Augmented Coral Health:

Linking heterotrophic Diets to the acquisition of

Probiotics in Symbiotic Scleractinia

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General Abstract

Tropical coral reefs represent biodiversity hotspots that support vital ecosystem services including habitat for thousands of marine species, coastline protection as well as economic drivers for fisheries, aquaculture, tourism, and recreation industries. The integrity of these ecosystems relies upon a group of calcifying foundation species, symbiotic scleractinian corals. Confronted with a myriad of anthropogenic and environmental stressors, understanding what underpins the resilience of individual coral colonies, species and communities is of growing importance globally as reef health declines.

Optimal nutrition is paramount to the health of all organisms yet is an overlooked aspect of coral health and resilience. Nutritionally robust corals could be reared in controlled captive environments to help re-populate denuded reefs and relieve pressure on wild sourced corals for aquarium trade industries. Nutritional bottlenecks; however, are an impediment to maintaining a large biomass of healthy corals in captive aquaculture environments. One approach to enhance aquaculture coral nutrition is through the use of live prey enriched with beneficial nutrients or probiotic bacteria. However, corals vary in their ability to capture and ingest live feeds which inevitably impacts the efficacy of delivering beneficial nutrients or probiotics to produce a beneficial effect on an organism's health. In this study the capture and ingestion rates of live prey across three Indo-Pacific coral species with varying polyp sizes were investigated. In addition, novel probiotic delivery protocols for corals were developed to assess the application of nutritionally and microbially enhanced feeds on a larger scale.

Coral fragments sourced from adult *Acropora millepora*, *Pocillopora acuta* and *Galaxea fascicularis* colonies were fed *Artemia salina* nauplii with and without 1 μm fluorescent polystyrene microbeads. Capture rates were calculated by the difference in initial and final prey counts normalized to the number of coral polyps. Ingestion rates were calculated by fluorescent prey enumerated within decalcified coral polyps using a stereomicroscope with a fluorescent filter. Fluorescent microbeads were useful in the accurate detection of ingested live feeds. The combined capture and ingestion rates (individual prey/polyp/hour ± s.e.) across replicates delivered prey at 3 ind./mL were as follows: *A. millepora,* 0.66 ± 0.10; *P. acuta,* 1.56 ± 0.31; and *G. fascicularis*, 38.85 ± 3.80. Feeding rates were positively correlated with polyp size across these species. In *A. millepora*, feeding rates varied significantly by genotype with the replicates sourced from an adult colony showing signs of stress containing the only polyps with prey detected in gut cavities. Capture rate calculations overestimated the average number of *Artemia* nauplii consumed per polyp in *A. millepora* as compared to ingested prey enumerated from gut cavities whereas capture rate calculations underestimated the average number of nauplii consumed per polyp in both *P. acuta* and *G. fascicularis*. Presumably, feeding behaviors, such as the production of mucus in *A. millepora* may explain these results and demonstrate why an ability to directly detect prey within gut cavities is important for more accurate quantification of feeding in corals.

Live prey represent one option as a vehicle of administration for delivering probiotics to corals. Visualizing the administration, uptake and retention of the bacteria added via feeds to corals is currently lacking. To address this, 22 plasmid transconjugant strains of *Vibrio alginolyticus* were constructed with plasmid inserted fluorescent protein genes of different colors. However,

detection of fluorescence was not successful possibly due to the nature of the growth-independent promoter. The growth-independent promoter was selected here due to its availability for use with delivery plasmids containing a range of colors which is important to provide contrast to with auto-fluorescent coral tissues. A growth-dependent ribosomal promoter, available with delivery plasmids containing only green fluorescent protein genes, could stimulate higher rates of protein synthesis, resulting in higher fluorescence levels. Regardless, the histological and fluorescence microscopy approaches developed in this study to track live prey incubated with fluorescent microbeads may be valuable in modeling and visualizing the acquisition, retention, and establishment of delivered bacteria through prey vectors. Real time observations have important implications for the execution of successful delivery of bacterial probiotics to corals first in captivity with the long-term goal being field-deployment strategies.

