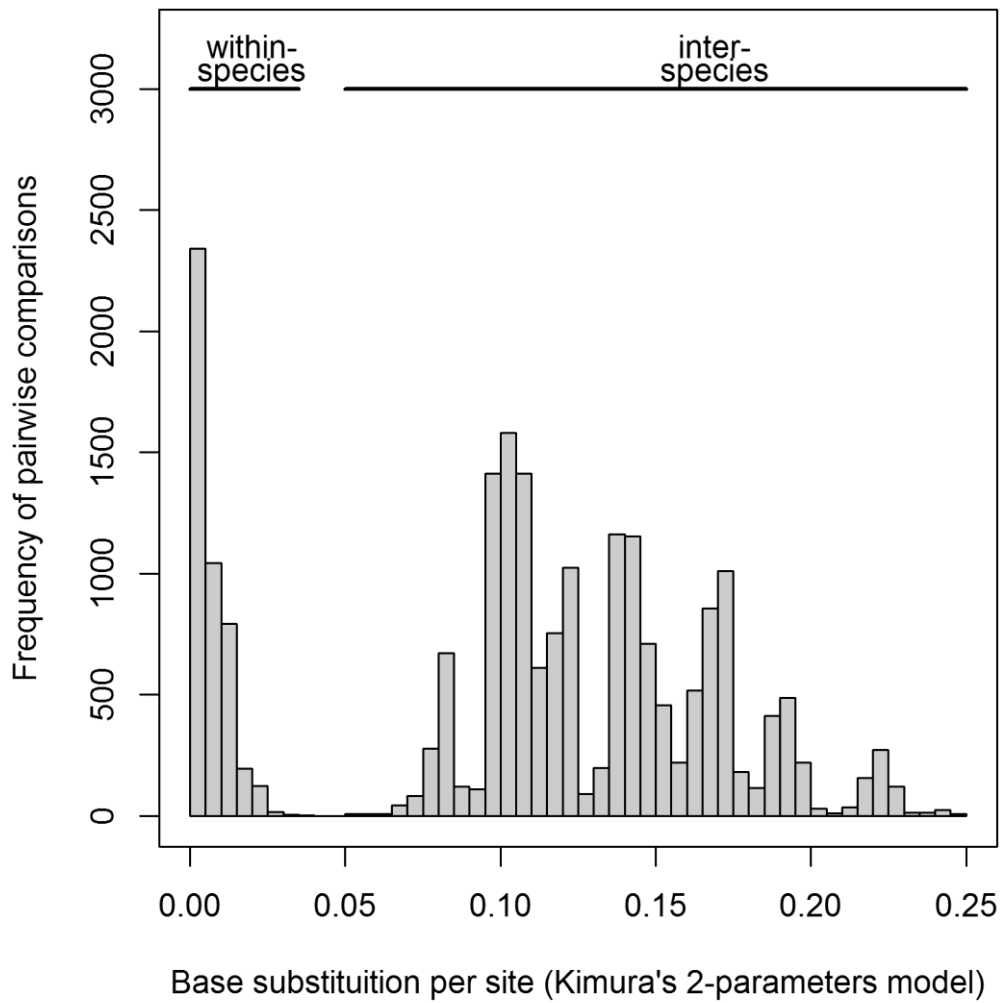
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## APPENDICES

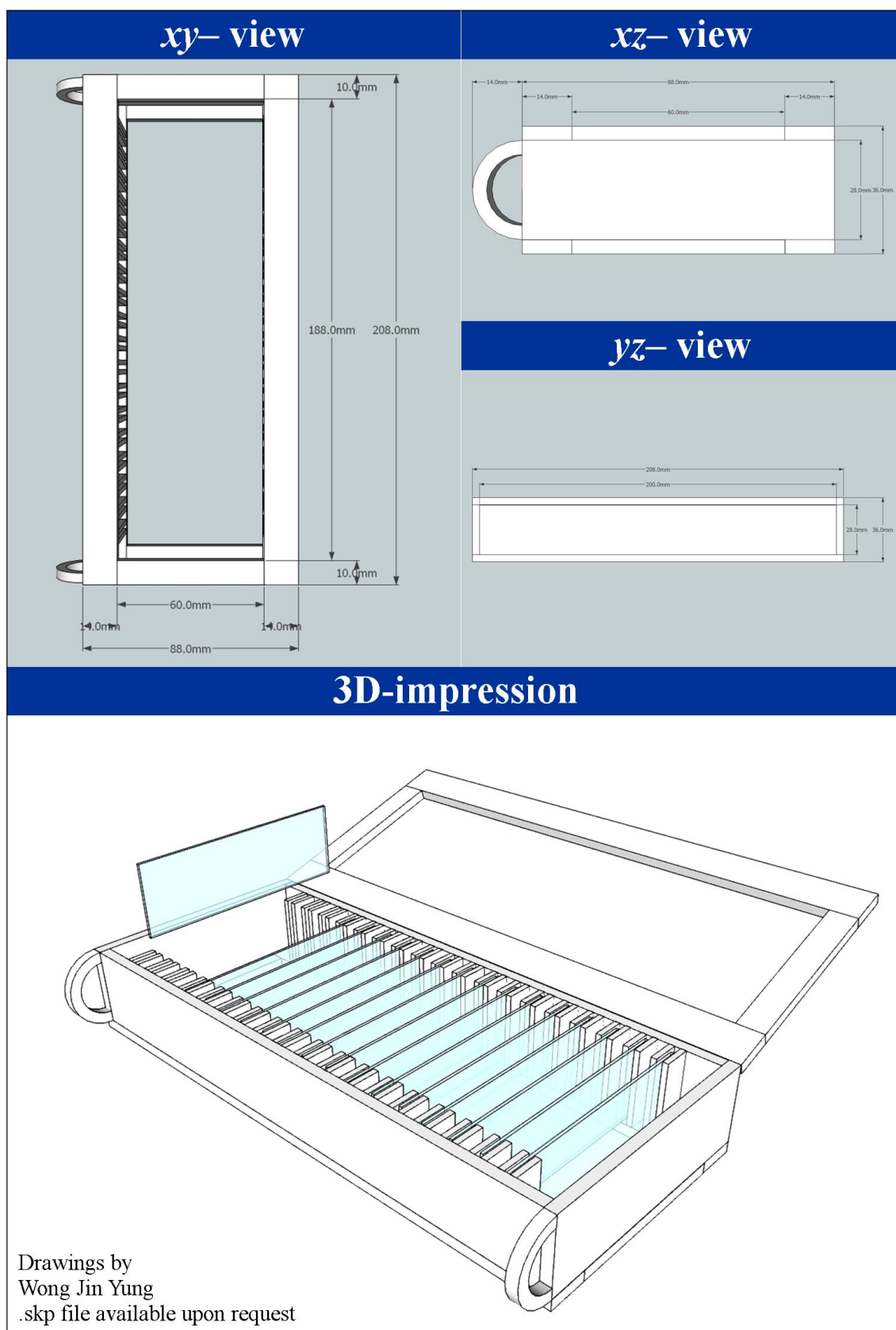
**Appendix A:** Histogram showing variations of pair-wise genetic distances computed from 12S-rRNA gene fragment sequences using Kimura 2-parameter model. Note the distribution of within-species variations does not overlap with that of inter-species variation.



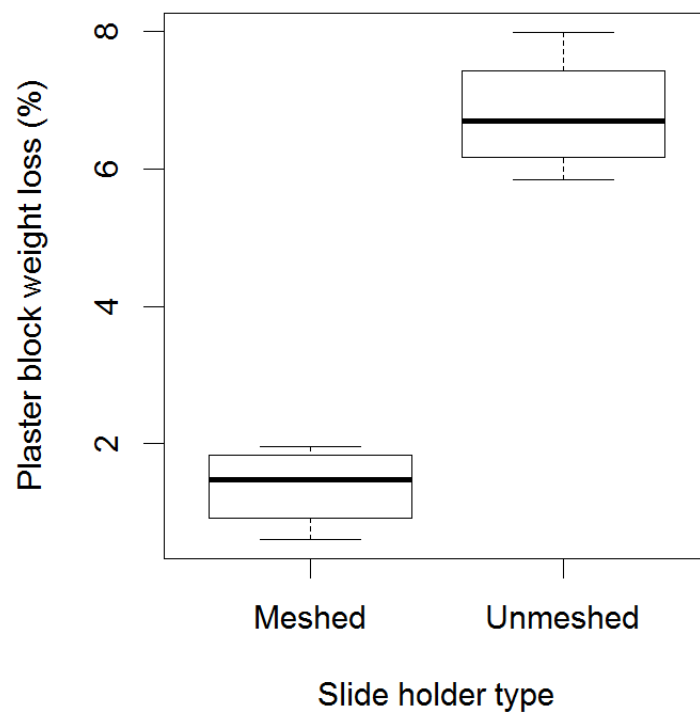
**Appendix B:** Preliminary study using glass slides as fouling substrate. Barnacles settled in large numbers within a month, with hundreds of them on each slide. (This is an earlier version of custom made slides holder).



## Appendix C: Detailed design of the glass slide holder prototype.



**Appendix D:** Effect of wire mesh cover on the water flow inside the slide holder. The slide holders, encased with wire mesh or without the wire mesh, were fixed with small blocks of Plaster of Paris and deployed in the field for three days ( $n=4$  in each type). Percentage loss of weight of the plaster blocks ((weight after – weight before)/weight before  $\times 100\%$ ) was measured as an indication of the strength of water flow (Doty, 1971).



**Appendix E:** Sampling scheme for 2012 sampling. Sacrificial sampling was performed by selecting the slides randomly from each slide holders. Good interspersation (of both the treatments within blocks, and slide purpose within each holder) was checked to avoid experimental bias. Analysis of wettability was abandoned as appropriate equipment was not available.

