Regulatory effects of mussel (*Aulacomya maoriana* Iredale 1915) larval settlement by neuroactive compounds, amino acids and bacterial biofilms

Andrea C. Alfaro, Tim Young, Annapoorna Maitrayee Ganesan

PII: S0044-8486(11)00686-7
DOI: doi: 10.1016/j.aquaculture.2011.08.038
Reference: AQUA 629854

To appear in: *Aquaculture*

Received date: 21 March 2011
Revised date: 29 August 2011
Accepted date: 29 August 2011

Please cite this article as: Alfaro, Andrea C., Young, Tim, Ganesan, Annapoorna Maitrayee, Regulatory effects of mussel (*Aulacomya maoriana* Iredale 1915) larval settlement by neuroactive compounds, amino acids and bacterial biofilms, *Aquaculture* (2011), doi: 10.1016/j.aquaculture.2011.08.038

Regulatory effects of mussel (*Aulacomya maoriana* Iredale 1915) larval settlement by neuroactive compounds, amino acids and bacterial biofilms

Running Head: Induction of larval settlement

ANDREA C. ALFARO

TIM YOUNG

ANNAPOORNA MAITRAYEE GANESAN

School of Applied Sciences
Faculty of Health and Environmental Sciences
Auckland University of Technology
Private Bag 92006
Auckland 1142
New Zealand

Corresponding author
Andrea C. Alfaro
Phone: +64-9-921-9999 ext. 8197
Fax: +64-9-921-9743
andrea.alfaro@aut.ac.nz

Regulatory effects of mussel (*Aulacomya maoriana* Iredale 1915) larval settlement by neuroactive compounds, amino acids and bacterial biofilms

Abstract

Larval settlement responses of the ribbed mussel, *Aulacomya maoriana* Iredale 1915, were investigated after exposure to various chemicals and mono-species bacteria. Identification of settlement inductive compounds assists in the elucidation of intermediary biochemical mechanisms involved in the neuronal control of settlement behaviour downstream from primary cue reception. Neuroactive compounds and amino acids (potassium ions, GABA, acetylcholine, L-Phenylalanine, L-Tyrosine, dopamine, epinephrine, L-Tryptophan, and 5-HTP) and planktonic bacteria, biofilms and biofilm exudates of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1, and *Pseudoalteromonas* sp. AMGP1 were tested for their abilities to induce larval settlement. Toxicity effects of each treatment also were simultaneously identified by recording larval mortalities. Results indicate that all chemicals used induced larvae to settle, with acetylcholine being the most effective (~24% at 10^{-6} M compared to <2% in control assays). Toxicities of treatment compounds were low at optimal settlement inducing concentrations, except for L-Tryptophan (~32%) and GABA (~59%). Our data suggest that catecholamines (and their precursors) play an important role in the biochemical mechanisms of settlement for *A. maoriana*. While serotonin precursors did induce low levels of larval settlement at some concentrations, high toxicity responses to 5-HTP at 10^{-5} M, combined with complete settlement inhibition indicate

that the mechanism of action may be more complex than can be elucidated in this study. Larval settlement responses to bacterial treatments were low for planktonic and biofilm phases across all three strains, and settlement inhibition was observed when larvae were exposed to biofilm exudates of all bacterial strains. Comparisons of *A. maoriana* responses to other endemic and worldwide distributed mussel species are provided as a means to highlight potential evolutionary differences in chemoreception mechanisms.

Highlights

1. Mussel larval settlement responses were tested with neuroactive compounds amino acids, and bacterial biofilms.
2. All chemicals induced larvae to settle, with acetylcholine being the most effective.
3. Catecholamines (and their precursors) play an important role in the biochemical mechanisms of settlement for *A. maoriana*.
4. Settlement responses to serotonin precursors require further investigation since there were high toxicity responses.
5. Larval settlement responses to bacterial treatments were low for planktonic and biofilm phases, and exposure to biofilm exudates resulted in settlement inhibition.

Keywords: Larval settlement, Ribbed mussels, *Aulacomya maoriana*, Chemical cues, Bacterial biofilms.

Introduction

A great majority of marine invertebrates has planktonic larvae, which after a period of minutes to months (Hadfield and Paul 2001) settle onto benthic substrates. A wide range of environmental and biological stimuli or cues mediate this settlement process (Pawlik 1992; Harder et al. 2002; Steinberg et al. 2002; Wikstrom and Pavia 2004; Hadfield 2011). Chemoreception involves the binding of chemicals to receptors in the neural tissues of larvae, which activate neuronal networks (Hay 2009). Factors which regulate larval settlement behavior have been investigated extensively for many marine taxa (reviewed by Hadfield and Paul 2001; Steinberg and De Nys 2002; Murthy et al. 2009), but the complex chemoreception process has yet to be elucidated. For example, within the Class Bivalvia, many cues have been found to induce larval settlement in oysters (Tamburri et al. 2008; Yu et al. 2008; Yu et al. 2010a), scallops (Leyton and Requelme 2008; Mesias-Gansbiller 2008), clams (García-Lavandeira 2005; Sumin et al. 2006; Neo et al. 2009), and mussels (Dobretsov and Qian 2003; Alfaro et al. 2006; Bao et al. 2007; Ganesan et al. 2010; Young 2009). Settlement responses to different cues appear to be genus-, species- and even intraspecies-specific (Rodríguez et al. 1993; Williams et al. 2008; Ritson-Williams et al. 2010). These differences suggest evolutionary variations in cue-binding receptors, endogenous biochemical processes, and the metabolites produced during the settlement process.

Chemical compounds that mediate larval settlement often are produced by bacteria or the biofilms they form on just about every surface in the marine environment (Hadfield and Paul 2001). The chemical cues generated by bacteria may be surface-bound

(bound to bacterial cells or exopolymeric substances) or water-soluble (produced by free-swimming planktonic bacteria or released by their biofilms) (Hadfield 2011). The surface-bound cues induce larval settlement only when larvae come into contact with the bacteria (Hadfield 2011). On the other hand, water-soluble cues (e.g., low and high molecular weight polyshaccharides [Dobretsov and Qian 2004; Harder et al. 2004] low molecular weight peptides [Tamburri et al. 1992; Zimmer-Faust and Tamburri 1994], and even neurotransmitters [Mountfort and Pybus 1992]) may regulate larval settlement without the need for them to contact the substrate (Tamburri et al. 1996; Browne and Zimmer 2001). For example, studies on the green-lipped mussel, *Perna canaliculus*, showed that water-soluble chemical cues produced by the biofilm of two bacterial strains (*Macrococcus* sp. [AMGM1] and *Bacillus* sp. [AMGB1]) induced larval settlement (Ganesan et al. 2010).

While settlement cues for various taxa include those derived from marine biofilms, the specific molecular characteristics of these inductors remain elusive. A commonly applied method for gaining insight into the endogenous mechanisms of cue reception is to agonise or antagonise particular endogenous receptor classes with pharmacologically active compounds. These chemicals are likely to act directly at some intermediate site downstream from primary chemoreceptors (Pechenik et al. 1995), or as precursors in the biosynthesis of neuroactive ligands (Young 2009). Some of these compounds include ions (Yu et al. 2008), amino acids (Kang et al. 2003; Young 2009), neurotransmitters (Faimali et al. 2003; Young 2009), choline derivatives (Dobretsov and Qian 2003; García-Lavandeira et al. 2005), and enzyme inhibitors (Mesías-Gansbiller et al. 2008). For example, potassium is a universal regulator of ion gradients across cell membranes, and is involved in depolarisation of neurons, causing formation of action potentials. Generally, potassium ions have been branded as the

universal inducer of metamorphosis and settlement for numerous taxa (Yool et al. 1986; Rodríguez et al. 1993), while GABA is a settlement and metamorphosis inducer for many gastropods (reviewed by Roberts et al. 2001) and a few bivalves (Gacrcia-Lavendeira et al. 2005; Mesías-Gansbiller et al. 2008).

The role of cholinergic neurotransmission in modulating larval behaviors and important life-history events, such as settlement and metamorphosis, are poorly understood. The ability of the neurotransmitter acetylcholine to induce settlement and metamorphosis has been shown to have highly variable results across marine invertebrate taxa (Coniglio 1998; Dobretsov and Qian 2003; Yu et al. 2007, 2008, 2010b; Young 2009). L-Phenylalanine, L-Tyrosine and dopamine are members of the epinephrine biosynthesis pathway (Fig. 1a). While some of these compounds have been investigated for their ability to induce settlement of bivalve larvae, the role of dissolved amino acids as precursors for epinephrine induction has yet to be identified.

The importance of catecholamines in bivalve life-history events is well recognized (Pani and Croll 2000). For these taxa, the biochemical pathways involved in catecholamine synthesis and catabolism are proving to be similar to those operating in vertebrate nervous systems (Pani and Croll 1995, 1998), and may highlight the conserved and critical role they play in early evolutionary development. In bivalves, these neurotransmitters and hormones are known to regulate spawning (Martínez et al. 1996), larval swimming behaviour (Beiras and Widdows 1995), larval settlement (Garcia-Lavandeira 2005), metamorphosis (Wang et al. 2006; O'Conner et al. 2009), feeding rates (Beiras and Widdows 1995), muscular activity (Aiello et al. 1981; Gies 1986), respiration (Catapane 1983) and digestion (Giard et al. 1995). Since the uptake of epinephrine precursors (L-Tyrosine, L-DOPA, and dopamine) into bivalve tissues from seawater is well documented (Shu-Ing et al. 1977; Brown et al. 1981; Manahan

1989), and they are endogenously converted into dopamine, norepinephrine and epinephrine in various mollusc taxa (Boadle-Biber and Roth 1972; Paparo and Finch 1972; Peng Loh and Jacklet 1977; Stefano 1990; Pani and Croll 1995, 1998; Filla et al. 2009), these compounds are good candidates to test the involvement of this pathway in larval settlement behavior.