The research findings presented herein refined our knowledge of the feeding capabilities across coral species from which further development of nutritional strategies can be implemented. The diversity of feeding behaviors, morphologies, and nutrient partitioning strategies can be used to design specific feeding and probiotic regimes for different types of corals. Better characterizations of the feeds a coral can and cannot consume depend upon improved assessment strategies and fluorescent microbeads may be an invaluable tool in future studies. Enhanced diets are critical to the success of commercial aquaculture operations and thus their inclusion in coral aquaculture has the potential to improve growth, health and resilience of captive corals allowing for expansion of coral aquaculture activities. Well-fed corals are likely to be more resilient to stressors (e.g., higher temperatures): therefore enhancing nutritional strategies for corals reared in captive environments can help re-populate denuded reefs with nutritionally

robust corals and support sustainable coral cultivation without relying on wild sources. Through controlled experimental trials conducted in this study, important foundational knowledge is provided from which to expand the inclusion of species-specific coral feeding regimes in captive coral aquaculture as a proposed reef restoration strategy and means of supplying cultured organisms for the marine ornamental aquarium industries.

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1 Chapter 1

1.1 Introduction to Coral Nutrition

Tropical coral reefs are often referred to as the rainforests of the sea. These biodiverse marine ecosystems support an estimated quarter of all known marine species (Bouchet et al. 2006; Plaisance et al. 2011). Coral reefs offer ecosystem services, such as protection from coastal erosion and storm surge, as well as directly sustain fisheries, aquaculture, pharmaceutical, tourism and recreation industries (Spurgeon & Lindahl, 2000; Osinga et al. 2011; Deliotte, 2017). The calcareous structures deposited by symbiotic scleractinian corals are what enable these important ecosystems to thrive, yet anthropogenic and environmental stressors have caused declines in coral cover globally (Hughes et al. 2016; Hughes et al. 2019). Now more than ever, an understanding of how to improve the health and resilience of reef-building organisms is paramount for the persistence of these environments and the ecologic and economic benefits dependent upon them.

A neglected potential driver of coral resilience is how heterotrophic feeding can support an organism's health. Flexible trophic strategies underpin the evolutionary success of symbiotic scleractinian corals. For example, primitive scleractinia which emerged 240 million years ago, relied on heterotrophy to meet their metabolic needs until the evolution of a nutritional partnership (symbiosis) with dinoflagellate algae, from the family Symbiodiniaceae (LaJeunesse et al. 2018).

This symbiotic association underpins the growth of modern coral in low nutrient, oligotrophic waters and facilitates the building of complex reef ecosystems such as the Great Barrier Reef (Dupraz & Strasser, 2002; Stanley & Van de Schootbrugge, 2009; LaJeunesse et al. 2018). Today, reef ecosystems are degrading globally due to the impacts of nutrients, sedimentation, over-fishing, wild sourcing for aquarium trade and ocean acidification (Souter et al. 2020). However, the most imminent threat corals face is thermal stress (i.e., bleaching) resulting directly from anthropogenic climate change (Palmer, 2018; Van de Water et al. 2018; Hughes et al. 2019; Morris et al. 2019). Thermal stress leads to the loss of photo-symbionts from the coral host leaving the coral skeleton completely white (Hughes & Grottoli, 2013; Hughes et al. 2019; Morrison et al. 2019). Bleaching deprives the host of its central energy source, photosynthates translocated from the photo-symbionts, and therefore severe bleaching can result in coral mortality. Heterotrophic feeding may provide a mechanism to promote resilience in corals by building up energy reserves to better withstand the energetic losses coupled with thermal stress.

The ability of a coral to derive benefits from heterotrophy depends upon their dominant nutritional strategy (Conti-Jerpe et al. 2020). Autotrophic dominant corals, such as those inhabiting shallow, light replete habitats, assimilate photosynthetically fixed carbon to meet up to 90% of their metabolic needs (Tremblay et al. 2012). Heterotrophic dominant corals, such as those inhabiting deeper or light limited habitats, assimilate externally sourced organic compounds to meet up to 60% of their metabolic needs (Falkowski et al. 1984). Mixotrophic corals, such as those inhabiting highly variable environments, co-vary across the resource acquisition spectrum (Houlbrèque et al. 2009). Regardless of the species-specific variation in

nutritional strategy, all symbiotic corals are mixotrophs. Symbiotic corals obtain autotrophic carbon (i.e., the photosynthates) as well as heterotrophic carbon, from live planktonic matter (LPM), suspended particulate matter (SPM) or dissolved organic matter (DOM) to drive key biological processes (Figure 1.1). Despite the importance of both nutritional pathways, the roles of autotrophy have been studied more extensively (Anthony & Fabricius, 2000; Furla et al. 2000; Little et al. 2004; Puill-Stephan et al. 2012) than the roles of heterotrophy in supporting coral health.