Slide no.	Block 1				Block 2				Block 3				Block 4				Block 5			
	Treatment				Treatment				Treatment				Treatment				Treatment			
	0	2	3	1	1	3	2	0	2	0	1	3	3	1	0	2	2	0	3	1
1	B	W	D	B	B	W	D		C	B	W	B	B	B		C	C		B	B
2		B	B	D	C	B	W	B	B		B	D	C	D	B	D	D	B	C	D
3	B	D	B	C	W	D	B		B		D	C	W	C	B	D	D	B	W	C
4		C	D	D	B	C	D	B	D		C	B	B	B		W	W		B	B
5		D	C	B	D	D	B		W	B	D	W	D	W		B	B		D	W
6		B	W	W	D	B	C		D		B	D	D	D		B	B		D	D
7		B	D	D	B	B	C	B	W	B	B	W	B	W	B	D	D		B	W
8	B	D	W	C	D	D	B		B		D	B	D	B		C	C		D	B
9		D	B	W	C	D	D		D		D	B	C	B		W	W	B	C	B
10		B	D	B	B	B	W	B	B		B	C	B	C	B	D	D		B	C
11	B	C	B	D	W	C	D		C		C	D	W	D		B	B	B	W	D
12		W	C	B	D	W	B		D	B	W	D	D	D		B	B		D	D
13	B	D	D	D	W	D	B		B		D	B	W	B		C	C	B	W	B
14		B	B	W	D	B	C		D		B	W	D	W	B	D	D		D	W
15		C	W	B	B	C	D	B	D	B	C	D	B	D		B	B		B	D
16	B	W	C	C	B	W	D	B	W	B	W	D	B	D		B	B		B	D
17		D	B	D	C	D	W		B		D	B	C	B		D	D		C	B
18		B	D	B	D	B	B		C		B	C	D	C	B	W	W	B	D	C

Slide no.	Block 6				Block 7				Block 8				Block 9			
	Treatment				Treatment				Treatment				Treatment			
	3	1	2	0	1	2	0	3	0	3	1	2	1	0	2	3
1	D	C	D	B	D	B		C		B	D	B	B		W	B
2	W	B	B		B	D	B	D	B	D	W	C	C		B	D
3	B	B	B		B	C		D		C	B	W	W	B	D	C
4	D	D	D	B	D	D	B	W		B	D	B	B		C	D
5	B	W	C		C	B		B	B	W	B	D	D	B	D	B
6	C	D	W		W	W		B	B	D	C	D	D		B	W
7	C	W	D	B	D	D	B	D		W	C	B	B		B	D
8	B	B	W		W	C	B	C		B	B	D	D		D	C
9	D	D	B		B	W		W		B	D	C	C	B	D	W
10	W	B	D		D	B		D	B	C	W	B	B		B	B
11	D	C	B		B	D		B	B	D	D	W	W	B	C	D
12	B	D	C	B	C	B		B		D	B	D	D		W	B
13	B	B	D		D	D	B	C	B	B	B	W	W	B	D	D
14	C	D	B		B	W		D	B	W	C	D	D		B	W
15	D	D	W	B	W	B		B		D	D	B	B		C	B
16	D	W	C	B	C	C		B	B	D	D	B	B		W	C
17	W	B	B		B	D	B	D		B	W	C	C	B	D	D
18	B	C	D		D	B		W		C	B	D	D		B	B

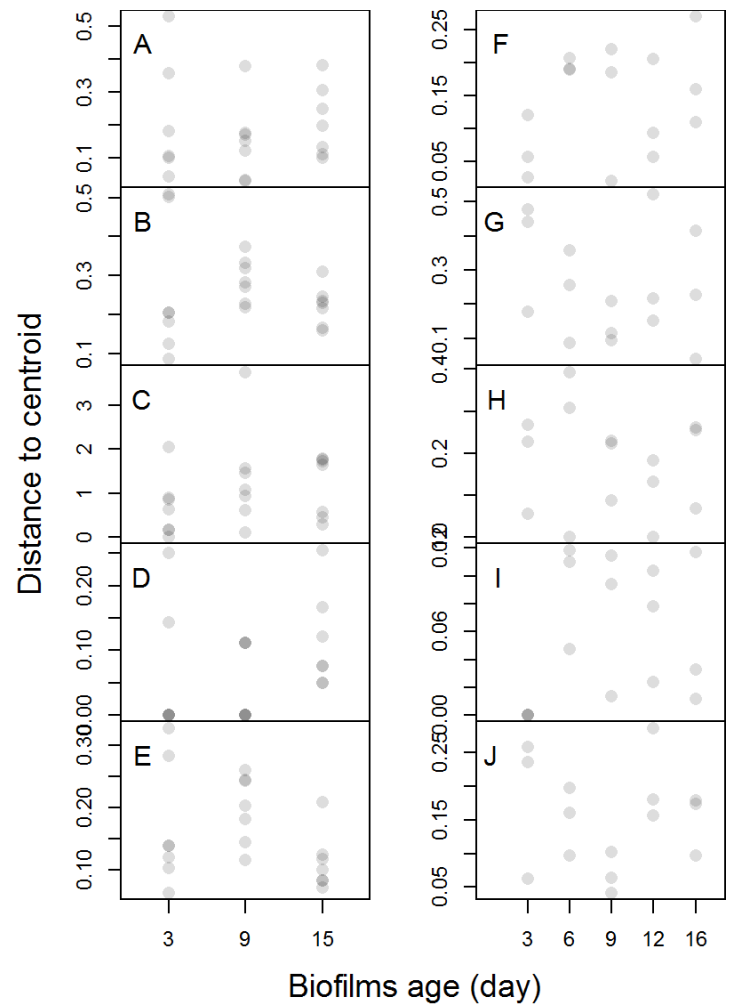
  

Legend	
Treatment	
0	Control
1	3 days
2	9 days
3	16 days
Slide purposes	
D	DGGE
C	CLSM
W	Wettability
B	Barnacle counts
	Empty

**Appendix F:** Summary of differences between 2011 sampling and 2012 sampling for experiment of effects of biofilm age on barnacle settlement (3.2.2.2) and analysis of biofilm structure (3.2.6).

	2011 sampling	2012 sampling	Remarks
Levels of biofilm age	5 (3-,6-,9-,12-,16-days)	3 (3-,9-,16-days)	Figure 3.3
Support frame for glass slide holder	single	multiple	Figure 3.4
Sampling period	NE monsoon	SW monsoon	-
Experimental design	CRD (completely randomised design)	RCBD (randomized complete block design)	-
CLSM analysis of biofilm	DAPI staining	SYTO 9 and WGA-TRITC staining	3.2.6

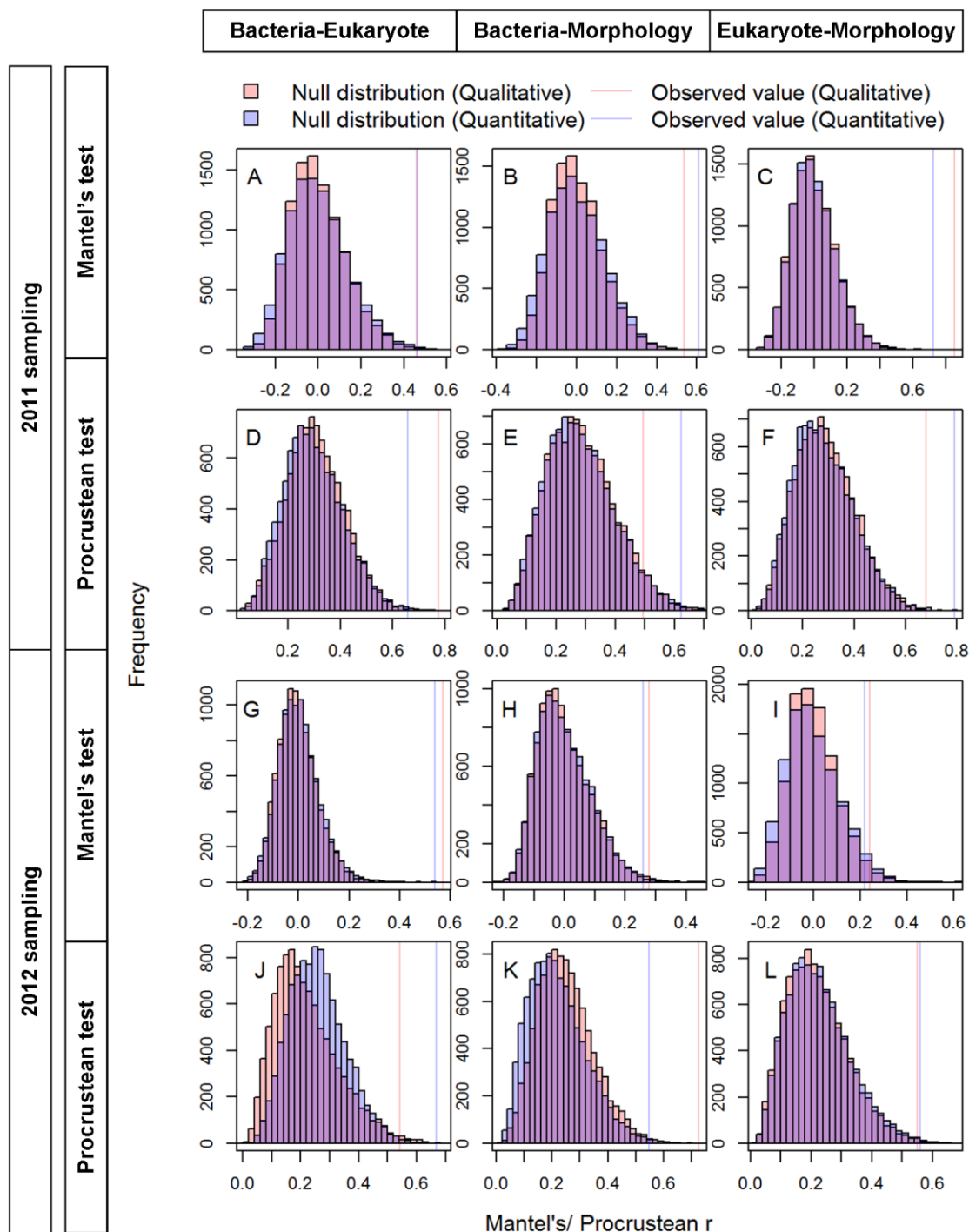
**Appendix G:** Distance to centroid of each age group calculated from dissimilarity distance matrices, as a diagnostic of within group dispersion for permutational MANOVA analysis. (A-E) 2012 sampling (F-J) 2011 sampling. (A, F) microeukaryotic semi-quantitative DGGE (B, G) bacterial semi-quantitative DGGE (C, H) CLSM (D, I) microeukaryotic qualitative DGGE (E, J) bacterial qualitative DGGE.



**Appendix H:** Procrustean test between qualitative and semi-quantitative DGGE profiles.

	2011		2012	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Qualitative vs Semi-quantitative bacterial DGGE profiles	0.97	<0.0001	0.96	<0.0001
Qualitative vs Semi-quantitative microeukaryotic DGGE profiles	0.95	<0.0001	0.94	<0.0001

**Appendix I:** Null distributions of Mantel's  $r$  and Procrustean  $r$  from 10000 permutations, and the observed values of the samples they were drawn from. Note that the overlapped parts of the histograms appear as purple colour. Refer to table 3.5 for summarized results.