Likewise, compounds within the serotonin pathway (i.e., L-Tryptophan, and 5-HTP; Fig. 1b) have received little attention for the role this pathway may play on larval settlement. Serotonin itself is involved in the neuromodulation of various marine invertebrate behaviours (Pires and Wollacot 1997; Kravitz 1988; Beiras and Widdows 1995). For example, serotonin has been implicated in the regulation of arthoropod aggressiveness (Kravitz 1988), Bryozoan larval phototaxis (Pires and Wollacot 1997), gastropod locomotion (Panchin et al. 1996), and is known to increase velar ciliary activity (Beiras and Widdows 1995) and mucus release in bivalves (Lent 1973). Most of the research to date on the involvement of serotonin during larval ontogeny has been focused on regulation of metamorphosis rather than larval settlement, with a few exceptions (e.g., barnacle [Yamamoto et al. 1996; 1999] and oyster [Yu et al. 2008; Grant 2009]). The ability of serotonin, or its precursors, to induce mussel larval settlement has not been demonstrated previously.

The ribbed mussel, *Aulacomya maoriana* (Iredale, 1915) is endemic to New Zealand (Gosling 2003), where it inhabits intertidal rocky shores (Kennedy 1976; Lachowicz 2005). Currently, this species is not of commercial value, but its cultivation potential is being investigated and evaluated (e.g., Gibbs et al. 2006). Studies on this species have been limited to its distribution (Lachowicz 2005; Phillips et al. 2008), genetics (Phillips et al. 2008), reproductive biology (Kennedy 1977; Tortell 1980), and general ecology (Kennedy 1976; Lachowicz 2005). However, little

is known about its settlement patterns, and there is no information about the biochemical mechanisms involved in larval settlement of *A. maoriana*. Thus, the aim of this paper is to identify the effect of a range of neuroactive compounds and amino acids (potassium ions, GABA, acetylcholine, L-Phenylalanine, L-Tyrosine, dopamine, epinephrine, L-Tryptophan, and 5-HTP) and planktonic bacteria, biofilms and biofilm exudates of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1, and *Pseudoalteromonas* sp. AMGP1 on settlement of *A. maoriana* larvae.

Methods and Materials

Chemical Treatments

The following neuroactive compounds and amino acids were tested simultaneously for their ability to induce settlement of *Aulacomya maoriana* larvae. These chemicals included potassium chloride (K^+), γ-aminobutyric acid (GABA), acetylcholine chloride, L-Phenylalanine, L-Tyrosine, dopamine hydrochloride, epinephrine, L-Tryptophan, and 5-hydroxytryptophan (5-HTP).

Stock solutions of each treatment were prepared by dissolving compounds in 0.45 μm filtered seawater (FSW) immediately prior to settlement assays. Solutions were diluted in FSW to prepare 10× concentrates of each treatment level to give the desired final assay concentrations (Table 1). These concentrations were chosen based on previous experiments with other mussel species (e.g., Ke at al. 1998; Dobretsov and Qian 2003; Young 2009).

Bacterial Treatments

Three marine bacterial strains previously isolated and identified as *Macrococcus* sp. (AMGM1), *Bacillus* sp. (AMGB1), and *Pseudoalteromonas* sp. (AMGP1) at the AUT laboratory also were tested for their ability to induce larval settlement. Planktonic bacteria, bacterial biofilms, and biofilm exudates were prepared as described by Ganesan et al. (2010). Briefly, bacterial colonies were drawn from log phase mono-specific bacterial cultures grown on Zobell marine agar media. Planktonic bacteria were produced by culturing the cells in 50 ml of 0.5% peptone autoclaved filtered seawater (AFSW) with constant agitation at 37 °C to prevent the formation of a biofilm. After 24 h, the planktonic cells were washed with AFSW and concentrated by centrifugation at 3500× g for 10 minutes. This planktonic bacterial solution (10^6 to 10^7 cells ml^{-1}) was used in the settlement assays (see below). Bacterial biofilms were produced by placing 1 ml of bacterial cell suspension (10^6 to 10^7 cells ml^{-1}) into polystyrene Petri plates. Then, 4 ml of AFSW containing 0.5% peptone were added to each plate. The plates were incubated for 24 h at 37 °C on a rotary incubator at 100 rpm to encourage biofilm growth. After incubation, the plates were washed thrice with a total of 30 ml of FSW, leaving the attached cells on the Petri plates. These plates were used as larval settlement substrates. Production of biofilm exudates was achieved by culturing another set of biofilm plates (as described above), and scraping off the attached cells with a clean glass cover slip into 1 ml of AFSW. Ten of these 1 ml samples were pooled and centrifuged at 3500× g for 10 minutes. The pellets containing the bacterial cells were discarded. The supernatant was filtered through a 0.22 μm acetate filter to obtain a cell-free extract. These pure biofilm exudates were used for the settlement assays.

Settlement Assays

Larvae of *Aulacomya maoriana* in their veligers stage (25 days post-fertilisation, and competent to settle) were obtained from Cawthron Institute, Nelson, New Zealand. The larvae were transported in moist and cold containers to the Auckland University of Technology (AUT) laboratory, Auckland, New Zealand. Upon arrival at AUT, the larvae were transferred into a 2 L beaker with 1 L of FSW. After 30–60 min, healthy swimming larvae were decanted into another beaker, and filled with FSW to make a concentration of 20–30 larvae ml^{-1}. Settlement assays were conducted in sterile polystyrene Petri plates (60 mm in diameter, and 14 mm in depth), with nine to ten replicates per treatment at 17±1ºC under ambient light conditions. Chemical treatment assays consisted of 8 ml FSW, 1 ml larval solution and 1 ml concentrated (10×) treatment solution. For the case of planktonic bacteria and biofilm exudates, a 1 ml solution was added to the 8 ml AFSW and 1 ml larval solution. Plates with bacterial biofilms were topped up with 9 ml AFSW and 1 ml larval solution. Controls consisted of 9 ml AFSW and 1 ml larval solution. The plates were monitored daily to determine the time at which a significant proportion (~ 5%) of larvae had settled on the control plates. This point was achieved after 120 h. Then, larval settlement and mortality were recorded for all plates. Under a dissecting microscope at 20–45× magnification, a 200 µl displacement pipette was depressed and brought within close proximity (0.5–1.5 mm) to each larva, and gentle suction was applied. Individuals that maintained firm attachment to the substratum were considered settled, and those moving freely with no resistance were considered unsettled. In many cases, settlement could be detected visually by the presence of thin transparent mucous-like threads, but settlement always was verified with suction.

Mortality Assays

Mortality was recorded to determine acute toxicity effects from all chemicals and bacterial treatments. Mortality was identified under a stereo microscope at 20–45× magnification. Larvae that showed signs of movement of the velum, foot, or gut were considered alive. Since live larvae often were inanimate for periods of more than 15 min, the neutral red vital stain was used to corroborate mortality detection (see Platter-Rieger and Frank 1987; Jacobson et al. 1993). A 120 ppm solution of neutral red was prepared in FSW and diluted in the experimental medium to give a final stain concentration of 20 ppm. After 30 min, larvae were again viewed at 20× magnification under a stereo microscope. Larvae that did not incorporate the stain into their tissues were considered dead.

Statistics

All percent larval settlement and mortality data were analyzed using the non-parametric Kruskal–Wallis *H*-test and followed by Dunn's multiple comparison tests. The level of significance chosen was 0.05 for all statistical tests. Data were analyzed using the Minitab version 15 statistical software package.

Results

Larval Settlement

Excess potassium ions (KCl) induced larvae to settle (±SE) with a peak induction of 21.0±4.0% after exposure to 20 mM solution (Fig. 2). Settlement responses revealed a typical dose response curve with significant differences detected for 20 and 30 mM compared to the control (Table 2). The neurotransmitters GABA and acetylcholine also displayed a typical dose response with maximum settlement (±SE) of 22.4±4.1% at 10^{-4} M and 24.2±4.3% at 10^{-6} M, respectively.

Results from the two catecholamines and two of the amino acids used in this study indicate a significant increase in larval settlement (±SE) from lowest to highest concentrations peaking at 21.3±3.2% (10^{-5} M), 11.3±1.7% (10^{-5} M), and 13.8±3.8% (10^{-4} M) for L-Phenylalanine, L-Tyrosine, and dopamine, respectively (Fig. 3, Table 2). The peak larval settlement (±SE) for exposure to epinephrine was 12.3±2.3% at 10^{-5} M, with a lower settlement of 4.9±1.0% at a concentration of 10^{-4} M.

Less than 12% larval settlement was achieved with either of the serotonin synthesis pathway compounds used in this experiment (Fig. 4). Compared to the control, treatment with L-Tryptophan resulted in a significant peak settlement (±SE) response of 11.8±1.7% at 10^{-5} M and 5-HTP had a significant maximum of 6.7±1.3% at 10^{-6} M (Fig. 4, Table 2).

Larval settlement responses to bacterial treatments showed no significant difference in settlement after exposure to planktonic bacteria of all three stains (< 10% settlement) compared to the control (Fig. 5, Table 3). Exposure to bacterial biofilms also resulted in non-significant differences between the treatments and the control. Significantly lower settlement responses were observed with biofilm exudates from all bacterial strain treatments (< 2%) compared to the control (10%).

Mortality

Results from the larval mortality assays indicate a low and non-significant toxicity effect across KCl concentrations (<3%), and significantly high toxicity effects for GABA at all concentrations (up to about 80% mortality at 10^{-3} M) and for acetylcholine at concentrations between 10^{-5} and 10^{-3} M (Fig. 2, Table 2). Exposure to catecholamines and their precursor compounds did not result in significantly high mortality, except for L-Phenylalanine at 10^{-6} and 10^{-5} M, L-Tyrosine at 10^{-5} M, dopamine at 10^{-5} M, and epinephrine at 10^{-5} M (Fig. 3, Table 2). The two precursor compounds in the serotonin biosynthesis pathway resulted in significantly high toxic effects of 32.2±5.3% at 10^{-5} M for L-Tryptophan and 100% at 10^{-5} and 10^{-3} M for 5-HTP (Fig. 4, Table 2).