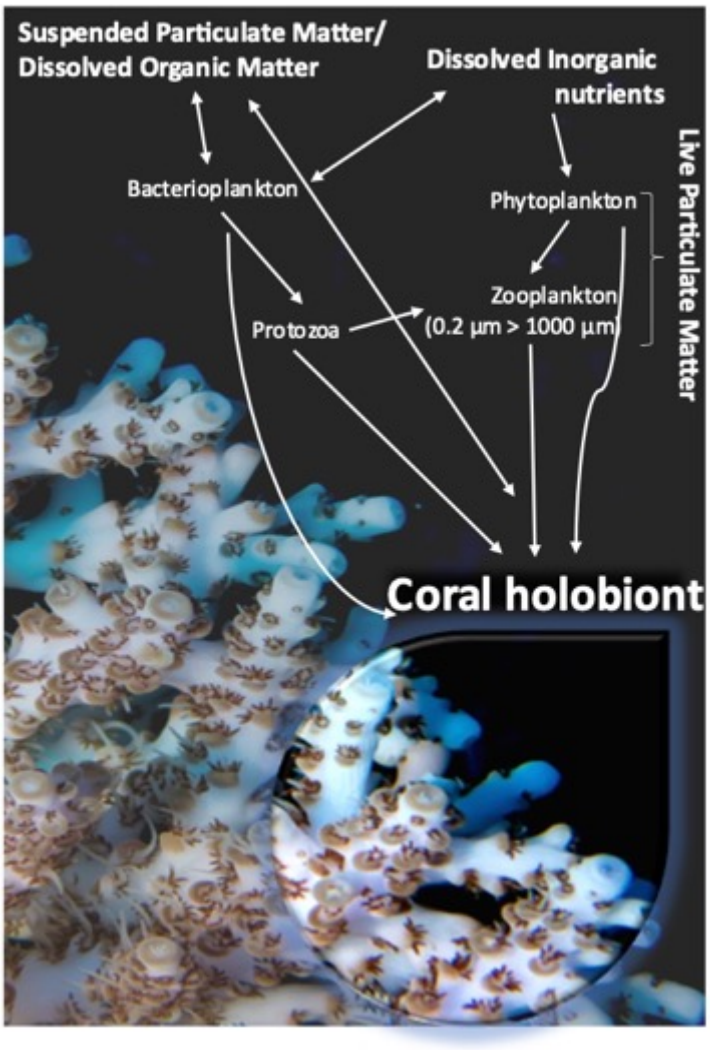

Figure 1.1 Coral trophic interactions. Adapted from figure 5 "Microbes in the coral holobiont: Partners through evolution, development, and ecological interactions," by Rivera et al. (2015) Frontiers in Cellular and Infection Microbiology.

Heterotrophy provides a wide-range of physiological and energetic functions to coral hosts, photo-symbionts and associated microorganisms. Field based studies identified a diversity of food sources able to be utilized by corals, as well as the relative importance of autotrophy versus heterotrophy in different species (Houlbrèque et al. 2004, 2009; Ferrier-Pagès et al. 2011; Hoogenboom et al. 2015). However, species-specific effects make it challenging to define a causal relationship between any specific food source and health in a diversity of ~ 800 symbiotic Scleractinia species (Veron et al. 2016). Field-based studies alone cannot elucidate the factors driving the variability in feeding strategies and derivative benefits to coral health due to confounding environmental factors (e.g., prey availability, depth, temperature, current, water quality). Understanding what roles various nutritional sources play requires an ability to test diets in controlled environments yet strictly controlled experimental studies still show inter- and intra-specific differences in the ability of corals to upregulate feeding and/or assimilate nutrients from feeds (i.e., feeding ability). Differences in feeding ability are likely driven by genotype (e.g., corals of the same species from different parent colonies) and physiological/morphological characteristics (e.g., polyp size) which should be investigated further. Determining the drivers dictating what external resources a coral can or cannot exploit could help improve feeds to meet the needs of different corals in aquarium systems.