**Appendix J:** List of studies on effect of biofilm on barnacle settlement

<b>Barnacle</b>	<b>Biofilm</b>	<b>Experiment</b>	<b>Effect</b>	<b>Factor influence biofilm effect</b>	<b>Reference</b>
<b>Monospecies biofilms</b>					
<i>Amphibalanus amphitrite</i>	Monospecies bacteria (18 different strains)	Laboratory	Inhibition/ no effect	Depends on biofilm age and bacteria strains	Maki <i>et al.</i> (1988)
<i>Amphibalanus amphitrite</i>	Monospecies bacteria (16 different strains)	Laboratory	Facilitation/ inhibition	Depends on biofilm age, cyprid age and substrate type	Maki <i>et al.</i> (1990)
<i>Amphibalanus amphitrite</i>	Monospecies bacterium (unidentified)	Laboratory	Inhibition	Depends on biofilm age	Holmström <i>et al.</i> (1992)
<i>Amphibalanus amphitrite</i>	Monospecies bacteria of <i>Aeromonas</i> , <i>Alcaligenes</i> , <i>Flavobacterium</i> , <i>Pseudomonas</i> , and <i>Vibrio</i> groups	Laboratory	Mostly Inhibition/ no effect	-	Mary <i>et al.</i> (1993)
<i>Elminius modestus</i>	Monospecies ( <i>Deleya marina</i> )	Laboratory	Facilitation	Depends on biofilm age	Neal & Yule (1994a)
<i>Balanus improvisus</i> , <i>Balanus eburneus</i>	Monospecies bacteria ( <i>Deleya marina</i> , <i>Alteromonas macleodii</i> , <i>Pseudomonas fluorescens</i> Migula)	Laboratory/ field	Facilitation/ no effect/ inhibition	Depends on cyprid age, salinity of settlement test, substrate type, bacteria strains	O'Connor & Richardson (1996)

## Appendix J (continued)

Barnacle	Biofilm	Experiment	Effect	Factor influence biofilm effect	Reference
<i>Amphibalanus amphitrite</i>	Monospecies bacteria ( <i>Deleya marina</i> , <i>Alteromonas macleodii</i> , <i>Pseudomonas fluorescens</i> Migula)	Laboratory	Facilitation/ no effect/ inhibition	Depends on cyprid age, substrate type, bacteria strains	O'Connor & Richardson (1998)
<i>Amphibalanus reticulatus</i>	Monospecies bacteria/ diatom (unidentified)	Laboratory	Facilitation/ inhibition	Depends on bacteria strain and density of bacteria	Thiyagarajan <i>et al.</i> (1999)
<i>Amphibalanus amphitrite</i>	Monospecies bacteria (12 different strains)	Laboratory	Inhibition/ no effect	Depends on bacteria strain	Lau & Qian (2000)
<i>Amphibalanus amphitrite</i>	Monospecies bacterium ( <i>Halomonas marina</i> )	Laboratory	Inhibition	Biofilm developed on petri dish is inhibitive but not EPS extracts	Maki <i>et al.</i> (2000)
<i>Amphibalanus amphitrite</i>	Monospecies bacterium ( <i>Halomonas marina</i> )	Laboratory	Inhibition	-	Perry <i>et al.</i> (2001)
<i>Amphibalanus amphitrite</i>	Monospecies bacteria (10 different strains from the genus <i>Pseudoalteromonas</i> )	Laboratory	Inhibition/ no effect	Depends on bacteria strain	Holmström <i>et al.</i> (2002)

## Appendix J (continued)

Barnacle	Biofilm	Experiment	Effect	Factor influence biofilm effect	Reference
<i>Amphibalanus amphitrite</i>	Monospecies bacteria ( <i>Pseudomonas aeruginosa</i> , <i>Bacillus pumilus</i> and <i>Citrobacter freundii</i> )	Laboratory	Facilitation/ no effect	Depends on bacteria strains (facilitation by <i>P. aeruginosa</i> while no effect for the rest), effect negated by lectin-binding	Khandeparker <i>et al.</i> (2003)
<i>Amphibalanus amphitrite</i>	Monospecies bacteria ( <i>Micrococcus</i> sp., <i>Rhodovulum</i> sp., and <i>Vibrio</i> sp.)	Laboratory	Inhibition	Depends on the bacteria density, effect remained even if the films were killed	Lau <i>et al.</i> (2003)
<i>Amphibalanus amphitrite</i>	Monospecies diatoms <sup>a</sup> ( <i>Amphora coffeaeformis</i> , <i>A. rostrata</i> , <i>Navicula transitans</i> var. <i>derasa</i> f. <i>delicatula</i> , <i>N. crucicula</i> and <i>N. subinflata</i> )	Laboratory	Facilitation/ no effect	Depends on diatom species	Patil & Anil (2005)
<i>Amphibalanus amphitrite</i>	Monospecies diatoms of 23 different strains	Laboratory	Facilitation/ Inhibition	Depends on the strain of diatoms, diatom density, presence of bacteria	Jouuchi <i>et al.</i> (2007)
<i>Amphibalanus improvisus</i>	Monospecies bacteria/ mixed	Field	Inhibition/ no effect	Depends on temperature and larval supply	Nasrolahi <i>et al.</i> (2012)

# Appendix J (continued)

Barnacle	Biofilm	Experiment	Effect	Factor influence biofilm effect	Reference
<b>Mixed biofilms</b>					
<i>Elminius modestus</i> , <i>Balanus perforates</i>	Mixed; developed with NSW	Laboratory	No effect/ inhibition	Depends on biofilms growing conditions (shear) and barnacle species ( <i>E. modestus</i> : no effect; <i>B. perforates</i> : inhibitive)	Neal & Yule (1994b)
<i>Amphibalanus variegatus</i> , <i>Elminius modestus</i>	Mixed; developed with NSW/ field developed	Field	Inhibition	Depends on biofilm age	Keough & Raimondi (1995) <sup>c</sup>
<i>Amphibalanus amphitrite</i>	Mixed; developed with NSW	Laboratory	Facilitation	Depends on biofilm age	Wieczorek <i>et al.</i> (1995)
<i>Semibalanus balanoides</i>	Mixed; field developed	Field/ laboratory	Facilitation	Depends on biofilm age, origin (tidal zones) of biofilms and presence of conspecific cues	Thompson <i>et al.</i> (1998) <sup>c</sup>
<i>Amphibalanus amphitrite</i>	Mixed; field developed	Laboratory	Facilitation	Depends on biofilm volume	Tsurumi & Fusetani (1998)
<i>Amphibalanus amphitrite</i>	Mixed; field developed	Field	Inhibition	Depends on biofilm age and origin (tidal zone)	Olivier <i>et al.</i> (2000) <sup>c</sup>

# Appendix J (continued)

Barnacle	Biofilm	Experiment	Effect	Factor influence biofilm effect	Reference
<i>Amphibalanus amphitrite</i>	Mixed; field developed	Laboratory	Facilitation	Depends on biofilm age, origin (tidal zone), correspond to the changes of bacterial communities	Qian <i>et al.</i> (2003)
<i>Amphibalanus amphitrite</i>	Mixed; developed with NSW	Laboratory	Inhibition	Depends on biofilm age	Famaili <i>et al.</i> (2004)
<i>Amphibalanus amphitrite</i> , <i>Balanus trigonus</i>	Mixed; developed with NSW	Laboratory	Facilitation/ no effect/ Inhibition	Depends on biofilms culture temperature (high-medium temperature: facilitation; low temperature: no effect/ inhibition)and barnacle species. Correspond to changes in bacterial communities	Lau <i>et al.</i> (2005)
<i>Balanus trigonus</i>	Mixed; field developed	Field/ laboratory	Facilitation/ no effect	Depends on biofilm origin (tidal zone; intertidal: no effect, subtidal: facilitation), correspond to difference in bacterial communities	Thiyagarajan <i>et al.</i> (2006)
<i>Fistulobalanus albicostatus</i>	Mixed; developed with natural sea water (NSW)	Laboratory	Facilitation	Depends on biofilm age and microbes size which the biofilms originated from	Chen <i>et al.</i> (2007)
<i>Amphibalanus amphitrite</i>	Mixed; field developed	Field/ laboratory	Facilitation	Depends on origin (sites) of biofilms, correspond to the difference in bacterial communities	Hung <i>et al.</i> (2007)

## Appendix J (continued)

Barnacle	Biofilm	Experiment	Effect	Factor influence biofilm effect	Reference
<i>Amphibalanus amphitrite</i>	Mixed; field developed	Laboratory	Facilitation	Depends on biofilm age and cyprid age. Young cyprids preferred biofilm of older age regardless of surface wettability, old cyprids could not differentiate	Hung <i>et al.</i> (2008)
<i>Amphibalanus amphitrite</i>	Mixed; developed with NSW	Laboratory	Facilitation <sup>b</sup>	-	Zardus <i>et al.</i> (2008)
<i>Amphibalanus amphitrite</i>	Mixed; cultured from natural biofilms using media	Laboratory	Facilitation/ no effect	Depends on biofilms origin (barnacle shell: facilitation, rock surface: no effect)	Bacchetti De Gregoris <i>et al.</i> (2012)

<sup>a</sup> Using extracts of biofilms only












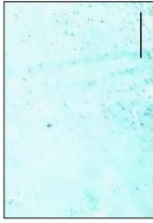



<sup>b</sup> Testing of the effect on the strength of larval adhesion rather than settlement

<sup>c</sup> Studies most similar to the present study in terms of biofilm type, experiment type, effect and factor (field developed biofilm, field settlement assay, facilitation effect and affected by biofilm age)

## Appendix K: Tree perimeter by treatment


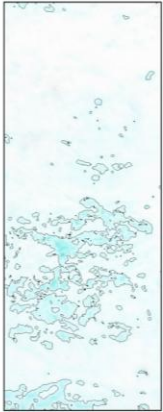









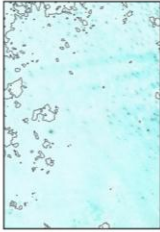



Index	Perimeter at 0.9m (cm)	Block
<i>Control</i>		
1	15.2	1
3	14.3	2
6	7	3
7	18.7	4
14	15.4	5
36	20.2	6
39	12.7	7
42	17	8
44	12	9
<i>Open</i>		
31	17.5	1
2	12.8	2
34	15.5	3
8	15	4
10	11	5
38	19	6
40	10.9	7
43	10.1	8
45	17.2	9
<i>Exclusion</i>		
13	11.4	1
32	13.8	2
33	12.4	3
11	18	4
35	12.8	5
37	19	6
41	12.1	7
17	18.8	8
25	15.6	9

**Appendix L:** The original images of the Alcian Blue stained artificial substrate in Exclusion experiment II. Scale bar denotes 2cm. Yellow arrows indicate possible mucus trails left by littorinids, which could be stained by Alcian Blue too.