Toxicity effects to the planktonic bacteria were relatively low (8-20%), and significantly different for all three bacterial strains compared to the control (Fig. 5, Table 3). For bacterial biofilms, only *Bacillus* sp. AMGB1 (15.8±2.3%), and *Pseudoalteromonas* sp. AMGP1 (42.6±6.0%) caused significantly higher mortalities relative to the control. Non-significant mortality effects for biofilm exudates were recorded for all bacterial strains compared to the control.

Discussion

Results from the larval settlement experiments after exposure to various neuroactive compounds and amino acids indicate that all the chemicals used induced

larvae to settle at some concentration with low toxicity. However, for some of the chemicals used, their ability to induce settlement was relatively low compared to the controls. In addition, larval settlement responses to bacterial treatments were low for planktonic and biofilm phases across all three strains. Settlement inhibition was observed when larvae were exposed to biofilm exudates of all bacterial strains. While the overall percent settlement was low in our assays, the relative differences between control and treatments provide a clear trends, which are comparable to published results for other species. Our findings indicate that the neuronal control of settlement behavior for *Aulacomya maoriana* differs from that of other mussel species, such as *Mytilus galloprovincialis* (Garcia-Lavandeira 2005) and *Perna canaliculus* (Young 2009; Ganesan et al. 2010). Furthermore, these differences support the notion of nerve system evolutionary divergence across taxa. Although comparative neurodevelopment in bilaterian invertebrates appears to have a high degree of conserved molecular architecture from a common ancestor (Arendt et al. 2008), our results corroborate the growing belief that critical differences exist.

Chemical Treatments

Potassium Ions

Larval settlement responses to potassium chloride followed a typical dose response, with maximum settlement at 20 mM with less than 3% mortality across concentrations. Previously, potassium ions (from KCl) have been shown to induce larval settlement in two species of mussel, *Perna viridis* (Ke et al. 1998) and *Perna canaliculus* (Young 2009). However, excess potassium ions do not induce settlement in the blue mussel *Mytilus edulis* (Eyster and Pechenik 1988; Dobretsov and Qian

2003), which may indicate that these species rely on different chemoreceptor types. Compared to another New Zealand native mussel species (*P. canaliculus*; Young 2009), *A. Maoriana* required double the concentration of KCl to reach peak settlement. It is uncertain why optimal concentrations for inducing larval settlement of these two endemic species differ. However, other marine invertebrate taxa stimulated to settle by KCl also display species-specific concentration responses (e.g., Gapasin and Polohan 2004; Yu et al. 2010b). Distinct differences in acute toxicity data also were observed between *P. canaliculus* and *A. maoriana* (Young 2009; this paper). The LC50 of KCl for *P. canaliculus* was reported to be just over 30 mM after 48hr of exposure (Young 2009). However, no toxic effects were observed in *A. maoriana* after being exposed to 30 mM KCl for 120 hrs. These toxicity differences suggest that *A. maoriana* is more resilient to KCl exposure, which agrees with their habitat distribution in rocky shores. Compared to *P. canaliculus*, *A. maoriana* has been reported to inhabit higher intertidal areas (Kennedy 1976; Lachowicz 2005), where increased salinity may occur following desiccation periods.

GABA

The larval settlement results show that GABA enhanced settlement in *A. maoriana*, with a maximum induction at 10^{-4} M. These results are highly comparable to those seen in *Mytilus galloprovincialis*, suggesting similarities in chemoreception mechanisms between the two mussel species. GABA is an amino acid and neurotransmitter, which can function as an inhibitory and excitatory neurotransmitter in molluscs (Yarowsky and Carpenter 1977; Bokisch and Walker 1986), and is widely recognized for its ability to induce settlement and metamorphosis of abalone, *Haliotis* spp. (reviewed by Roberts 2001). While the settlement inducing effects of GABA

have now been extensively investigated for various marine invertebrate taxa, there are only three reports of its effect on mussel larvae. GABA does not induce settlement in *P. canaliculus* (Young 2009) and *M. edulis* (Dobretsov and Qian 2003), but is an effective inducer for *M. galloprovincialis* (Garcia-Lavandeira et al. 2005). It has been suggested that the activity of this compound for *Haliotis* spp. is due to its close structural relationship with naturally occurring inducer molecules isolated from crustose corraline algae (Morse and Morse 1984a, b). This settlement induction is likely to be mediated by epithelia-bound chemoreceptors *via* an excitory depolarisation mechanism (Baloun and Morse 1984). While the specific mode of action of GABA is yet to be elucidated for bivalves, it is likely that different mechanisms are involved. While GABA did induce settlement in *A. maoriana*, the effective concentrations were all highly toxic to larvae. This implies that GABA is unlikely to be a natural settlement cue for this species. The observed induction of settlement by GABA may be a result of the endogenous release of other metabolites important to the signaling pathway. Further experiments with GABA analogs and other pharmacological compounds would be needed to improve our understanding of the specific mechanisms at play for different marine invertebrate taxa.

Acetylcholine

Exposure of larvae to acetylcholine induced highest settlement (>24%) at the lowest concentration assayed (10^{-6} M), with no significant acute toxicity effects. Acetylcholine is an ester of acetic acid and choline, which modulates numerous biological processes. While the complete structural and functional diversity of acetylcholine receptors (AChR's) has not yet been fully characterised, studies have shown that some molluscan receptor sub-types are highly unique (van Neirop et al.

2005, 2006). Acetylcholine has been identified previously as a larval settlement inducer of two other mussel species: *P. canaliculus* (Young 2009) and *M. edulis* (Dobretsov and Qian 2003). In addition to these studies, our findings for *A. maoriana* highlight the importance of AChR's in early molluscan development. Acetylcholine may act by innervating velar cilia, affecting swimming behavior. Acetylcholine is known to inhibit lateral ctenidial cilia in adult *M. edulis* (Jones and Richards 1993), and it is likely that such responses to pharmacological modulations are similar between adult ctenidial cilia and larval velar cilia (Beiras and Widdows 1995). Furthermore, acetylcholine has demonstrated an ability to reduce swimming of larvae in *Crassostrea virginica* (Grant 2009). However, future studies will be needed to identify the direct mode of action of acetylcholine in *A. maoriana* and other mussel species.

Epinephrine Pathway

In this study, we selected two amino acids (L-Phenylalanine and L-Tyrosine) and two catecholamines (dopamine and epinephrine) within the epinephrine biosynthesis pathway (Fig. 1a) to test for larval settlement responses. All of these compounds induced *A. maoriana* to settle at various concentrations (10^{-5} to 10^{-4} M), suggesting that catecholamines are directly involved in neuromodulation of settlement responses for this species. L-DOPA has previously been identified as a settlement inducer for *M. edulis* (Dobretsov and Qian 2003). While this result may suggest a catecholamine involvement, it is uncertain if the L-tyrosine derivatives between L-DOPA–epinephrine have inductive activities. On the other hand, while epinephrine has been shown to have an ability to induce larval settlement in *M. galloprovincialis* (Garcia-Lavandeira 2005), substantiating a catecholamine involvement in mytilids, the effects of all five precursor compounds (L-Phenylalanine to norepinephrine) are

unknown. Our study is the first to test the inductive effects of four metabolites in the epinephrine biosynthesis pathway on mussel larval settlement. In addition, this is the first published report of the induction of settlement of any bivalve species by L-Phenyalanine and L-Tyrosine, and of any mussel species by dopamine. Since all the compounds we tested induced larval settlement in *A. maoriana*, it is likely that the epinephrine biosynthesis pathway plays an important role in signal transduction mechanisms downstream from primary cue reception. Furthermore, the amino acid precursors also may serve as naturally occurring xenobiotic cues for larval settlement in this species. Dopamine, norepinephrine and epinephrine have been demonstrated to inhibit velar cilia in *M. edulis* (Beiras and Widdows 1995). In the case of *A. maoriana*, it is likely that the settlement process was, at least partially, stimulated by cilioinhibitory action of catecholamines on velar cilia, which would have caused cessation of swimming.

While mortality levels across the treatments for testing the epinephrine pathway were low, a slightly higher mortality was observed at high concentrations of the two amino acids (L-Phenylalanine and L-Tyrosine) compared to the catecholamines (dopamine and epinephrine). These results may reflect conversion of the amino acids to high levels of toxic derivatives *via* non-catecholamine L-Tyrosine metabolism, such as tyramine, octopamine, morphine or codeine.

Serotonin Pathway

Larval settlement responses to serotonin biosynthesis precursors L-Tryptophan and 5-HTP were generally low (<12% and <7%, respectively), but significantly higher than controls. However, exposure of larvae to both compounds at 10^{-5} M resulted in

high mortality (32% for L-tryptophan and 100% for 5-HTP). We selected the serotonin precursors over serotonin itself as a treatment since bivalve tissues have a limited ability to uptake serotonin dissolved in seawater, whereas intracellular diffusion of 5-HTP is considerably greater (Aiello and Guideri 1966). In addition, conversion of the precursors to serotonin in gastropod neural tissues is well established. For example, *M. edulis* pedal ganglia bathed in ^{14}C labelled L-Tryptophan leads to synthesis of ^{14}C serotonin (Boadle-Biber and Roth 1972). Also, gastropod cerebropleural and pedal ganglia and whole larvae exposed to exogenous 5-HTP significantly increases serotonin levels (Fickbohm et al. 2005; Filla et al. 2009).