Coral aquaculture faces nutritional bottlenecks that must be resolved in order to improve production (Leal et al. 2016). Culture organisms must be provided with supplementary nutrition to help with growth and disease resistance, which is particularly important for organisms reared in high density. Emerging literature on the composition and levels of lipids and fatty acids in corals provides useful baseline data on the energetic status of nutritionally competent corals

(Conlan, Bay, et al. 2018; Conlan, Humphrey, et al. 2018). Despite clear physiological benefits of feeding (Box 1), it is unclear how to manipulate feeds in ways which can significantly improve the composition of nutritional profiles in coral tissues (Conlan et al. 2017; Tagliafico et al. 2017; Conlan, Bay, et al. 2018). Energetic metrics, such as analyzing lipid and constituent fatty acid profiles may provide a better understanding of a coral's health status, the mechanisms of which are not yet fully understood and should be prioritized after improved characterizations of the diverse feeding abilities, preferences and behaviors of captive corals (Cárdenas et al. 2012; Sweet & Bulling, 2017). With improved feed delivery to corals with diverse nutritional needs, we can then augment diets with nutritionally enhanced feeds.

Microbiome manipulations of symbiotic corals suggest that beneficial microorganisms for corals (BMCs) could be used to buffer against disease and or nutritional losses (Damjanovic et al. 2019; Rosado et al. 2019). Feeding may be able to modulate coral microbiome health through the delivery of putatively probiotic inocula. Interactions between feeding and (i) the acquisition of microbial (e.g., prokaryotic) inputs for direct consumption, (ii) the establishment of nutritionally symbiotic communities and (iii) introduction of microorganisms with antimicrobial traits preventing colonization of opportunistic pathogens needs to be resolved. Controlled experiments may answer the question of how microbial partners are acquired, whether they establish symbiotic communities which serve a specific, beneficial function within hosts and how long these beneficial functions are maintained.

Given the concerning decline in the state of reefs globally, restoration efforts are critical and require an improved understanding of basic life history strategies of symbiotic scleractinian corals, the building blocks of these ecosystems. Results from experimental studies can be directly applied to coral cultivation strategies in aquaculture for both reef restoration and the growing ornamental aquarium trade, predicted to put additional pressures on wild corals (Wabnitz et al. 2003; Leal et al. 2016). Understanding energy acquisition and probiotic delivery strategies are central to the development of successful propagation methodologies which could impart benefits to a diversity of industries in three broad-ranging sectors: (i) coral aquaculture, (ii) ornamental and public aquarium industries (estimated annual net worth of US$60 million–US$1 billion with demand for coral growing 12-30% in the USA per annum) (Government of Western Australia Dept of Fisheries, 2009; Wood et al. 2012; Barton et al. 2015) and (iii) reef restoration projects with the accompanying benefits to recreational activities, fisheries and coastal protection, worth an estimated annual net worth of $6.4 billion (AUD) to the Great Barrier Reef region (Spurgeon & Lindahl, 2000; Osinga et al. 2011; Barton et al. 2015; Deliotte, 2017).

The research conducted in this thesis focuses on the characterization of the feeding ability of captive corals to develop delivery strategies of enhanced feeds to augment health. Although not investigated experimentally here, an understanding of how digested prey are assimilated into useful nutrients, as well as how feeding relates to microbiome maintenance is critical to identify effective feeding regimes. The aim of this review is to summarize our current understanding of the importance of heterotrophy in Scleractinia by highlighting the known links between feeding ability, diet, energetics and microbial profiles. This review evaluates the mechanisms and

derivative benefits of heterotrophy in symbiotic scleractinian corals. Firstly, I will provide background information on the energy budgets and nutritional modes of corals (section 1.2). Secondly, I will describe the morphological traits evolved for capturing and digesting prey items and summarize previous *ex situ* feeding studies (section 1.3). Lastly, I will discuss the emerging fields of research on coral energetics (section 1.4) and coral probiotics (section 1.5) and the potential to integrate results to improve the efficacy of coral aquaculture for the purpose of reef restoration. An improved characterization of the feeding abilities of corals is a critical stepping-stone from which to investigate the complex associations between external inputs, nutritional status, and microbiome function of corals for the inclusion of enhanced feeds for captive corals.