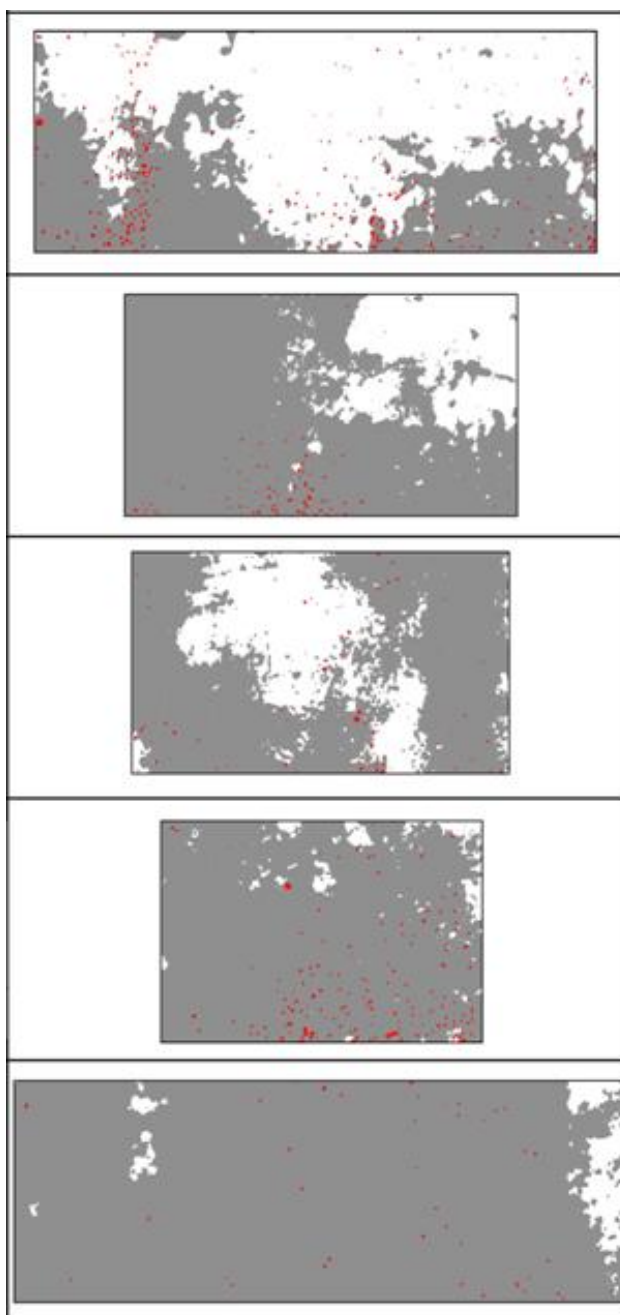
	Control	Open Cage	Exclusion
Block 4			
Block 5			
Block 7			
Block 8			
Block 9			



**Appendix L (continued):** Images overlaid with thresholding selection

	Control	Open Cage	Exclusion
<b>Block 4</b>			
<b>Block 5</b>			
<b>Block 7</b>			
<b>Block 8</b>			
<b>Block 9</b>			

**Appendix M:** Positions of the barnacle juveniles/ cyprids (red spots) on the substrates and the area covered by biofilms (grey shades), as in Exclusion treatment for exclusion experiment II.



## **Appendix N:** List of publication and presentations.

### *Publication:*

Wong, J. Y., Chen, H.- N., Chan, B. K. K., Tan, I. K. P., & Chong, V. C. (2014). A combined morphological and molecular approach in identifying barnacle cyprids from the Matang Mangrove Forest in Malaysia: essentials for larval ecology studies. *Raffles Bulletin of Zoology*, 62, 317-324.

### *Oral presentation:*

Wong, J. Y., Chen, H.- N., Chan, B. K. K., Tan, I. K. P., & Chong V. C. (2014, Jan). *Large scale identification of barnacle cyprids*. Presented at the 18<sup>th</sup> Biological Science Graduate Congress, University of Malaya, Kuala Lumpur, Malaysia.

### *Poster presentations:*

Wong, J. Y., Kong, C. S. Y., Tan, I. K. P., & Chong, V. C. (2014, Jul). *Effect of littorinid grazers on biofilm growth and recruitment of barnacles: implications to mangrove rehabilitation*. Presented at 2<sup>nd</sup> International Symposium “Current Topics on Barnacle Biology”, National University of Singapore, Singapore.

Wong, J. Y., Chong, V. C., & Tan, I. K. P. (2011, Dec). *Successional concordance among structure, bacterial and microeukaryotic communities of natural biofilm*. Presented at the 16<sup>th</sup> Biological Science Graduate Congress, National University of Singapore, Singapore.

Wong, J. Y., Tan, I. K. P., & Chong, V. C. (2011, Jun). *Hubungan antara ‘microfouling’ oleh biofilem dan ‘macrofouling’ oleh teritip*. Presented at Seminar Kebangsaan Projek-projek Penyelidikan & Pembangunan (R&D) bagi Hutan Pesisiran Pantai di Malaysia, Institut Penyelidikan Perhutanan Malaysia (FRIM), Kuala Lumpur, Malaysia.

## A combined morphological and molecular approach in identifying barnacle cyprids from the Matang Mangrove Forest Reserve in Malaysia: essentials for larval ecology studies

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**Abstract.** Identification of larval mesoplankton is essential to the study of the supply-side ecology of marine benthic or sessile organisms, such as barnacles. Combined morphological and molecular identifications of wild-caught barnacle cyprids from Matang Mangrove Forest Reserve (MMFR), Malaysia were studied based on mitochondrial 12S-rRNA gene sequences of the unidentified larvae and identified adults. Six species of barnacle adults and cyprids had matched DNA sequences. These included *Fistulobalanus pattellaris*, *Fistulobalanus* sp., *Amphibalanus reticulatus*, *Amphibalanus variegatus*, *Amphibalanus amphitrite*, and *Euraphia withersi*. Morphological characters of the identified cyprids were described, and used to develop a morphology-based classification tree. Carapace sculpturing pattern on the cyprids was the most important morphological discriminator. Preliminary analysis of the diversity of barnacle cyprids in MMFR showed that the dominant species could be morphologically classified with high accuracy.

**Key words.** larval ecology, barnacle cyprids, classification, DNA barcoding, mangrove

### INTRODUCTION

Thoracican barnacles are important filter feeders in the mangrove food web (Fry & Smith, 2002) and can play a role in the filtration function of mangroves (Soares-Gomes et al., 2010). They are common on the surface of roots, trunks, and leaves of mangrove plants, fallen propagules and plant debris, and shells of crustaceans and molluscs. In replanted mangrove system, barnacles are considered as pests because their settlement on the stems and leaves can result in mortality or reduced fitness of mangrove seedlings (Perry, 1988; Li & Chan, 2008; Li et al., 2009). In fact, barnacle infestation on newly replanted mangrove seedlings is recognised as one of the important problems in mangrove rehabilitation (Angsupanich & Havanona, 1996; Primavera & Esteban, 2008).

The life cycle of thoracican barnacles is composed of both planktonic larval and sessile adult stages. The planktonic larvae include six naupliar stages and a final cyprid stage prior to settlement. The distribution of the barnacle cyprids in the water column is patchy on spatial and temporal scales (Pineda, 2000) which can affect the subsequent recruitment

dynamics of adults (Grosberg, 1982; Pineda et al., 2002), including those that inhabit the mangrove ecosystem (Ross & Underwood, 1997; Satumanatpan et al., 1999; Ross, 2001; Satumanatpan & Keough, 2001). In replanted mangroves at Ban Don Bay, Thailand (Angsupanich & Havanona, 1996), and Haji Dorani, Malaysia (Tan, 2013), the pulse recruitment of barnacle cyprids is often intense, resulting in rapid cover by barnacles on the replanted mangrove. The supply-side ecology of barnacle cyprids is, therefore, important to understanding the distribution and larval settlement processes of barnacles in mangroves. However, the remarkable similarity of cyprid morphology among species (Elfimov, 1995) and lack of detailed morphological descriptions of larvae of many species make identification difficult and pose a major obstacle to the study of barnacle supply-side ecology.

At present, descriptions of barnacle cyprids are mostly dependent upon laboratory-reared larvae. There are very few morphological keys for the identification of wild caught barnacle cyprids. Such keys are often limited in their usefulness. For instance, the guide developed by Standing (1981) pertains to only the cyprids of Oregon waters in U.S.A. A guide has yet to be developed for barnacle cyprids for any particular region in the tropics. Moreover, larval culture itself poses several challenges in terms of suitability of larval feed and rearing conditions to ensure sufficient larval survival. Molecular techniques which enable accurate species identifications could dispense with the need for larval culture. For example, DNA barcoding has been extensively used for species identification in recent years. By matching a chosen region of DNA fragments from the specimen with known reference specimens, identification can be achieved (Hebert et al., 2003). The method is very useful for the identification of species with different life stages, if the adult

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can be confidently identified by morphology but not for the young stages. Chen et al. (2013) have shown that DNA barcoding based on mitochondrial COI sequences is suitable for identifying wild-caught barnacle cyprids including those from possible invasive species. Other markers used to resolve barnacle taxonomic problems and biodiversity surveys include the 12S and 16S rRNA genes and nuclear ITS1 region (e.g., Chan et al., 2007a–c, 2009; Tsang et al., 2009; Chen et al., 2012; Cheang et al., 2012).

The Matang Mangrove Forest Reserve (MMFR) is the largest mangrove forest in the peninsular Malaysia and has attracted extensive ecological and scientific interest (Shaharuddin et al., 2007). The numerous creeks and channels with diverse water conditions, from the upper estuary to near shore waters, provide suitable habitats for barnacle colonisation on the fringing mangrove vegetation as well as on the numerous fish stakes, jetty pilings, and floating fish cages. Barnacle diversity in the MMFR waters has not been reported except for one biofouling study on floating fish cages, where *Balanus amphitrite* (= *Amphibalanus amphitrite*) was identified as the only species (Madin et al., 2009). The present study aimed to use a combined morphological and molecular approach to identify and describe the barnacle cyprids of MMFR, and to provide diagnostic morphological characters to identify barnacle cyprids in MMFR in Malaysia.