In our study, the inductive effects of serotonin precursors on larval settlement of *A. maoriana*, while low, were unexpected. Serotonin is known to increase activity of different types of cilia in molluscs (Audesirk et al. 1979; Beiras and Widdows 1995; Uhler et al. 2000 Carroll and Catapane 2007). For example Beiras and Widdows (1995) showed increased swimming activity of *M. edulis* larvae after exposure to serotonin, which enhanced movement of velar cilia. During our settlement experiments (0-3 days), *A. maoriana* larvae were observed swimming more vigorously in 5-HTP treatments compared to controls. The low settlement responses and high mortalities observed after larvae were exposed to L-Tryptophan and 5-HTP (10^{-5} M) suggest that the involvement of serotonin, if any, in *A. maoriana* larval settlement is more complex than previously thought. Neuronal integration between serotonin and catecholamines is known to take place in some molluscan tissues (Stefano et al. 1976; Hiripi and Stefano 1980; Filla et al. 2009). For example, increased serotonin levels enhance dopamine synthesis in the pond snail *Lymnaea stagnalis* (Filla et al. 2009). This upregulation may be an intermediate step in the negative feedback of serotonin, since *M. edulis* ganglia exposed to dopamine downregulates serotonin synthesis by

reducing tryptophan hydroxylase activity (Stefano et al. 1976; Hiripi and Stefano 1980). Based on our results, the role of serotonin and its precursors in the larval settlement of mussels warrant further investigation and may present a unique opportunity for identification of novel neurotransmitter functions for this class of taxa.

Bacterial Treatments

Larval settlement of *A. maoriana* in response to *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1 in their planktonic and biofilm phases and their exudates revealed that all strains did not produce settlement inducing cues for this species of mussel. These results differ from previous work on *P. canaliculus*, which demonstrated that bacterial biofilms and exudates from *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1produced significantly higher larval settlement (over 60% more settlement compared to controls; Ganesan et al. 2010). Conversely, *Pseudoalteromonas* sp. AMGP1 biofilms and exudates resulted in non-significant *P. canaliculus* settlement differences with controls. However, in the present study, exudates from all three bacterial strains inhibited *A. maoriana* settlement, suggesting that the inhibiting compound was not surface-bound.

Mortality results indicate that all three bacterial strains may produce toxic compounds during their planktonic and biofilm phases. If the acute toxic responses were caused by such secondary bacterial metabolites, the metabolites likely would be surface-bound to the bacterial cells, since no significant mortality was observed when larvae were exposed to exudates alone. Alternatively, it is possible that respiring bacterial cells (in planktonic or biofilm phases) could have lowered dissolved oxygen concentrations in the seawater, causing significant larval mortality. In addition,

bacterial biofilms of *Pseudoalteromonas* sp. AMGP1 were highly toxic to *A. maoriana* larvae. These results were paralleled to those of *P. canaliculus* (Ganesan et al. 2010). However, high mortality also was observed when *P. canaliculus* was exposed to biofilm exudates of *Pseudoalteromonas* sp. AMGP1, suggesting different susceptibilities to potential toxins.

The comparative results between these two endemic mussel species suggest that the mechanisms of settlement cue chemoreception is diverse, substantiating claims of previous studies on other marine invertebrates (reviewed by Morse 1990; Rodríguez et al. 1993; Hadfield and Paul 2001; Hay 2009; Hadfield 2011). For these two mussel species, the differences in larval settlement responses to mono-specific bacterial biofilms indicate that bacterial community structure on marine surfaces may differentially influence larval substrate preferences. This may partially explain why *A. maoriana* is found in the mid to low intertidal and *P. canaliculus* inhabits low intertidal to subtidal habitats. However, further studies would be needed to elucidate this relationship.

Evolutionary Trends and Future Directions

The effect of neuroactive compounds and their precursors on larval settlement of marine invertebrates can assist in the elucidation of endogenous signalling pathways. Researchers are continually finding that marine invertebrate larval settlement and metamorphosis can be differentially regulated by various combinations of these compounds across widely, and closely, related taxa. While of great interest to scientists, the evolutionary implications of such highly conserved life history events possibly being governed by very different biochemical pathways have not yet been commented on in the literature. However, recent advances in '-omics' based approaches (e.g., transcriptomics, proteomics, metabolomics) provide us with

significant scope to further understand the molecular processes involved in cue reception, and may yield evidence to identify evolutionary trends.

Characterisation of compounds, and their biochemical modes of action, which have the ability to regulate marine invertebrate fouling has significant commercial applications. The development of novel technologies to promote, or inhibit, larval attachment would be highly valuable for various industry sectors, such as aquaculture (e.g., enhancement of wild larval catch and reseeding of juveniles), sea transportation and maintenance of underwater structures (e.g., reducing hull and structural fouling).

The relatively new and rapidly escalating fields of comparative and evolutionary neurobiology will benefit from research advances in larval settlement induction. Some of these future directions lead on from our results and include: a) experiments to test the effect of specific reuptake inhibitors for GABA, acetylcholine, dopamine and catecholamines on larval settlement, b) the use of specific inhibitors to inhibit enzymes involved in epinephrine and serotonin biosynthesis to determine settlement responses, c) the use of specific agonists/antagonists of receptor sub-types within GABA, acetylcholine, dopamine, adrenergic and serotonin receptor classes to identify settlement responses, d) determination of temporal metabolite flux in larvae induced to settle by xenobiotic and endogenous occurring chemical cues, and e) investigation of gene and protein expression before, during and after the larval settlement event under normal and induced states.

Acknowledgements

We are thankful to Dan McCall from SPATnz and the Cawthron Institute, Nelson New Zealand, for providing mussel larvae for these experiments. We are grateful to the laboratory technicians in the School of Applied Sciences, Auckland University of Technology (AUT), for their ongoing assistance. We also thank the staff and students of the Aquaculture Biotechnology Group at AUT for assisting with the laboratory experiments and for the many fruitful discussions which made this work possible. This research was supported by an AUT Research Grant to A.C. Alfaro.

References

Aiello, E., Guideri, G. (1966). Relationship between 5-hydroxytryptamine and nerve stimulation of ciliary activity. *Journal of Pharmacology and Experimental Therapeutics* 154(3):517-523.

Aiello, E., Stefano, G. B., Catapane, E. J. (1981). Dual innervation of the foot and the control of foot movement by the central nervous system in *Mytilus edulis* (Bivalvia). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 69(1):25-30.

Alfaro, A. C., Copp, B. R., Appleton, D., Kelly, S., Jeffs, A. G. (2006). Chemical cues promote settlement in larvae of the green-lipped mussel, *Perna canaliculus*. *Aquaculture International* 14(4):405-412.

Arendt, D., Denes, A. S., Jekely, G., Tessmar-Raible, K. (2008). The evolution of nervous system centralization. *Philosophical Transactions of the Royal Society B* 363:1523-1528.

Audesirk, G., McCaman, R. E., Willows, A. O. D. (1979). The role of serotonin in the control of pedal ciliary activity by identified neurons in *Tritonia diomedea*. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 62(1):87-91.

Baloun, A. J., Morse, D. E. (1984). Ionic control of settlement and metamorphosis in larval *Haliotis rufescens* (Gastropoda). *Biological Bulletin* 167:124-138.

Bao, W. Y., Satuito, C., Yang, J. L., Kitamura, H. (2007). Larval settlement and metamorphosis of the mussel *Mytilus galloprovincialis* in response to biofilms. *Marine Biology* 150(4):565-574.

Beiras, R., Widdows, J. (1995). Induction of metamorphosis in larvae of the oyster *Crassostrea gigas* using neuroactive compounds. *Marine Biology* 123:327-334.

Boadle-Biber, M. C., Roth, R. H. (1972). Factors modifying the synthesis of dopamine from tyrosine in pedal ganglia of *Mercenaria mercenaria* (Mollusca). *Comparative and General Pharmacology* 3(9):61-74.

Bokisch, A. J., Walker, R. J. (1986). The ionic mechanism associated with the action of putative transmitters on identified neurons of the snail, *Helix aspersa*. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 84(2):231-241.

Brown, M., Burrell, D. E., Stefano, G. B. (1981). Analysis of monoamine accumulations in the neuronal tissues of *Mytilus edulis* (bivalvia)- II: Pharmacological alteration of pedal ganglia monoamine uptake. *Comparative*

Biochemistry and Physiology Part C: Comparative Pharmacology 70(2):215-221.

Browne, K. A., Zimmer, R. K. (2001). Controlled field release of a waterborne chemical signal stimulates planktonic larvae to settle. *Biological Bulletin* 200:87-97.

Carroll, M. A., Catapane, E. J. (2007). The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 148(2):445-450.

Catapane, E. J. (1983). Comparative study of the control of lateral ciliary activity in marine bivalves. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 75(2):403-405.

Coniglio, L., Morale, A., Angelini, C., Falugi, C. (1998). Cholinergic activation of settlement in *Ciona intestinalis* metamorphosing larvae. *Journal of Experimental Zoology* 280(4):314-320.

Dobretsov, S. V., Qian, P. Y. (2003). Pharmacological induction of larval settlement and metamorphosis in the blue mussel *Mytilus edulis* L. *Biofouling* 19(1):57-63.

Dobretsov, S., Qian, P. Y. (2004). The role of epibotic bacteria from the surface of the soft coral *Dendronephthya* sp. in the inhibition of larval settlement. *Journal of Experimental Marine Biology and Ecology* 299(1):35-50.

Eyster, L. S., Pechenik, J. A. (1988). Attachment of *Mytilus edulis* L. larvae on algal and byssal filaments is enhanced by water agitation. *Journal of Experimental Marine Biology and Ecology* 114(2-3):99-110.

Faimali, M., Falugi, C., Gallus, L., Piazza, V., Tagliafierro, G. (2003). Involvement of acetylcholine in settlement of *Balanus amphitrite*. *Biofouling* 19(1):213-220.

Fickbohm, D. J., Spitzer, N., Katz, P. S. (2005). Pharmacological manipulation of serotonin levels in the nervous system of the opisthobranch mollusc *Tritonia diomedea*. *Biological Bulletin* 209(1):67-74.