1.2 Overview of Energy Acquisition in Scleractinia

1.2.1 Energy Budget of host-algal symbiosis

Symbioses are ubiquitous in nature shaping ecosystems by cooperative and competitive interactions between their species. Many mutually beneficial symbioses exist between a host coral and its' associated microorganisms but the primary symbiosis within the holobiont is the bi-directional exchange of nutrients between hosts and associated photo-symbionts (Equation 1.1) (Table 1.1) (Llewellyn, 1982; Borneman, 2009; Brafield & Tremblay et al. 2012, 2016; Alldredge et al. 2013;). Products from photosynthesis (C_A), glucose and oxygen, drive cellular respiration, providing metabolic currency for coral hosts (R_H) and in exchange, the hosts provide shelter and carbon dioxide to drive algal photosynthesis (R_A). Both partners recycle key carbon, nitrogen and phosphorus nutrients to one another that are required for the production of biomass (P_A and P_H). Excess nutrients are either excreted (E), often as mucus, or, if useful, allocated towards gametogenesis (i.e., reproduction) (G) (equations adapted from Borneman, 2009;

Brafield & Tremblay et al. 2012, 2016). An understanding of coral health first depends upon an in depth understanding of the benefits of both autotrophic and heterotrophic nutrients as well as trade-offs in nutritional strategies.

Box 1. Advantages of increased heterotrophy in mitigating thermal stress

Heat stress events are predicted to become more common based on the current trajectory of fossil fuel emissions and therefore studies predicting how corals can acclimate or adapt to future climate scenarios are critical. Feeding ability and strategy may be a key factor of coral thermal tolerance (Grottoli et al 2006; Tremblay et al. 2016; Conte-Jerpe et al 2020). Corals with adequate energy reserves tend to possess higher tolerance to temperature changes (Anthony, 1999; Borell & Bischof, 2008; Forsman et al. 2012; Hughes & Grottoli, 2013; Osinga et al. 2012; Leal et al. 2016; Tagliafico et al. 2018). In bleached corals, not only does the amount of translocated carbon drop due to loss of Symbiodiniaceae, but also due to an increased retention of autotrophic carbon by the remaining symbionts. The coral host must rely solely on its carbon stores and heterotrophic feeding to survive. Feeding can promote resilience by (i) promoting photosynthesis in depleted Symbiodinaceae cells, (ii) providing useful nutrients to replenish lipid stores and (iii) stimulating recovery of symbioses following heat stress events (Hoogenboom et al. 2010; Hughes & Grottoli, 2013; Tagliafico et al. 2017; Tremblay et al. 2016), and other nutrients like metals.

In field experiments, sustained levels of increased heterotrophy in *Favia fragum* (Grottoli et al. 2006), *Porites compressa* and *Montipora capitata* (Hughes & Grottoli, 2013) under elevated temperatures resulted in lower mortality and greater recovery, despite overall declines in biomass, energy reserves (Grottoli et al. 2006), symbiont and chlorophyll densities (Hughes & Grottoli, 2013). To empirically elucidate changes to carbon budgets in thermally stressed corals, isotope markers were used to assess the metabolic adjustments of fed and unfed *Stylophora pistillata* colonies during a normal (25°C) and an acute (28 d) heat stress event (31°C). During heat stress, fed and unfed corals prioritized maintaining energy homeostasis by increasing energy expenditure to sustain tissue biomass (Tremblay et al. 2016). Higher rates of respiration indicated that corals began relying upon energy rich lipid reserves and/or exogenous food sources. Less energy was then allocated to growth and biomineralization, both energy intensive processes (Tremblay et al. 2016). Fed corals were able to resume normal nutritional exchanges when the acute heat stress subsided, suggesting that heterotrophy can promote the recovery of bleached *S. pistillata* corals (Tremblay et al. 2016). Fed, captive *Stylophora pisillata* colonies, in a long-term heat stress experiment (28 d at 31°C) were able to partially buffer against energetic losses yet suppressed levels of translocated carbon inhibited calcification and skeletal growth.