## MATERIAL AND METHODS

**Collection of barnacle cyprids and adults.** Specimen collections were made from the upper estuary in the Matang Mangrove Forest Reserve (MMFR) as far as the coastal waters (< 12 km offshore) on two separate sampling occasions, one on 20–21 April 2011 and the other on 25–26 June 2012 (see Fig. 1 for the location of sampling sites). Adult barnacles were collected as species references, using a hammer and chisel to detach the animals from their substrates, including mangrove tree trunks and roots, and buoy for oyster culture. Multiple surface plankton samples were collected by a standard plankton net of 160 µm mesh size (45 cm mouth diameter) towed for either 5 or 10 min each. All collected specimens were immediately preserved in 95% ethanol for further analyses.

**Morphological analyses.** The adult barnacles were identified to species level based on their morphology and served as the adult reference collection for subsequent comparison. All barnacle cyprids were first sorted out from the plankton samples under a stereo microscope (Olympus SZX7). Approximately 250 cyprids that could represent the full range of observed morphological variations were selected for analysis. Photos of the lateral view of the selected set of cyprids were taken under normal bright field of a compound microscope (Zeiss Axio Scope A1) equipped with a camera (Panasonic Lumix G1). A series of photos at differential focus were taken for each larva and integrated into an extended-focus image using the iSolution Lite image processing software (i-Solution Inc., Vancouver, Canada) for optimal viewing and measurement. Morphometric measurements of the carapace of each cyprid were then

taken from the extended-focus images using ImageJ (version 1.44; U.S. National Institute of Health, available at <http://imagej.nih.gov/ij/>). The measurements included carapace length (maximum distance between anterior and posterior margin), carapace height (maximum distance between dorsal and ventral margin), posterior carapace angle (angle formed by extension of dorsal and ventral margin), and calculated ratio of length-to-height (Fig. 2; also see Chen et al., 2013). Carapace sculpturing was examined, described, and recorded in addition to the morphometric measurements. Since not all the cyprids had their antennules and thoracic appendages extended, measurements were restricted to the carapace only.

**Scanning Electron Microscopy (SEM).** Morphology and carapace sculpturing patterns of cyprids initially observed under light microscopy were further observed by scanning electron microscopy (SEM). Cyprids preserved in 95% ethanol were transferred into acetone, critical point dried, and coated with gold palladium before observation with a FEI Quanta 200 Scanning Electron Microscope (methods follows Chan & Leung, 2007). Measurements related to the carapace sculpturing pattern were made on SEM images. Maximum

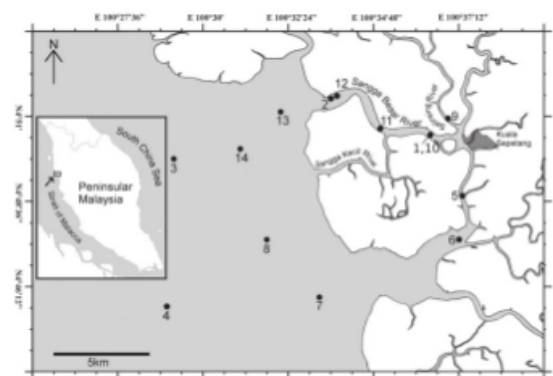


Fig. 1. Map of sampling locations at Matang Mangrove Forest Reserve (MMFR) in Perak, Malaysia. Sampling was carried out in April 2011 at sites 1–8 and in June 2012 at sites 9–14.

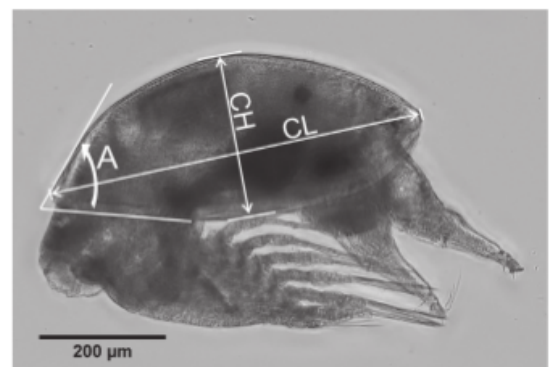


Fig. 2. Lateral view of cypris larvae of barnacle showing measurements used for morphometric analysis. CL: carapace length; CH: carapace height; A: posterior carapace angle. Ratio of CL/CH was also calculated.

feret diameter (largest distance between two parallel planes restricting an object) was used to measure the size of the ultrastructures if the use of diameter was not appropriate.

**DNA extraction, PCR and sequencing.** Total genomic DNA from adult and larval tissue was extracted using DNeasy blood and tissue extraction kit (Qiagen GmbH, Germany) after the cirripedes were identified and morphological measurements made. A faster alternative extraction method using extraction buffer containing 5% (w/v) Chelax®-100 resin (Bio-Rad, California, USA) was used only for cyprids DNA extraction (Walsh et al., 1991). For DNA extraction using the tissue extraction kit, soft tissue (~25 mg) of adult barnacle or whole barnacle cyprids were used for DNA extraction following the manufacturer's instructions. Polymerase chain reaction (PCR) was used to amplify a region of the mitochondrial 12S-rRNA gene from the DNA using forward primer 5'-GACCGTGCTAAGGTAGCATAATC-3' (Tsang et al., 2009) and reverse primer 5'-CCGGTCTGAACTCAAATCGTG-3'. Amplification was performed using reaction mixture containing 2 µL of template DNA, 12 µL Taq master mix (1.5 mM MgCl<sub>2</sub> type; Ampliqon, Denmark), 0.05 µM of each primer, and ddH<sub>2</sub>O to a total volume of 20 µL. PCR conditions were set as follows: 2 min and 30 sec at 94°C for initial denaturation, then 30 cycles of 30 s at 95°C, 30 s at 48°C, and 1 min at 72°C, with final extension for 5 min at 72°C. Sequencing was performed using an ABI 3730 XL DNA analyser with BigDye terminator cycle sequencing reagents kit (Applied Biosystems, California, USA).

**Sequence analyses.** Cyprids were identified through comparison of their 12S-rDNA sequences with that of the identified adult barnacles. All sequences (including 207 cyprid sequences successfully obtained from the selected set, 16 adult sequences and three outgroup sequences from GenBank) were first aligned using MUSCLE (Edgar, 2004) using the default settings, and these were then manually inspected. The three outgroups used were *Verruca laevigata* (JX083933.1), *Metaverruca recta* (JX083931.1), and *Rostratoverruca krugeri* (JX083932.1). A neighbour-joining tree was constructed from the aligned sequences using MEGA 5.05 (Tamura et al., 2011), with a Kimura 2-parameter (K2P) model used to compute the genetic distances. Bootstrapping was conducted with 1000 replicates to estimate the reliability of the inferred tree. When the sequences of cyprids and adult references formed a "monophyletic" clade with high bootstrap support, it was considered to be the same species. Monophyletic groups that failed to cluster with any adult references were then considered as an operational taxonomy unit (OTU). To assess the strength of the current 12S sequence fragments for DNA barcoding purposes, the pair-wise genetic distances of all of the sequences (except outgroups) computed from the K2P model were also summarised to show the between—and among—clade genetic divergence.

**Statistical analyses and construction of morphology-based classifier.** Of 207 sequenced cyprids from the selected set, only 183 were used for the morphological analysis due to the exclusion of cyprids with low quality images.

With species identity determined from the DNA barcoding analysis, morphology-based classification models were then constructed. The classification models would be used for quick preliminary classification and to show the effect of adding carapace sculpturing as a morphological variable to differentiate cyprids. The classification tree algorithm method was chosen over LDA (linear discriminant analysis) because it can handle mixed inputs of predictor variables (both quantitative and qualitative variables), and is easier to interpret (De'ath & Fabricius, 2000). Furthermore, classification trees are not limited by the number of samples used in each group (i.e., species), whereas LDA requires the number in each group to be not less than the number of variables. This is a problem for the present study as the specimen numbers of *A. amphitrite*, OTU 1 and OTU 2 were low in the training dataset. Two models of classification tree were constructed and compared, i.e., one with only quantitative morphological characters, and the other with both quantitative and qualitative (carapace sculpturing) morphological characters. The performance of the tree classifiers was evaluated using multiple runs of 5-fold cross validation. Within each run, the dataset of the selected cyprids was randomly partitioned into five subsets; four subsets were used as training sets and one subset was used as a validation set. This process was repeated until each subset had been used once as a validation set. The cross validation was then repeated for 100 runs and the misclassification rate of the classifier was estimated from the average over the 100 runs. All statistical analyses were conducted using R (version 2.13.0; R Development Core Team, 2011). R package 'tree' (Ripley, 2011) was used for classification trees.

**Application of morphology-based classifier.** The decision tree classifier was then utilised to aid the classification of the remaining cyprid specimens to give a preliminary view of the species composition of cyprids at different locations in MMFR. To achieve this, the remaining collection was identified and counted under a compound microscope, and photos were taken as measurements which were needed before a decision on species identity could be made for individual species/OTU. The decision on species identity was assisted by the tree classifier.

## RESULTS

**Identification of adult barnacle.** Six species of adult barnacle from MMFR were identified to species level, namely *Fistulobalanus patellaris*, *Fistulobalanus* sp. (an undescribed species), *Amphibalanus reticulatus*, *Amphibalanus variegatus*, *Amphibalanus amphitrite*, and *Euraphia withersi*.