Filla, A., Hiripi, L., Elekes, K. (2009). Role of aminergic (serotonin and dopamine) systems in the embryogenesis and different embryonic behaviors of the pond snail, *Lymnaea stagnalis*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 149(1):73-82.

Ganesan, A. M., Alfaro, A. C., Brooks, J. D., Higgins, C. M. (2010). The role of bacterial biofilms and exudates on the settlement of mussel (*Perna canaliculus*) larvae. *Aquaculture* 306(1-4):388-392.

Gapasin, R. S. J., Polohan, B. B. (2004). Induction of larval settlement in the donkey-ear abalone, *Haliotis asinina* Linnaeus, by chemical cues. *Hydrobiologia* 519:9-17.

Garcia-Lavandeira, M., Silva, A., Abad, M., Pazos, A. J., Sanchez, J. L., Perez-Paralle, M. L. (2005). Effects of GABA and epinephrine on the settlement and metamorphosis of the larvae of four species of bivalve molluscs. *Journal of Experimental Marine Biology and Ecology* 316(2):149-156.

Giard, W., Favrel, P., Boucaud-Camou, E. (1995). *In vitro* investigation of α-amylase release from the digestive cells of the bivalve mollusc *Pecten maximus*: effect of second messengers and biogenic amines. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology,* 164(7):518-523.

Gibbs, M., Pilcher, O., Keeley, N., Heasman, K., Webb, S. (2006). Aquaculture stocking density guidelines for alternative bivalve species. Cawthorn Report No. 1192.

Gies, A. (1986). Serotonin and dopamine as regulators of adenylate cyclase and relaxation in a smooth muscle of the mussel *Mytilus edulis*. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 84(1):61-66.

Gosling, E. (2003). *Bivalve molluscs: biology, ecology and culture*. Blackwell Science, Oxford, U.K. 443 pp.

Grant, M. N. (2009). Chemical induction of settlement in larvae of the Eastern oyster *Crassostrea virginica* (Gmelin). Masters Thesis, University of Maryland, College Park, MD, United Sates.

Hadfield, M. G. (2011). Biofilms and marine invertebrate larvae: what bacteria produce that larvae use to choose settlement sites. *Annual Review of Marine Science* 3(1):453-470.

Hadfield, M. G., Paul, V. J. (2001). Natural chemical cues for settlement and metamorphosis of marine-invertebrate larvae. In J. B. McClintock and B. J. Baker, (Eds.) *Marine Chemical Ecology*. CRC Press, Florida, USA.

Harder, T., Dobretsov, S., Qian, P. Y. (2004). Waterborne polar macromolecules act as algal antifoulants in the seaweed *Ulva reticulata*. *Marine Ecology Progress Series* 274:133-141.

Harder, T., Lam, C., Qian, P. Y. (2002). Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues. *Marine Ecology Progress Series* 229:105-112.

Hay, M. E. (2009). Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science* 1:193-212.

Hiripi, L., Stefano, G. B. (1980). Dopamine inhibition of tryptophan hydroxylase in molluscan nervous tissue homogenates: evidence for intracellular site of action. *Life Sciences* 27(13):1205-1209.

Jacobson, P. J., Farris, J. L., Neves, R. J., Cherry, D. S. (1993). Use of neutral red to assess survival of juvenile freshwater mussels (Bivalvia: Unionidae) in bioassays. *Transaction of the American Microscopical Society* 112(1):78-80.

Jones, H. D., Richards, O. G. (1993). The effects of acetylcholine, dopamine and 5-hydroxytryptamine on water pumping rate and pressure in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology* 170(2);227-240.

Kang, K. H., Kim, B-H., Kim, J. M., Kim, Y. H. (2003). Effects of amino acids on larval settlement and metamorphosis in *Haliotis discus hannai*. *Korean Journal of Malacology* 19(2):95-106.

Ke, C. H., Li, S. J., Li, F. X., Zheng, Z. (1998). Ionic control of attachment and metamorphosis in the green mussel *Perna viridis* (Linnaeus) larvae. *Oceanologua et Limnologia Sinica* 29:128-134.

Kennedy, V. S. (1976). Desiccation, higher temperatures and upper intertidal limits of three species of sea mussels (Mollusca: Bivalvia) in New Zealand. *Marine Biology* 35:127-137.

Kennedy, V. S. (1977). Reproduction in *Mytilus edulis aoteanus* and *Aulacomya maoriana* (Mollusca: Bivalvia) from Taylors Mistake, New Zealand. *New Zealand Journal of Marine and Freshwater Research* 11(2):255-267.

Kravitz, E. A. (1988). Hormonal control of behavior: amines and the biasing of behavioral output in lobsters. *Science* 241(4874):1775-1781.

Lachowicz, L. S. (2005). Population biology of mussels (*Aulacomya maoriana,Mytilus galloprovincialis* and *Perna canaliculus*) from rocky intertidal shores in Wellington Harbour, New Zealand. PhD Thesis, Victoria University of Wellington.

Lent, C. M. (1973). Retzius cells: neuroeffectors controlling mucus release by the Leech. *Science* 179(4074):693-696.

Leyton, Y. E., Riquelme, C. E. (2008). Use of specific bacterial-microalgal biofilms for improving the larval settlement of *Argopecten purpuratus* (Lamarck, 1819) on three types of artificial spat-collecting materials. *Aquaculture* 276(1-4):78-82.

Manahan, D. T. (1989). Amino acid fluxes to and from seawater in axenic veliger larvae of a bivalve (*Crassostrea gigas*). *Marine Ecology Progress Series 53*:247-255.

Martínez, G., Saleh, F., Mettifogo, L., Campos, E., Inestrosa, N. (1996). Monoamines and the release of gametes by the scallop *Argopecten purpuratus*. *Journal of Experimental Zoology* 274(6):365-372.

Mesías-Gansbiller, C., Bendimerad, M. E. A., Roman, G., Pazos, A. J., Sanchez, J. L., Perez-Paralle, M. L. (2008). Settlement behavior of black scallop larvae in

response to GABA, epinephrine and IBMX. *Journal of Shellfish Research* 27(2):261-264.

Morse, D. E. (1990). Recent progress in larval settlement and metamorphosis: closing the gaps between molecular biology and ecology. *Bulletin of Marine Science* 46(2):465-483.

Morse, A. N. C., Morse, D. E. (1984a). GABA-mimetic molecules from *Porphyra* (Rhodophyta) induce metamorphosis of *Haliotis* (Gastropods) larvae. *Hydrobiologia* 116-117(1):155-158.

Morse, A. N. C., Morse, D. E. (1984b). Recruitment and metamorphosis of *Haliotis* larvae induced by molecules uniquely available at the surfaces of crustose red algae. *Journal of Experimental Marine Biology and Ecology* 75(3):191-215.

Mountfort, D. O., Pybus, V. (1992). Effect of pH, temperature and salinity on the production of gamma aminobutyric acid (GABA) from amines by marine bacteria. *FEMS Microbiology Letters* 101(4):237-244.

Murthy, P. S., Venugopalan, V. P., Nair, K. V. K., Subramoniam, T. (2009). Larval settlement and surfaces: implications in development of antifouling strategies. In Flemming, H-C., P. S. Murthy, R. Venkatesan, K. E. Cooksey (Eds.) *Marine and Industrial Biofouling* (pp. 233-263). New York: Springer-Verlag New York, USA.

Neo, M., Todd, P., Teo, S., Chou, L. (2009). Can artificial substrates enriched with crustose coralline algae enhance larval settlement and recruitment in the fluted giant clam (*Tridacna squamosa*)? *Hydrobiologia* 625(1):83-90.

O'Connor, S., Moltschaniwskyj, N., O'Connor, W. (2009). Use of neuroactive catecholamines to chemically induce metamorphosis of hatchery-reared flat oyster, *Ostrea angasi*, larvae. *Aquaculture Research* 40(14):1567-1577.

Panchin, Y. V., Arshavsky, Y. I., Deliagina, T. G., Orlovsky, G. N., Popova, L. B., Selverston, A. I. (1996). Control of locomotion in the marine mollusc *Clione limacina* XI. Effects of serotonin. *Experimental Brain Research* 109(2):361-365.

Pani, A. K., Croll, R. P. (1995). Distribution of catecholamines, indoleamines, and their precursors and metabolites in the scallop *Placopecten magellanicus*: (Bivalvia, Pectinidae). *Cellular and Molecular Neurobiology* 15(3):371-386.

Pani, A. K., Croll, R. P. (1998). Pharmacological analysis of monoamine synthesis and catabolism in the scallop, *Placopecten magellanicus*. *General Pharmacology* 31(1):67-73.

Pani, A. K., Croll, R. P. (2000). Catechol concentrations in the hemolymph of the scallop, *Placopecten magellanicus*. *General and Comparative Endocrinology* 118(1):48-56.

Paparo, A., Finch, C. E. (1972). Catecholamine localization, content, and metabolism in the gill of two lamellibranch molluscs. *Comparative and General Pharmacology* 3(11):303-309.

Pawlik, J. R. (1992). *Chemical ecology of the settlement of benthic marine invertebrates* (Vol. 30). Boca Raton, FL, ETATS-UNIS: CRC Press.

Pechenik, J. A., Hadfield, M. G., Eyster, L. S. (1995). Assessing whether larvae of the opisthobranch gastropod *Phestilla sibogae* Bergh become responsive to three chemical cues at the same age. *Journal of Experimental Marine Biology and Ecology* 191(1):1-17.

Peng Loh, Y., Jacklet, J.W. (1977). Catecholamine and protein synthesis in the eyes of *Aplysia californica*. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 57(2):159-163.

Phillips, N. E., Wood, A. R., Hamilton, J. S. 2008. Molecular species identification of morphologically similar mussel larvae reveals unexpected discrepancy between relative abundance of adults and settlers. *Journal of Experimental Marine Biology and Ecology* 362:90-94.