However, fed *S. pistillata* colonies recovered pre-bleaching nutritional exchanges once the temperature stress subsided, whereas unfed corals remained bleached. Similarly, heterotrophic dominant *Turbinaria, Favites* and *Platygyra* species were observed to take longer to bleach (~ 7.5 degree heating weeks) than auto-trophic dominant species (Conti-Jerpe et al. 2020). Although feeding can help with the re-establishments of photosynthate translocation after an acute heat stress episode, the benefits derived via heterotrophic pathways are not likely to be sufficient to buffer against cumulative impacts of climate change over time (Tremblay et al. 2016). Heterotrophic ability or plasticity will likely factor in thermal tolerance of corals under future climate scenarios (Hoogenboom et al. 2010; Imbs, 2013; Tagliafico et al. 2017).

Table 1.1 Simplified coral energy budget. Excludes microbial inputs and energetic outputs from competition

$C_A + C_H = (P_A + P_H) + (R_A + R_H) + E + G$ (1.1)	
Inputs	**Outputs**

C_A = Autotrophic inputs; photosynthates
• > 90% of energy budget
• Inefficient energy source
  o ≥80% C_A lost via R_A and E
• High in glucose, glycerol and lipids
• Low or insufficient ratios of nitrogen, phosphorus of essential elements
  o Limits assimilation into host tissue biomass
 	
C_H = Heterotrophic inputs; food intake
• > 60% of energy budget
• Efficient energy source
• Increases high energy lipid stores for P_A, P_H, and G
	P_A = Translocated carbon allocated to skeletal or tissue production
	• + correlation with calcification, a light-mediated process (Furla et al. 2000)
	
	P_H = Heterotrophically sourced carbon allocated to skeletal or tissue production
	• + correlation with tissue synthesis (Houlbrèque et al. 2004)
	
	R_A = Respiration by photo-symbionts; metabolic maintenance
	• Dependent on inputs from C_H
	
	R_H = Respiration by coral animal; metabolic maintenance
	• Referred to as CTAR; contribution of total acquired carbon to animal respiration (Grottoli et al. 2006)
	
	E = Excretion
	• DOC and POC; includes mucus
	• Drives benthic/pelagic coupling
	
	G = Gonad production energy allocations
	• If the sum of C_A and C_H is decreased, G is typically the first to disappear

1.2.2 Autotrophic mode

Autotrophic dominant corals benefit from fast growth rates which result in high surface area to volume ratios (S/V). Branching and tabulate corals belonging to the genus *Acropora* are widely considered the fastest growing taxa, dominating large expanses of reef flats (Huettel et al. 2006; Gold & Palumbi, 2018). The trade off to fast growth in *Acropora* corals is that they tend to have lower energy reserves and less resilience to external stressors (Houlbreque et al. 2004; Houlbrèque et al. 2009; Ferrier-Pagès et al. 2011). In addition, the fast skeletal growth in autotrophic dominant corals may come at the expense of compromised nutritional status (Alldredge et al. 2013).

1.2.3 Heterotrophic mode

Heterotrophic dominant corals can access and accumulate energy from wide-ranging and diverse nutrient sources, not able to be accessed by photosynthesis alone (*see review* Houlbreque & Ferrier-Pages, 2009). Feeding studies tend to focus on macro-zooplankton (~ 200 – 1000 μm), but dominant planktonic fractions (i.e., pico- and nanoplankton (0.2 - 100 μm) may be more important sources of LPM. In simulated benthic assemblages, uptake of picoplankton (0.2 – 2 μm) by corals corresponded with a reduction of 92% of the nitrogen content in the closed system (Ribes et al. 2003). Similarly, consumption of pico- and nanoflagellates contributed up to 94% of the total carbon and 85% of the nitrogen ingested by *Stylophora pistillata* and *Galaxea fascicularis* colonies (Houlbrèque et al. 2004). Although active capture of live prey is a significant energy source and a focus of this thesis, the contributions of DOM and SPM to coral health are also important. DOM sources, including dissolved free amino acids (DFAA), carbohydrates and urea, can contribute up to 75% of daily nitrogen needs for *S. pistillata* (Grover

et al. 2008). DOM uptake, as opposed to active feeding, is a non-selective, diffusion mediated process. Lastly, SPM sources, such as suspended detrital sediments, biofilms, microalgae and protozoans, may be useful for corals in near-shore, nutrient rich environments, subjected to increased rates of active sedimentation (Anthony, 1999).