**Molecular analyses.** Partial sequences of 12S-rRNA gene were successfully obtained from 207 individuals of cyprids and 16 individuals of barnacle adults. A neighbour-joining tree constructed from the sequences is shown in Fig. 3. Eight distinct clades were observed and six clades (including 195 cyprids sequences) had the sequences from the identified adult references. Two of the clades (comprising 12 of the cyprid sequences) with no matching adult sequence were

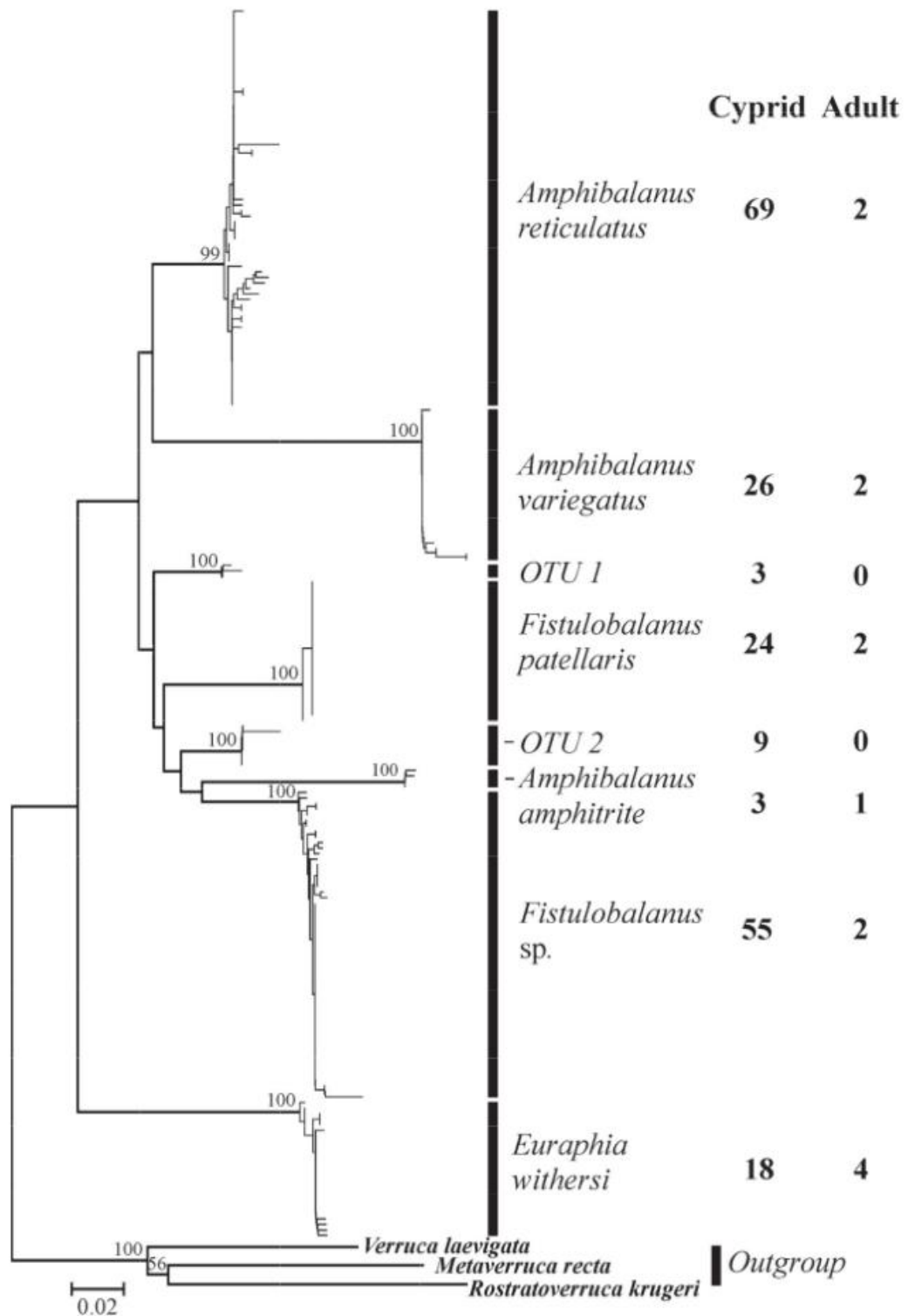


Fig. 3. Neighbour-joining tree constructed from partial 12S-rRNA gene fragment sequences of cyprids and adults of barnacle. The sequences were clustered into eight clades, and species name were labelled at the clades containing sequence(s) of identified adult of barnacle. Clades with no sequence of identified barnacle adult clustered within were designated as OTU (Operational Taxonomic Unit). Number of sequences in each clade were also shown. Scale bar denotes 0.02 base substitution per site.



designated as Operational Taxonomic Unit or OTU 1 and OTU 2. The mean within-species pairwise K2P distance was 0.6% (ranged from 0–3.5%) while the mean between-species distance was 13.5% (ranged from 5.4–25%). The non-overlapping ('barcode gap') of frequency distribution of pairwise K2P distance for within- and between-species suggests the suitability of the approach for barcoding purposes (Fig. 4).

**Morphological analyses.** In the present study, the range of carapace length from all cyprids collected was 439–685  $\mu\text{m}$ , and the range of carapace height was 199–329  $\mu\text{m}$ . The variations in the four quantitative morphometric characters of the carapace, namely length, height, angle and length-to-height ratio among species/ OTU are shown in Table 1. The carapace length and height data were also compared to those previously reported in the literature (Table 1). The sculpturing patterns were categorised into five types (details in Table 2). These carapace sculpturing patterns were not observable under a dissecting microscope and only at  $>100\times$  magnification under a compound microscope (henceforth referred to as CM). SEM which provided morphological details of high resolution confirmed the pattern classification based on the type of sculpturing pattern (Fig. 5–7). Four barnacle species (*Fistulobalanus* sp., *Fistulobalanus patellaris*, *Euraphia withersi*, and *Ampibalanus variegatus*) showed diagnostic carapace sculptures (Table 2). However, four other taxa (*A. amphitrite*, *A. reticulatus*, OTU1 and OTU2) showed no sculpturing pattern (i.e., smooth carapace). The honeycomb pattern of type A (*Fistulobalanus* sp.; Fig. 5A–D) is readily identifiable under CM. Type B pattern (*Fistulobalanus patellaris*; Fig. 5E–J) was not very apparent under CM, but was revealed under SEM. Due to their larger size, lunular pores on the ventral side were easier to observe under CM (Fig. 5G) compared to the punctae on the dorsal side (Fig. 5F). For type C pattern (*Ampibalanus variegatus*, Fig. 6A–H), the punctate pattern was observed on the ventral aspect of the carapace (Fig. 6C) but was absent on its dorsal aspect. Differentiation between the punctae of type C and the lunules of type B on the ventral aspect could only be identified under SEM (Figs. 5C & 6C, respectively). However, under CM, type C can be differentiated from type B based on the presence of punctae on both the anterior and posterior ends of the carapace in type C, whereas punctae in type B are absent in both positions. Type D is featured by ridges or folds at the posterior end of the carapace of *Euraphia withersi* (Fig. 6J). These folds extend into the ventral aspect of the carapace (not shown). *Euraphia withersi* also has unique reddish pigmentation scattered around the ventral edge of carapace (Fig. 6I, highlighted by arrows) and a dark rounded pigmentation spot posterior to the cyprid eye (Fig. 6I, circled). The reddish pigmentation, however, faded after prolonged preservation in 95% ethanol. Four other species/ OTU (*Ampibalanus reticulatus*, *Ampibalanus amphitrite*, OTU 1 and OTU 2) do not have any carapace sculpturing and were named as type E (Fig. 7). Classification of these taxa depends on their carapace size and shape, where *A. reticulatus* and OTU 1 are longer than *A. amphitrite* and OTU 2, while OTU 2 has a higher posterior carapace angle

than *A. amphitrite*. Discrimination between *A. reticulatus* and OTU 1 is difficult.

**Morphology-based classifier.** The performance of morphology-based tree classifier increased dramatically when the carapace sculpturing character was added. The estimated misclassification rate for the tree classifiers decreased from  $35.0 \pm 11.1\%$  ( $\pm\text{SD}$ ) to  $5.7 \pm 5.0\%$  ( $\pm\text{SD}$ ) respectively for datasets without and with carapace sculpturing characters. This decrease is mainly due to the increased accuracy of classification of species that have unique carapace sculpturing in the latter dataset. Low accuracy was especially a problem for species/OTU present at low abundances in the training dataset (*A. amphitrite*, OTU 1, and OTU 2).

**Assessment of cyprids distribution in MMFR.** The morphological classification model which was obtained in preceding steps (Fig. 8) was used to identify the cyprids collected at different stations in MMFR (Fig. 9). A total of 1124 and 736 cyprids were classified for the 2011 and 2012 collections, respectively. Marked differences in species composition were observed between 2011 and 2012 collections. The 2011 (April) collection was dominated by *E. withersi* and *A. reticulatus* while the 2012 (June) collection was dominated by *Fistulobalanus* sp. and *F. patellaris*. All species were found in both years except *E. withersi* which was not found in the 2012 samples. The within-year variations in species composition among stations were smaller compared to annual variability. However some differences were observed between stations, in particular the composition between the upper estuary and the rest of the stations.

## DISCUSSION

The adult barnacle species identified in this study belong to three genera, namely *Ampibalanus*, *Fistulobalanus*, and *Euraphia*, which are commonly found in tropical and sub-tropical mangrove habitats (Rainbow et al., 1989; Prabowo & Yamaguchi, 2005; Crona et al., 2006; Marques-Silva et al., 2006; Li & Chan, 2008). This indicates the importance of mangrove habitat for these barnacle genera. However, two of the clades (OTU1 and OTU2) derived from the cyprid data did not match any of the identified adult barnacle sequences by barcoding analysis. This suggests that the larvae may either be advected cyprids from offshore adult species which are not resident in MMFR, or the cyprids came from adults not sampled in the MMFR. Hence, the identity of these two unknown species awaits further detailed surveys of adult barnacles within and outside the MMFR. Non-matched results are common in barcoding analyses especially for areas that are not sufficiently surveyed. Barber & Boyce (2006) used COI fragments to study the diversity of coral reef stomatopods. They reported 22 distinct OTUs that could not be matched with any adult stomatopod references. Chen et al. (2013) also reported 10 unidentified OTUs from wild collection of barnacle cyprids and suggested the possible invasion of cyprids from neighbouring regions. The presence of OTUs in the absence of their adults shows the apparent disconnectedness between the presence of



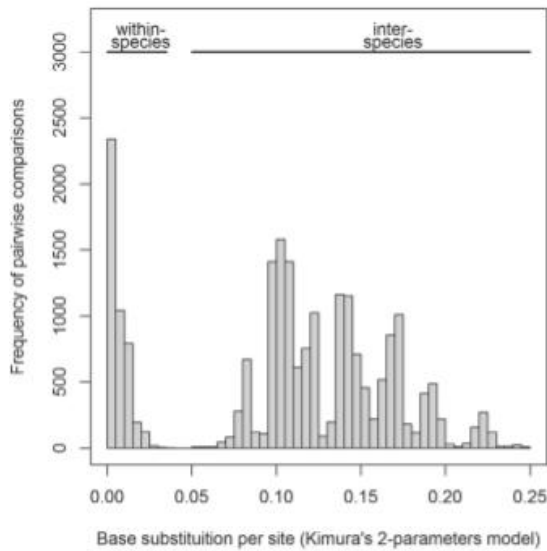


Fig. 4. Histogram showing variations of pair-wise genetic distances computed from 12S-rRNA gene fragment sequences using Kimura 2-parameter model. Note the distribution of within-species variations does not overlap with that of inter-species variation.

larval and their adults, which could be due to a reasonably long larval phase (10–45 days, Lohse & Raimondi, 2012) and hence, the potential to be widely dispersed by ocean current. Nonetheless, cyprids in the absence of settlement cues will not likely settle or survive on unsuitable substrates (Pawlik, 1992).