Pires, A., Woollacott, R. M. (1997). Serotonin and dopamine have opposite effects on phototaxis in larvae of the bryozoan *Bugula neritina*. *Biological Bulletin* 192(3):399-409.

Platter-Rieger, M. F., Frank, S. B. (1987). An assessment of tributyltin (TBT) effects on mussel larvae (*Mytilus edulis*) using a vital stain technique (abstract). Society of Environmental Toxicology and Chemistry, 8th Annual Meeting; 9-12th; Pensacola, Florida. USA.

Ritson-Williams, R., Paul, V. J., Arnold, S. N., Steneck, R. S. (2010). Larval settlement preferences and post-settlement survival of the threatened Caribbean corals *Acropora palmata* and *A. cervicornis*. *Coral Reefs* 29(1):71-81.

Roberts, R. (2001). A review of settlement cues for larval abalone (*Haliotis* spp.). *Journal of Shellfish Research* 20(2):571-586.

Rodríguez, S. R., Ojedal, F. P., Inestrosa, N. C. (1993). Settlement of benthic marine invertebrates. *Marine Ecology Progress Series* 97:193-207.

Shu-Ing, Y., Malanga, C. J., Azzaro, A. J. (1977). The disposition of tritiated dopamine by gills of *Mytilus edulis* and *Modiolus demissus*. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 58(1):49-55.

Stefano, G. B. (1990). Norepinephrine: presence and interaction with endogenous biogenic amines. In Stefano, G. B. (Ed.), *Neurobiology of Mytilus edulis* (pp. 93-101). Manchester, UK: Manchester University Press.

Stefano, G. B.,Catapane, E., Aiello, E. (1976). Dopaminergic agents: influence on serotonin in the molluscan nervous system. *Science* 194(4264):539-541.

Steinberg, P. D., De Nys, R. (2002). Chemical mediation of colonization of seaweed surfaces. *Journal of Phycology* 38(4):621-629.

Steinberg, P. D., de Nys, R., Kjelleberg, S. (2002). Chemical cues for surface colonization. *Journal of Chemical Ecology* 28(10):1935-1951.

Sumin, L., Zhenmin, B., Hui, L., Jianguang, F. (2006). Effect of epinephrine on the settlement and metamorphosis of Manila clam larvae. *Journal of Ocean University of China (English Edition)* 5(2):141-145.

Tamburri, M. N., Zimmer-Faust, R. K., Tamplin, M. L. (1992). Natural sources and properties of chemical inducers mediating settlement of oyster larvae: a re-examination. *Biological Bulletin* 183(2):327-338.

Tamburri, M. N., Finelli, C. M., Wehtey, D. S., Zimmer-Faust, R. K. (1996). Chemical induction of larval settlement behavior in flow. *Biological Bulletin* 191:367-373.

Tamburri, M. N., Luckenbach, M. W., Breitburg, D. L., Bonniwell, S. M. (2008). Settlement of *Crassostrea ariakensis* larvae: effects of substrate, biofilms, sediment and adult chemical cues. *Journal of Shellfish Research* 27(3):601-608.

Tortell, P. (1980). Preliminary experimental rearing of mussel (*Mytilus edulis aoteanus,Aulacomya maoriana* and *Perna canaliculus*) larvae in the laboratory. *Mauri Ora* 8:21-33.

Uhler, G. C., Huminski, P. T., Les, F. T., Fong, P. P. (2000). Cilia-driven rotational behavior in gastropod (*Physa elliptica*) embryos induced by serotonin and putative serotonin reuptake inhibitors (SSRIs). *Journal of Experimental Zoology* 286(4):414-421.

van Nierop, P., Bertrand, S., Munno, D. W., Gouwenberg, Y., van Minnen, J., Spafford, J. D., et al. (2006). Identification and functional expression of a family of nicotinic acetylcholine receptor subunits in the central nervous system of the mollusc *Lymnaea stagnalis*. *Journal of Biological Chemistry* 281(3):1680-1691.

van Nierop, P., Keramidas, A., Bertrand, S., van Minnen, J., Gouwenberg, Y., Bertrand, D., et al. (2005). Identification of molluscan nicotinic acetylcholine receptor (nAChR) subunits involved in formation of cation- and anion-selective nAChRs. *The Journal of Neuroscience* 25(46):10617-10626.

Wang, G., Liu, B., Tang, B., Zhang, T., Xiang, J. (2006). Pharmacological and immunocytochemical investigation of the role of catecholamines on larval metamorphosis by [beta]-adrenergic-like receptor in the bivalve *Meretrix meretrix*. *Aquaculture 258*(1-4):611-618.

Wikstrom, S. A., Pavia, H. (2004). Chemical settlement inhibition *versus* post-settlement mortality as an explanation for differential fouling of two congeneric seaweeds. *Oecologia* 138:223-230.

Williams, E. A., Craigie, A., Yeates, A., Degnan, S. M. (2008). Articulated coralline algae of the genus *Amphiroa* are highly effective natural inducers of settlement in the tropical abalone *Haliotis asinina*. *Biological Bulletin* 215:98-107.

Yamamoto, H., Shimizu, K., Tachibana, A., Fusetani, N. (1999). Roles of dopamine and serotonin in larval attachment of the barnacle, *Balanus amphitrite*. *Journal of Experimental Zoology* 284(7):746-758.

Yamamoto, H.,Tachibana, A., Kawaii, S., Matsumura, K., Fusetani, N. (1996). Serotonin involvement in larval settlement of the barnacle, *Balanus amphitrite*. *Journal of Experimental Zoology* 275(5):339-345.

Yarowsky, J., Carpenter, D.O. (1977). GABA mediated excitatory responses on neurons. *Life Sciences* 20(8):1441-1447.

Yool, A. J., Grau, S. M., Hadfield, M. G., Jensen, R. A., Markell, D. A., Morse, D. E. (1986). Excess potassium induces larval metamorphosis in four marine invertebrate species. *Biological Bulletin* 170(2):255-266.

Young, T. Y. (2009). Pharmacological induction of larval settlement in the New Zealand mussel, *Perna canaliculus*. Masters Thesis, Auckland University of Technology, Auckland, New Zealand.

Yu, X., Yan, Y., Ji-Dong, G. (2007). Attachment of the biofouling bryozoan *Bugula neritina* larvae affected by inorganic and organic chemical cues. *International Biodeterioration and Biodegradation* 60(2):81-89.

Yu, X., Yan, Y., Li, H. (2010b). The effect of chemical cues on larval settlement of the abalone, *Haliotis diversicolor supertexta*. *Journal of the World Aquaculture Society* 41(4):626-632.

Yu, X., He, W., Gu, J. D., He, M.,Yan, Y. (2008). The effect of chemical cues on settlement of pearl oyster *Pinctada fucata martensii* (Dunker) larvae. *Aquaculture* 277(1-2):83-91.

Yu, X., He, W., Li, H., Yan, Y., Lin, C. (2010a). Larval settlement and metamorphosis of the pearl oyster *Pinctada fucata* in response to biofilms. *Aquaculture* 306(1-4):334-337.

Zimmer-Faust, R. K., Tamburri, M. N. (1994). Chemical identity and ecological implications of a waterborne, larval settlement cue. *Limnology and Oceanography* 39:1075-1087.

Figure Captions

1. Chemical structure of treatment compounds used in settlement assays: A) Epinephrine biosynthesis pathway; B) Serotonin biosynthesis pathway.

2. Percent (±SE) settlement (graphs on left) and mortality (graphs of right) of mussel larvae exposed to different concentrations of chemical treatments. Notation includes C for control, 5 to 30 mM for potassium chloride, and 10^{-6} to 10^{-3} M for GABA and acetylcholine. Asterisks (*) denote significant differences against controls resulting from Dunn's multiple comparisons.

3. Percent (±SE) settlement (graphs on left) and mortality (graphs of right) of mussel larvae exposed to different concentrations of chemical treatments. Notation includes C for control, 10^{-7} to 10^{-5} M for L-Phenylalanine and L-Tyrosine, and 10^{-6} to 10^{-4} M for Dopamine and Epinephrine. Asterisks (*) denote significant differences against controls resulting from Dunn's multiple comparisons.

4. Percent (±SE) settlement (graphs on left) and mortality (graphs of right) of mussel larvae exposed to different concentrations of chemical treatments. Notation includes C for control, 10^{-7} to 10^{-5} M for L-Tryptophan, and 10^{-6} to 10^{-4} M for 5-Hydroxytryptophan. Asterisks (*) denote significant differences against controls resulting from Dunn's multiple comparisons.

5. Percent (±SE) settlement (graphs on left) and mortality (graphs of right) of mussel larvae exposed to planktonic bacteria, bacterial biofilms and biofilm exudates of three bacterial strains (M = *Macrococcus* sp. [AGMM1], B = *Bacillus* sp. [AGMB1], and P = *Pseudoalteromonas* sp. [AGMP1]), and controls (C). Asterisks (*) denote significant differences against controls resulting from Dunn's multiple comparisons.