1.2.4 Positive feedback between nutritional modes

Despite species-specific variability in trophic strategies, the mixotrophic ability of symbiotic corals is paramount to their success. In addition to the assimilation of host CO_2, symbionts either depend on, or at least benefit from, nutrients derived from heterotrophic feeding, namely nitrogen (Ferrier-Pagès et al. 2011; Tremblay et al. 2016). Tracing of stable nitrogen isotopes demonstrate that nitrogen from ingested zooplankton can be transferred from host to photo-symbionts in under ten minutes (Piniak et al. 2003). This transfer of nutrients from hosts to photo-symbionts may explain elevated chlorophyll concentrations, symbiont densities and rates of photosynthesis in fed versus unfed corals, although these results vary inter-specifically (Zhukova & Titlyanov, 2003; Ferrier-Pagès et al. 2011). For example, in *Acropora* sp., feeding on zooplankton significantly increased both chlorophyll concentrations and symbiont densities; however, in fed *Turbinaria* sp., chlorophyll concentrations increased independently of symbiont densities (Hoogenboom et al. 2015). Nevertheless, feeding does seem to positively impact photo-symbiont productivity which in turn, can increase the net carbon translocated to the hosts. The ability of corals to acquire enough energy to deposit calcium carbonate structures in oligotrophic waters, is made possible by the photosynthetic production of adenosine triphosphate (ATP). In summary, heterotrophy may increase photo-symbiont fitness

which then enhances photosynthesis and skeletal growth via a positive feedback loop. Although the focus of this review is on heterotrophy, the two nutritional modes are inextricably linked.

1.3 Physiological factors influencing prey capture and digestion in corals

Understanding how heterotrophic feeding can be used to improve the health status of captive corals requires an understanding of how corals capture and digest food items. On reefs, the heterotrophic component of the coral energy budget is various and diverse. The application of feeding in captive systems must utilize a subset of diets to be tested experimentally to address factors influencing feeding in corals. This section provides an overview of the anatomy of external and internal feeding appendages as well as summarizes the methods and insights gained from captive feeding experiments.

1.3.1 External anatomy

Corals evolved specialized appendages used for predation and defense. Extended tentacles directly intercept planktonic food and upon receiving chemical cues from amino acids and/or in response to touch, stinging cells called cnidocytes, deploy toxic, harpoon-like organelles called nematocysts to stun and capture prey. The success of tentacular interception depends on interacting environmental (e.g., currents, nutrient availability) and morphological factors such as tentacle length, nematocyst function (e.g., prey glutinants and penetrants or defensive volvents), and density (Pires & Pitombo, 1992; Fautin, 2009). Due to the use of the same type of nematocysts for feeding as well as for defense, it is not feasible to assess a corals' feeding ability by studying this trait alone (Fautin, 2009). Furthermore, different types of

nematocysts may be present in closely related species which occupy similar trophic niches. For example, differences in nematocysts present in Brazilian Mussidae corals had enough taxonomic value to class them as four distinct species (Pires & Pitombo, 1992). Instead of morphology, location of the nematocyst is a better proxy for their functional importance in prey subduction and digestion. High nematocyst cell densities lining endodermal, mesenterial filaments may indicate a digestive function whereas high nematocyst cell densities lining outer epithelial surfaces of tentacles (e.g., characteristic sweeper tentacles of *Galaxea fascicularis*) may indicate a defensive function (Hidaka & Yamazato, 1984, 1985; Fautin, 2009).

In addition to tentacles, surface mucosal layer secretions, regulated by the coral animal, trap and accumulate particulate matter. Nutrients adhering to mucus may either be directed via ciliary action to the polyp's mouth or released to the surrounding seawater, supporting benthic/pelagic coupling (Naumann et al. 2009). Corals, such as *Mycetophyllia reesi,* that lack tentacles rely on ciliary action to transport mucus entrapped food particles to be collected by digestive mesenterial filaments (Goldberg, 2002). Large quantities of mucus secretions for many coral species are derived from excess photosynthates and released to surrounding reef areas where the mucus can rapidly accumulate organic particles, leading to the formation of marine snow (Wild et al. 2004; Hadaidi et al. 2019). Mucosal enrichment from *Acropora* species on Heron Island, located in the southern Great Barrier Reef, drove significant increases in the particulate organic carbon (POC), organic nitrogen and phosphorus in the surrounding environment (Wild et al. 2004). Another study found that enrichment of POC from newly released, free floating coral mucus increased carbon (C) and nitrogen (N) content per unit volume of seawater by nearly 2 orders of magnitude in just 2 to 3 hours (Huettel et al. 2006). Although mucus plays other physiological functions

(e.g., preventing excess deposition of sediments, anti-microbial control) release of mucus could be an adaptive mechanism certain corals employ to increase the amount of nutrients available to them (Wild et al. 2004; Huettel et al. 2006). The increased marine snow and enhanced productivity via benthic/pelagic coupling derived from mucosal excretions can be recycled to the coral through active feeding on enriched particulate matter.