The 12S-rDNA region has proven to be successful and reliable for barnacle identification in this study. The 12S-rDNA fragments are shorter and relatively easier to amplify than COI fragments (unpublished data) and these are commonly used for species identification. Barnacle cyprids are usually small in size and their DNA can be easily degraded after a period of preservation. It is suggested that 12S-rDNA fragments can be obtained from small cyprids or cyprids that have been preserved for a prolonged period of time. However, the 12S-rDNA fragments have a smaller representation in online databases than COI fragments.

In the present study, the quantitative characters (carapace length, height, posterior angle and length-to-height ratio) have a low discriminating power. This problem is exacerbated by closely related species or genera within this study. Carapace length and carapace height are two common morphological measurements used for cyprids in previous reports, and

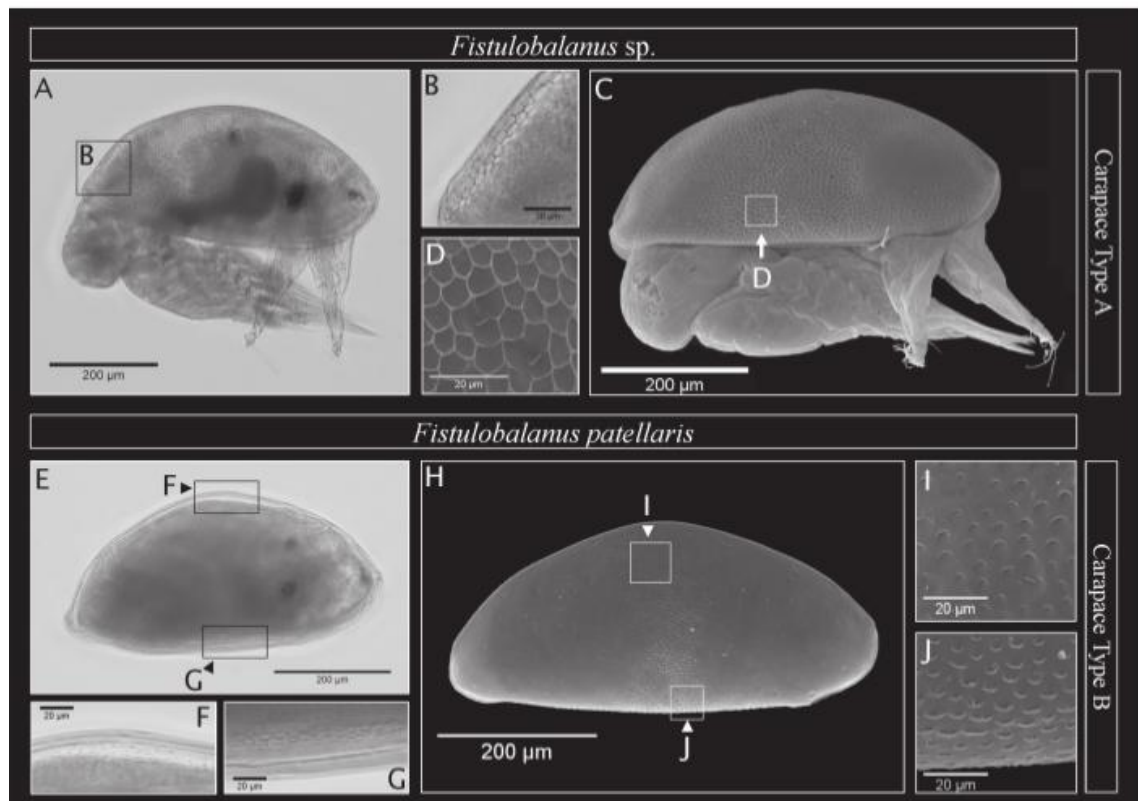


Fig. 5. Light and scanning electron micrograph of cyprids of: A–D, *Fistulobalamus* sp.; and E–J, *Fistulobalamus patellaris*. Details of specific carapace sculpturing patterns in each species are shown at higher magnification.

limit the usefulness of any cyprid identification key based on morphometrics to large geographical regions.

important character for



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Table 1. Quantitative morphological characters and comparison to other studies

Species	Carapace Length (µm)	Carapace Height (µm)	Posterior Carapace Angle (°)	Length-to-Height Ratio	Remarks	Reference
<i>Amphibalanus amphitrite</i>	480.75±38.04	227.79±18.03	65.72±1.13	2.12±0.27	Field collected; mean±SD	This study
	510	N/A	N/A	N/A	lab-reared; single measurement	Karande (1974)
	450±20	N/A	N/A	N/A	lab-reared; mean±95% CI	Egan & Anderson (1986)
	550	250	N/A	N/A	lab-reared; single measurement	Glenner & Hoeg (1995)
	421–480	211–230	N/A	N/A	lab-reared; range	Anil et al. (2001)
<i>Amphibalanus reticulatus</i>	605±21.13	275.72±16.67	60.95±6.56	2.20±0.14	Field collected; mean±SD	This study
	547±26.3	250±25.5	N/A	N/A	lab-reared; mean ± unspecified bar	Thiyagarajan et al. (1997)
	613±21	245±17	N/A	N/A	lab-reared; mean±SD	Lee et al. (1999)
<i>Amphibalanus variegatus</i>	636.46±24.91	284.17±17.41	65.16±6.79	2.25±0.14	Field collected; mean±SD	This study
	520±40	N/A	N/A	N/A	lab-reared; mean±95% CI	Egan & Anderson (1986)
	615	275	N/A	N/A	lab-reared; single measurement	Karande (1974)
<i>Fisulobalamus sp.</i>	555.44±13.49	270.78±14.25	67.79±5.12	2.06±0.11	Field collected; mean±SD	This study
<i>Fisulobalamus patellaris</i>	554.31±19.45	281.19±14.41	71.69±6.56	1.97±0.09	Field collected; mean±SD	This study
<i>Euraphia withersi</i>	482.42±14.88	230.77±7.17	72.57±4.40	2.09±0.09	Field collected; mean±SD	This study
OTU 1	598.01±17.03	256.15±11.32	57.48±0.49	2.34±0.17	Field collected; mean±SD	This study
OTU 2	522.86±20.18	226.95±16.22	54.71±10.94	2.31±0.19	Field collected; mean±SD	This study



Table 2. Types of carapace sculpturing patterns

Type	Description	Species/ OTU
Type A	'Honeycomb' pattern of raised pentagonal and hexagonal units. Maximum feret diameter of the combs is $7.6 \pm 1.2 \mu\text{m}$ (mean $\pm$ SD, n=67)	<i>Fistulobalanus</i> sp.
Type B	Sculpturing spans through dorso-ventral axis, with punctae on the dorsal aspects and lunular pores on the ventral aspects. Maximum feret diameter of the punctae is $2.4 \pm 0.5 \mu\text{m}$ (mean $\pm$ SD, n=65), and $5 \pm 0.9 \mu\text{m}$ (mean $\pm$ SD, n=70) for the pores.	<i>Fistulobalanus patellaris</i>
Type C	Rounded punctae on ventral side, anterior and posterior ends. Diameter of the pits is $2.8 \pm 0.7 \mu\text{m}$ (mean $\pm$ SD, n=65)	<i>Amphibalanus variegatus</i>
Type D	3–4 distinct ridges or folds at posterior end	<i>Euraphia withersi</i>
Type E	No sculpturing of carapace	<i>Amphibalanus reticulatus</i> , <i>A. amphitrite</i> , OTU 1 and OTU 2

reported that the carapace of *Amphibalanus reticulatus* is covered with numerous small denticles but this was not supported by SEM evidence in the present study. Neither LM nor SEM in the present study showed any denticles. Instead, the species, whose identification was confirmed by molecular analysis has a smooth carapace. Thiagarajan et al. (1997) also did not observe any denticles for *Amphibalanus reticulatus*. Such variation in the denticles on the carapace may be due to the presence of cryptic species. Although the sculpturing pattern appears to be species-specific for the cyprids in Matang mangrove waters, the type of pattern shows no generic affinity. This supports the findings of Standing (1981), where he described carapace sculpturing in *Pollicipes polymerus*, *Balanus improvisus*, and *Balanus glandula*, but none in *Chthamalus dalli*, *Balanus crenatus*, *Balanus nubilus*, and *Semibalanus cariosus*. The function and evolutionary history of carapace sculpturing in cyprids is presently unknown.

The combination of quantitative characters with carapace sculpturing characters gave better classification accuracy. This suggests that a combination of both qualitative and quantitative characters in classification problems should be considered especially when few characters are available. The use of classification trees is suitable for combined characters, and a good alternative to LDA (Feldesman, 2002). The other advantage is variable selection. This is automatically performed by the classification tree algorithm, because the variables that are not useful in reducing the misclassification errors are not used. This could remove the variable selection step, and simplify the models for quick classification. The classification tree based on the complete data with carapace sculpturing (Fig. 8) did not use carapace length and length-to-height ratio as predictors, which is simpler than using all of the variables. It has to be noted that the selected variables may differ when a different statistical package is used to compute the classification tree. Classification trees have previously been used in the taxonomic identification of fish (Guisande et al., 2010) and pollen grains (Lindbladh et al., 2002).

Morphological characters besides those described in the current study may be used to discriminate species that do not have any sculpturing. Chen et al. (2013) showed that the antennular morphology provides higher inter-species variations than carapace morphology, which would appear very useful for species identification, but is beset by the problem that not all preserved cyprids showed extended antennules. Kamiya et al. (2012) proposed a promising auto-fluorescence pattern approach to identify cyprids, but the method works only with fresh and unpreserved samples. Recent advances in image acquisition and processing have shown good promise in the development of large scale automated classification of planktons (Culverhouse et al., 2006), and such tools could be adapted to the specific purpose of cyprid classification in the future.

Field samples showed that cyprid composition, dominated by four species, varied spatially (between stations) and temporally (between sampling years), indicating the dynamic nature of their supply in MMFR waters. Cirripede nauplii were observed to be most abundant in the inshore waters of MMFR (<15 km off shore) compared to estuarine and offshore waters, being consistently found throughout the year but with peak abundance in May and October during the intermonsoon months (Chew, 2012). Thus, the difference in composition of cyprid samples in the present study is likely a result of temporal variability. Nonetheless, the present field study is preliminary and future studies requiring more exhaustive sampling over larger spatial and temporal scales are necessary to elucidate the supply-side ecology of barnacle larvae in the estuary.