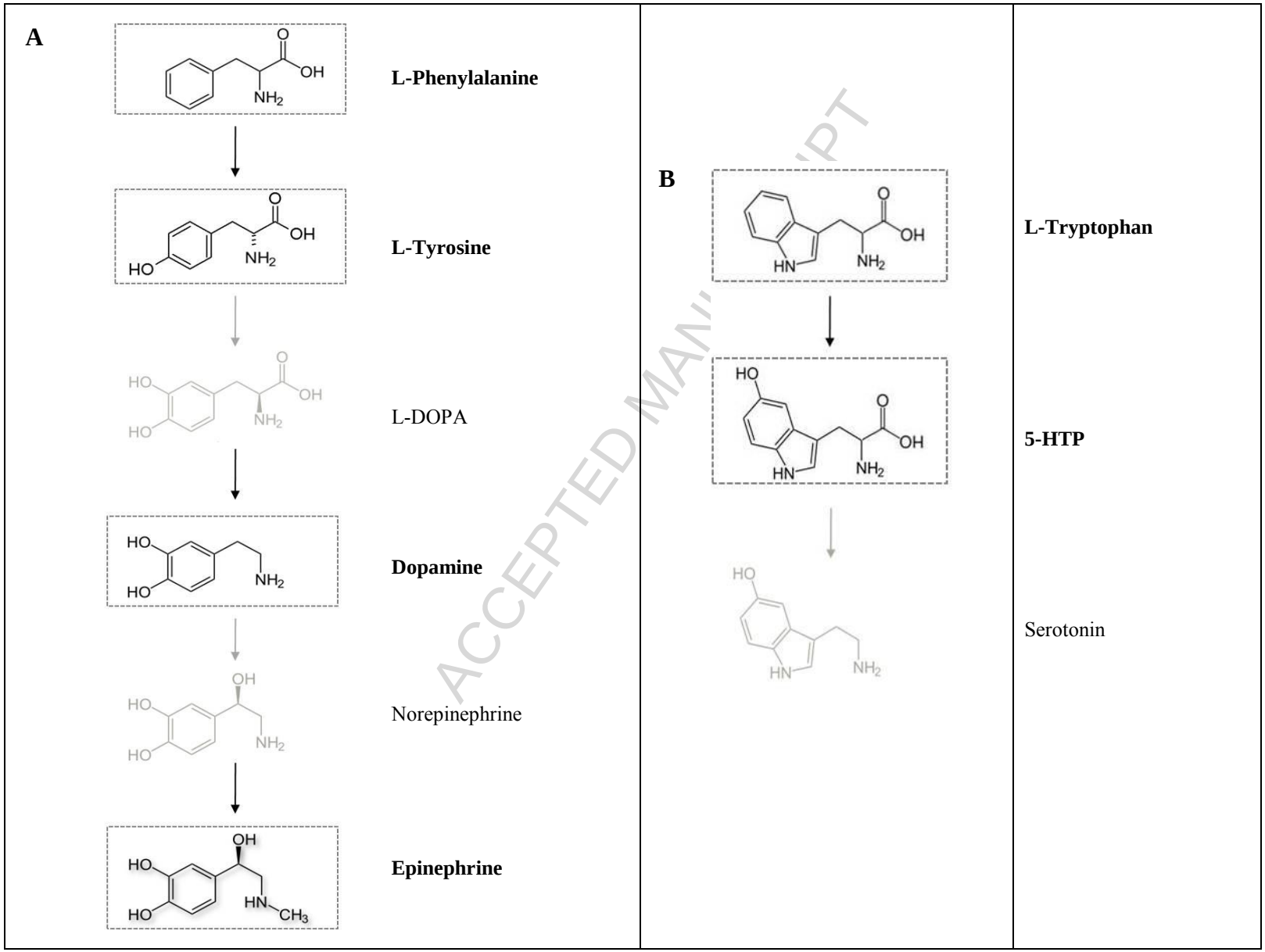

Figure 1

Figure 2

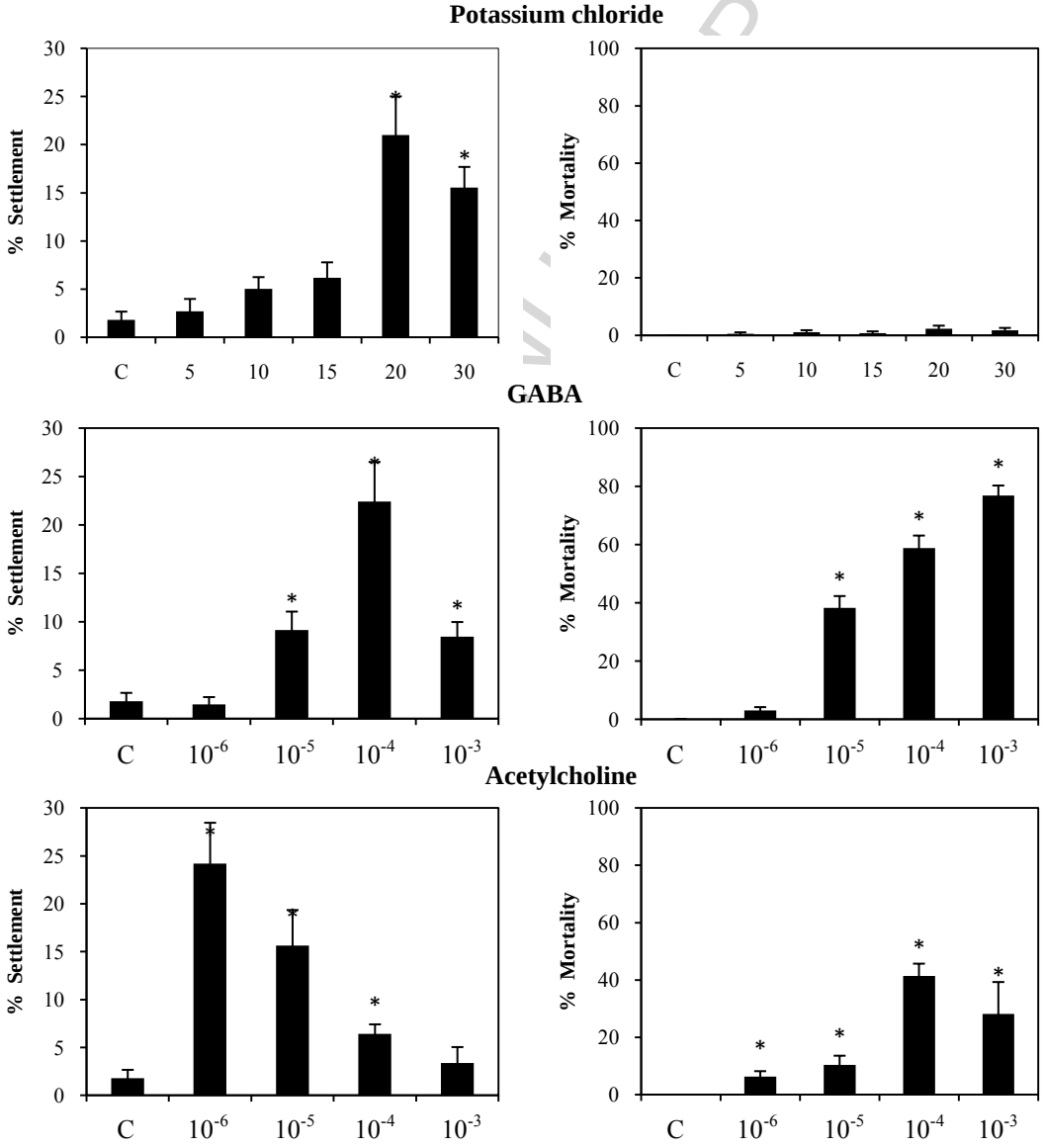

Figure 3

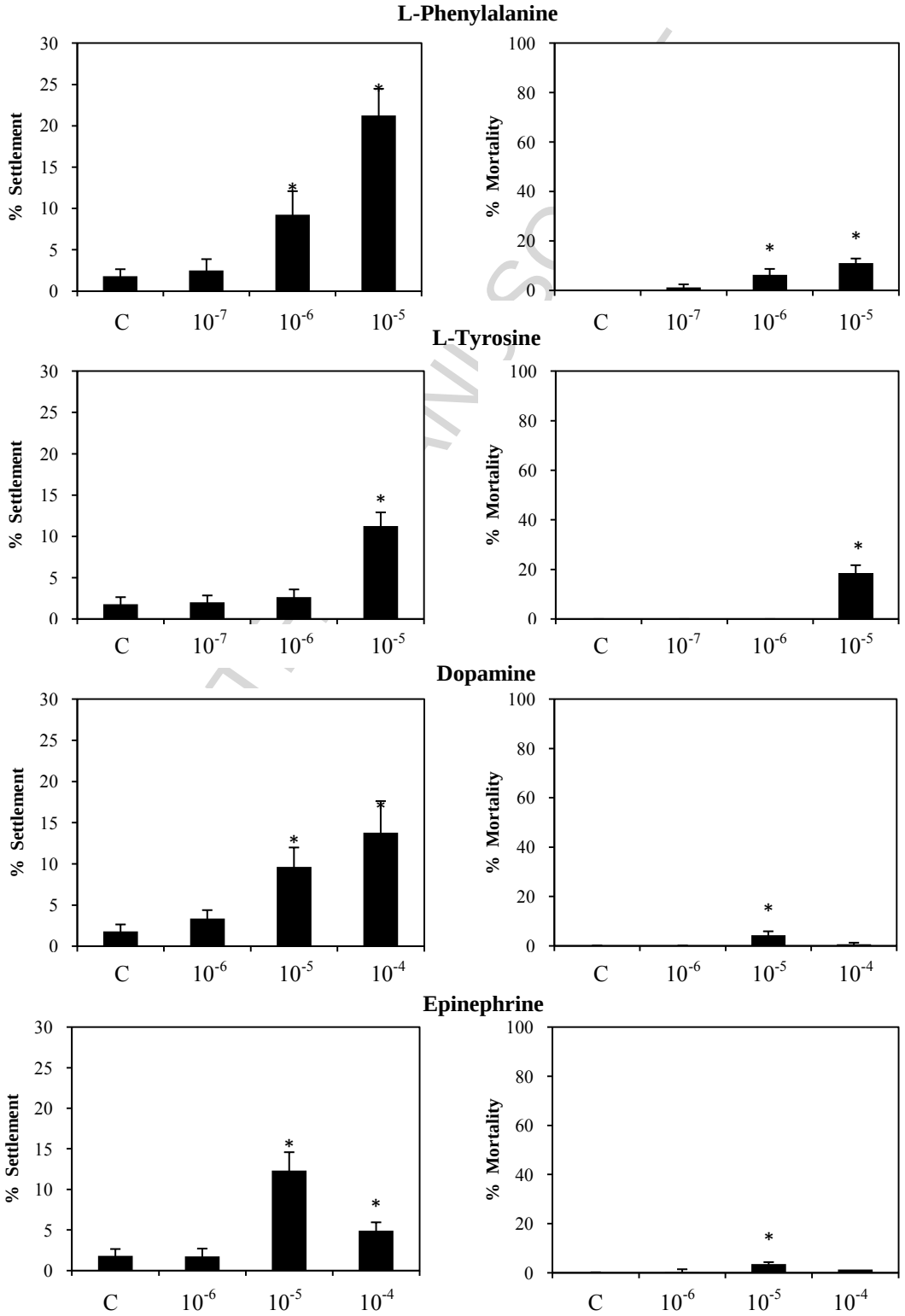

Figure 4

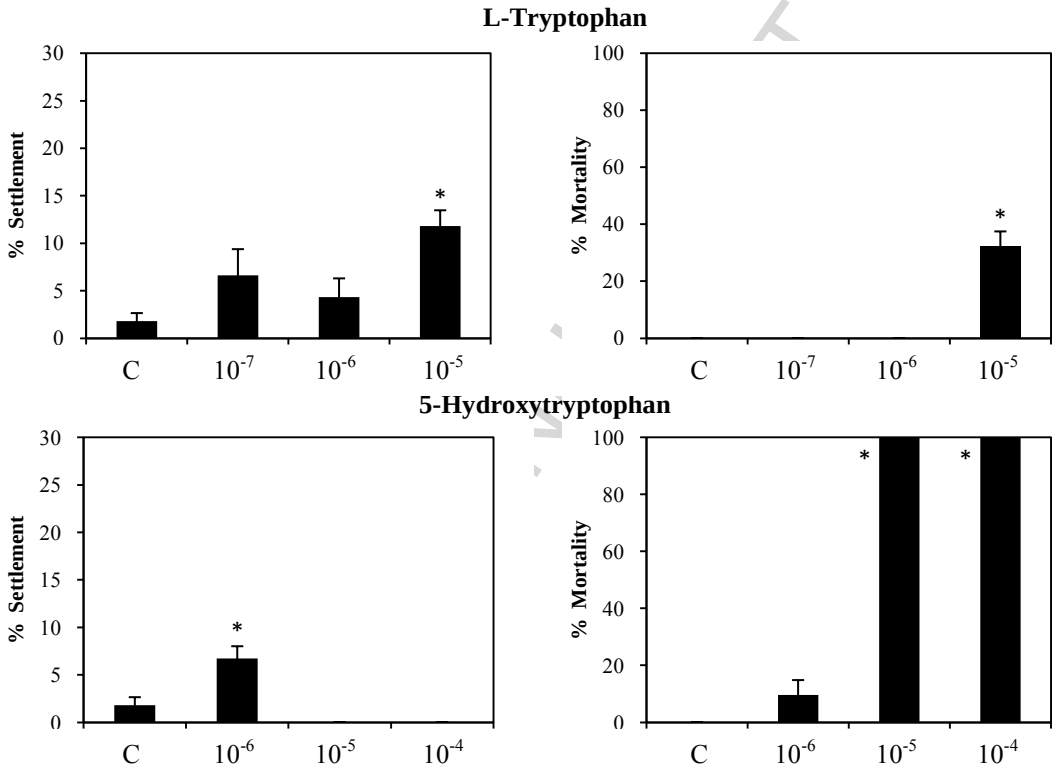

Figure 5

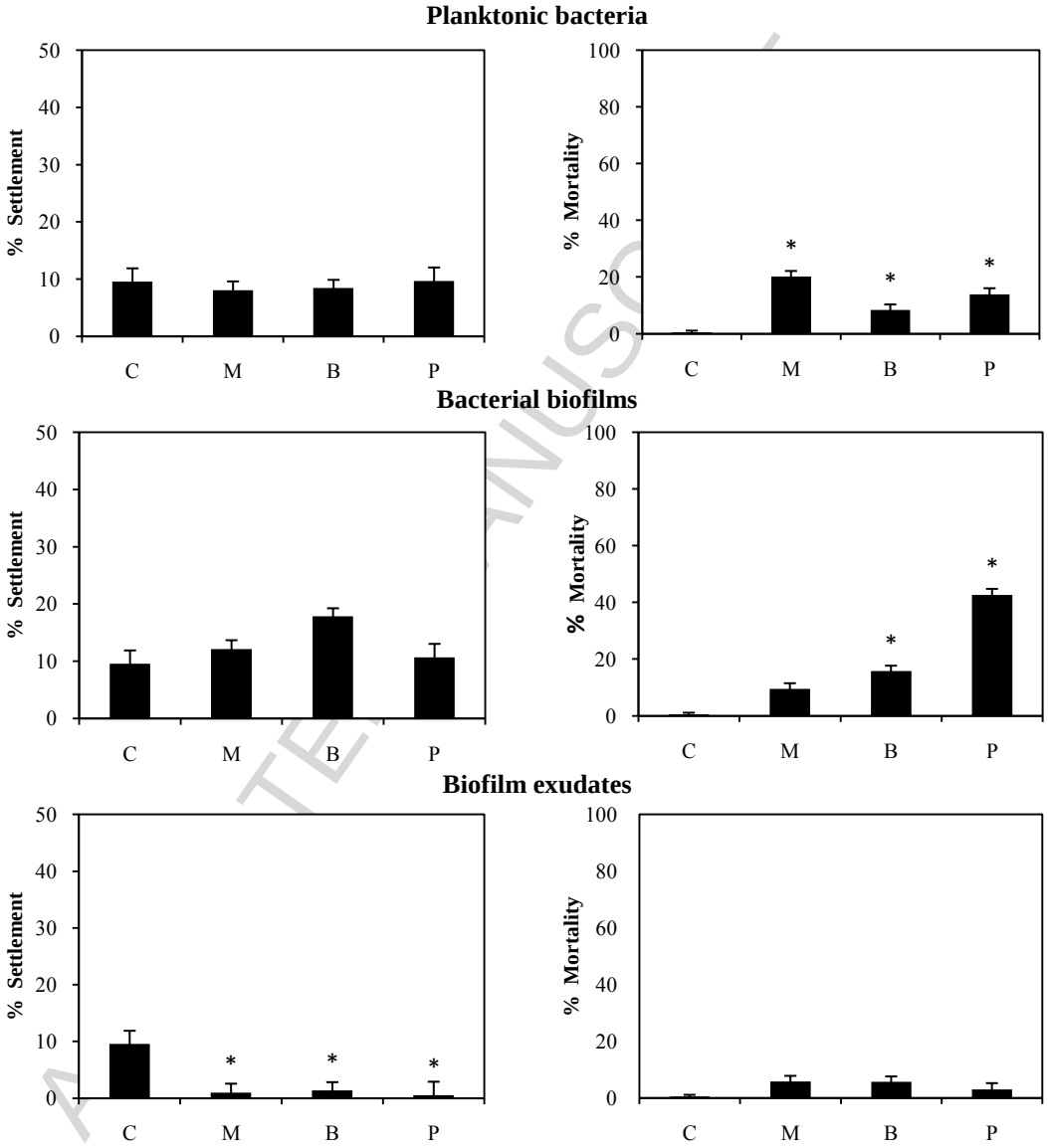

Table 1: Final treatment concentrations used in settlement experiments.

Treatment	Stock concentration	Exposure concentrations
KCl	300 mM	5, 10, 15, 20, 30 mM
GABA	10^{-2} M	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M
Acetylcholine	10^{-2} M	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M
L-Phenylalanine	10^{-3} M	10^{-7}, 10^{-6}, 10^{-5} M
L-Tyrosine	10^{-3} M	10^{-7}, 10^{-6}, 10^{-5} M
Dopamine	10^{-3} M	10^{-6}, 10^{-5}, 10^{-4} M
Epinephrine	10^{-3} M	10^{-6}, 10^{-5}, 10^{-4} M
L-Tryptophan	10^{-3} M	10^{-7}, 10^{-6}, 10^{-5} M
5-HTP	10^{-3} M	10^{-5}, 10^{-4}, 10^{-3} M

Table 2: Kruskal-Wallis tests of larval settlement and mortality after exposure to different concentrations of chemical treatments (potassium chloride, GABA, acetylcholine, L-Phenylalanine, L-Tyrosine, dopamine, epinephrine, L-Tryptophan, and 5-HTP). Bold *p*-values indicate significant differences across all treatments and control.

	Settlement								
	KCl	GABA	Acetylcholine	L-Phenylalanine	L-Tyrosine	Dopamine	Epinephrine	L-Tryptophan	5-HTP
H	33.38	30.70	24.11	24.32	16.76	12.57	18.24	12.17	18.67
df	5	4	4	3	3	3	3	3	3
p-value	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.006**	**0.001**	**0.007**	**0.001**
	Mortality								
	KCl	GABA	Acetylcholine	L-Phenylalanine	L-Tyrosine	Dopamine	Epinephrine	L-Tryptophan	5-HTP
H	7.95	44.42	25.53	28.43	42.08	16.59	12.20	27.68	39.18
df	5	4	4	3	3	3	3	3	3
p-value	0.159	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.007**	**0.001**	**0.001**

Table 3: Kruskal-Wallis tests of larval settlement and mortality within different bacterial treatments (planktonic bacteria, bacterial biofilms and biofilm exudates) using three bacterial strains (*Macrococcus* sp. [AGMM1], *Bacillus* sp. [AGMB1], and *Pseudoalteromonas* sp. [AGMP1]). Bold *p*-values indicate significant differences across all treatments and control.

	Settlement			*Mortality*		
	Planktonic bacteria	Bacterial biofilms	Biofilm exudates	Planktonic bacteria	Bacterial biofilms	Biofilm exudates
H	0.41	5.68	12.98	25.53	24.26	7.00
df	3	3	3	3	3	3
p-value	0.938	0.128	**0.005**	**0.001**	**0.001**	0.072