In summary, the molecular approach used in this study, i.e., 12S-rDNA sequence-matching of larval and adult barnacles, has successfully identified most of the sequenced cyprids (195 out of 207 sequences, six species out of eight clades). A morphology-based classifier has been developed with good classification accuracy for the dominant species of barnacle cyprids (*Fistulobalanus* sp., *F. patellaris*, *A. reticulatus*, *E. withersi*) in the MMFR. However, the identification of

cyprids/OTU classified with lower accuracy still requires molecular tools. At present, there is still no single approach that can provide identification of barnacle cyprids with high accuracy, high speed, and low cost at the same time. The selection of the best approach will largely depend on the research question. The approach used in the current study achieves a balance of these three criteria. Future global or regional-scale cyprid identification keys are likely to use an automated integrated approach combining the usage of carapace sculpturing features, geometric morphometrics and cyprid appendicular features.

# **KEY TO THE COMMON BARNACLE CYPRIDS IN MATANG MANGROVE FOREST RESERVE (MMFR)**

1. Carapace sculpturing absent, i.e., smooth (carapace Type E).2
  - Carapace sculpturing present. ....3
2. Carapace length less than 550  $\mu\text{m}$ . *Amphibalanus amphitrite*
  - Carapace length more than 550  $\mu\text{m}$ . *Amphibalanus reticulatus*
3. Carapace punctate at anterior, posterior and ventral aspects (carapace Type C) and large in size (carapace length more than 600  $\mu\text{m}$ ). .... *Amphibalanus variegatus*
  - Carapace not punctate with carapace length less than 600  $\mu\text{m}$ . ....4
4. Carapace with honeycomb sculpturing pattern (carapace Type A). .... *Fistulobalanus* sp.

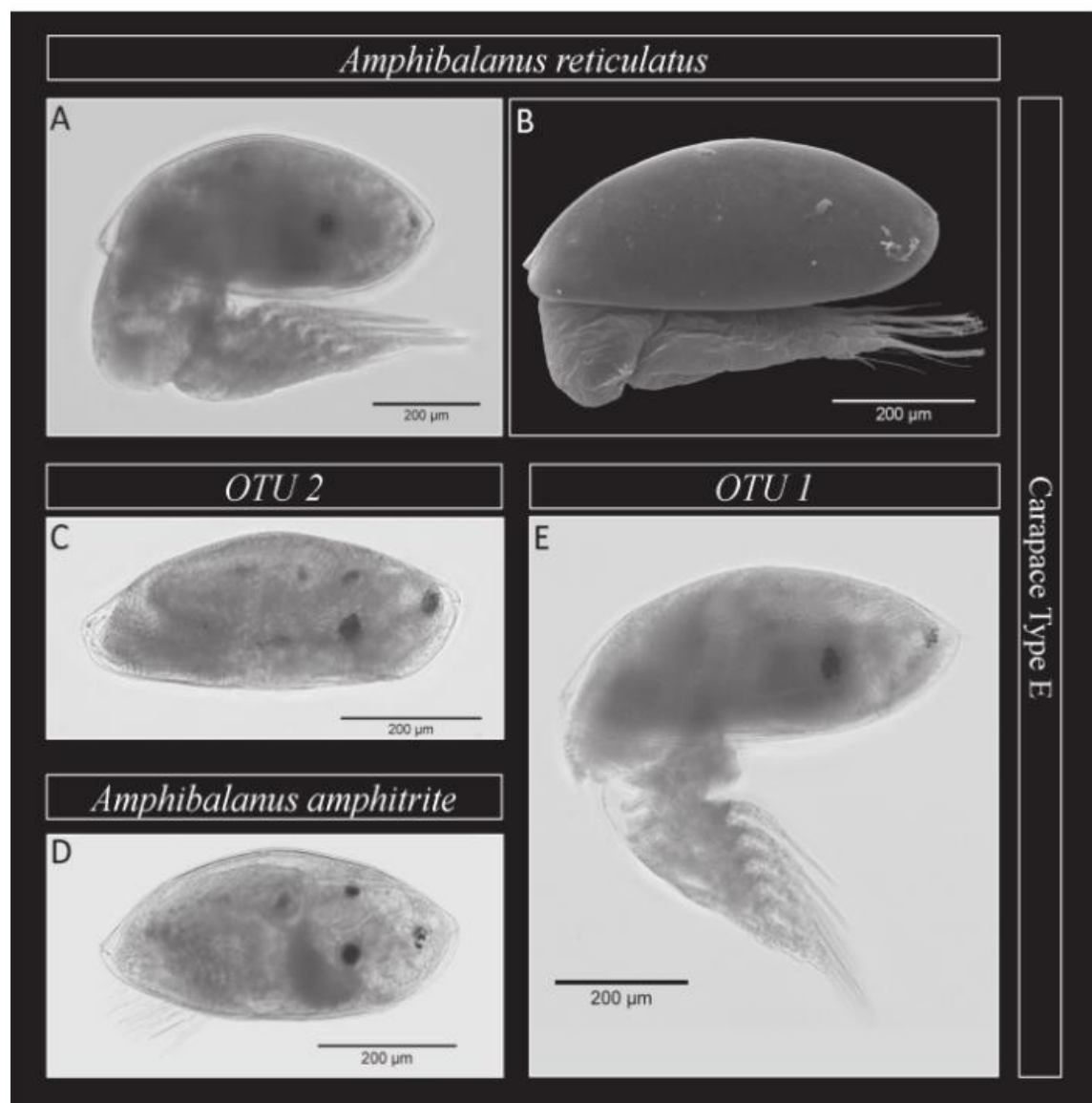


Fig. 7. Light and scanning electron micrograph of cyprids of: A, B, *Amphibalanus reticulatus*; C, OTU 2; D, *Amphibalanus amphitrite*, and E, OTU 1. Carapace sculpturing were absent in this group of cyprids.

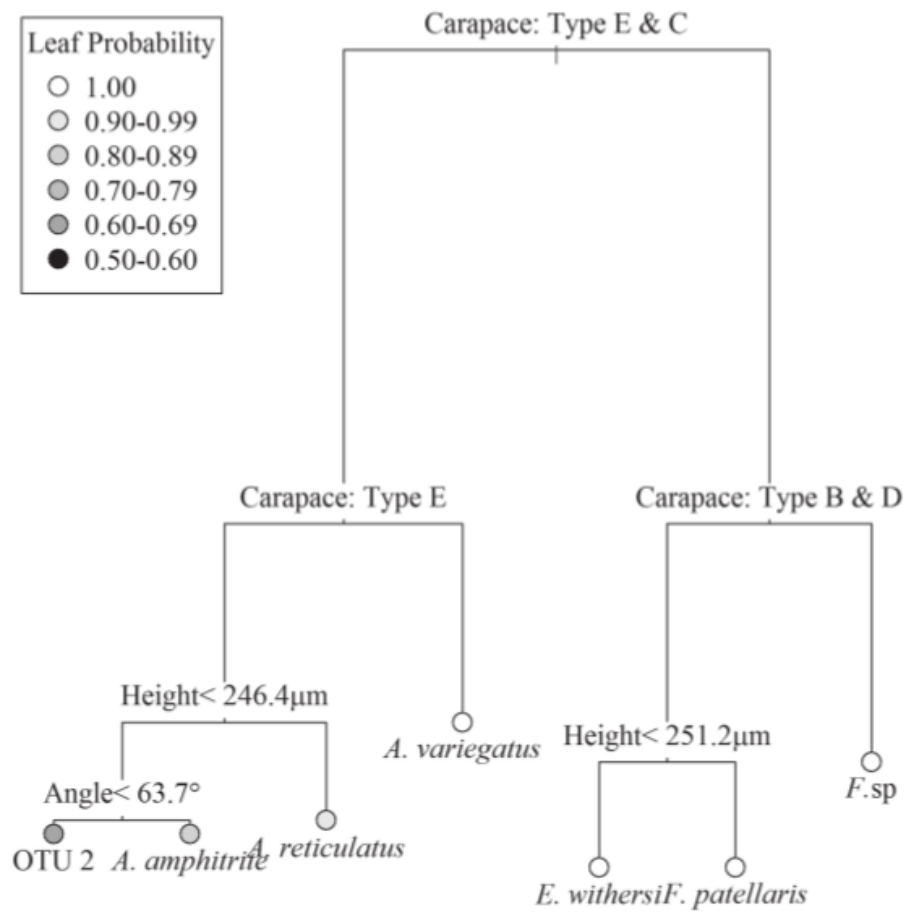


Fig. 8. Classification tree model computed from the morphometric characters of complete specimen data (with carapace sculpturing). A binary decision is made at each node, where 'true' for the node description lead to branch at left and 'false' to right. Probability of correct prediction ('recall') at each terminal node ('leaf') is also shown.

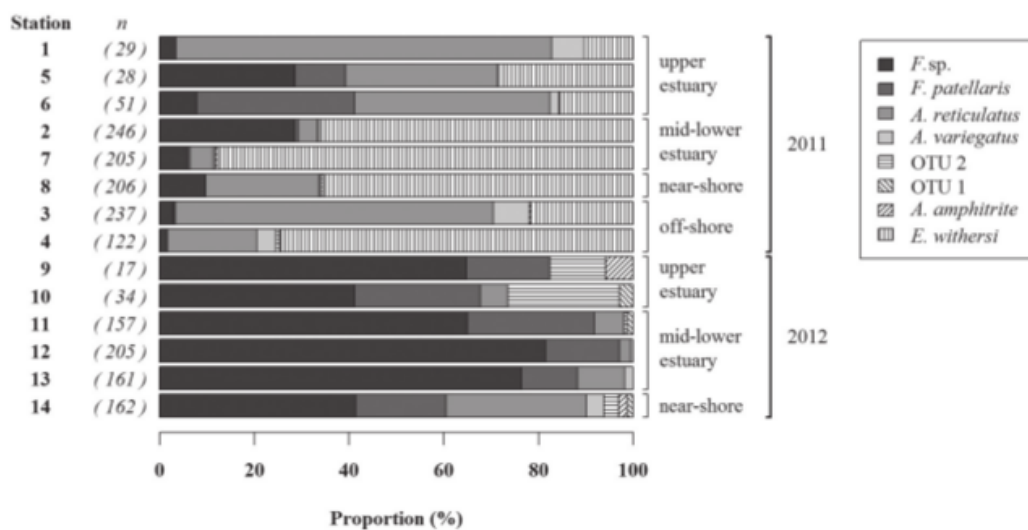


Fig. 9. Composition of barnacle cyprid diversity at different stations and different year of collection.



- Carapace without honeycomb sculpturing pattern. ....5
- 5. Carapace with dark rounded pigmentation spot posterior to cyprid eye, ridged sculpturing on the posterior end (carapace Type D), height less than 250 µm. .... *Euraphia withersi*
- Carapace with rounded and lunular sculpturing at dorsal and ventral aspects, respectively (carapace Type B), height more than 250 µm. .... *Fistulobalanus patellaris*

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