Polyp size is arguably the best predictor of a coral's trophic strategy (Falkowski et al. 1984; Conti-Jerpe et al. 2020). It is intuitive that smaller polyp corals (e.g., *Acropora* spp., *Porites* spp.~ < 1mm diameter) would not be as well-adapted to capture and consume the same potential prey as a larger polyp species (e.g., solitary mushroom coral, *Fungia scruposa*, < 300 mm diameter). This hypothesis was first posed by Porter in 1976 who plotted the S/V ratio against the polyp diameter of Caribbean reef corals to predict the "autotrophic and heterotrophic resource axes", arguing that greater S/V ratios in branching and tabulate corals optimize light interception, reducing the need for prey capture (Porter, 1976). The connection between polyp diameter and heterotrophy is corroborated by stable nitrogen isotope analysis (Alamaru et al. 2009; Ezzat et al. 2017; Conti-Jerpe et al. 2020). Actively feeding corals have enriched levels of nitrogen in their tissues due to nutrients that can only be derived from prey. Tissues sampled from large polyp *Favia fragum* and *Galaxea fascicularis* had higher levels of nitrogen (δ 15N) than those sampled from smaller polyp *S. pistillata* (Alamaru et al. 2009; Hoogenboom et al. 2015). Similarly, autotrophic dominant corals require more intense nitrogen recycling between host and algae and would be expected to exhibit higher degrees of overlap in nitrogen isotope values (Conti-Jerpe et al. 2020). A comparison of dominant coral taxa near Hong Kong revealed that smaller polyp corals (e.g., *Acropora* and *Goniopora*) had more overlap in host and algal δ

15N values whereas larger polyp corals (e.g., *Platygyra* sp., *Turbinaria* sp. and *Favia* sp.) had less overlap in δ 15N values, indicating a stronger reliance of heterotrophy (Conti-Jerpe et al. 2020). In this way, polyp size is generally, though potentially not uniformly, a good metric for trophic strategy and feeding ability.

1.3.2 Prey capture studies

Coral husbandry requires a fundamental understanding of what cultured corals are capable of capturing given the diversity of morphological factors indicative of varied feeding strategies (Osinga, Schutter, et al. 2012; Leal et al. 2016). All factors equal, captive corals exhibit increased capture success when the concentration of food increases, due to higher chances of encountering prey. Conversely, beyond a certain threshold, excess nutrients will begin to degrade the water quality and, in some cases, lead to tissue necrosis (Osinga et al. 2012; Tagliafico et al. 2018). With the goal of determining the effect of experimental variables (e.g., prey density, flow speed, morphological variables, temperature and light), clearance rate and video methods help better understand prey capture by captive corals.

The clearance rate method is the most commonly employed metric to measure zooplankton capture in *ex situ* feeding experiments (Sebens et al. 1998; Petersen et al. 2008; Hii et al. 2009; ; Osinga et al. 2012; Kuanui et al. 2016; Orejas et al. 2016)(Table 1.2A). The clearance rate concept calculates uptake rates from counts of homogenously distributed prey in a fixed volume of water (V_{water}) before (C_0) and after (C_t) an allocated time of feeding using the following equation (1.2):

$$(C_0 - C_t) \times V_{water}/polyp) \quad (1.2)$$

A drawback to using the clearance rate method is the inability to differentiate between capture and ingestion, thus caution needs to be taken when evaluating feeding abilities using prey cleared (Wijgerde et al. 2011; Osinga, Van Delft, et al. 2012). For example, *G. fasicularis* colonies that were fed high densities of *Artemia* (> 2000 nauplii polyp $^{-1}$), began excreting large amounts of mucus, trapping excess prey items without ingesting them (Wijgerde et al. 2011; Osinga, Van Delft, et al. 2012). Additionally, certain species of corals may secrete mucus at lower prey concentrations in response to nutrient stress or as a way of enriching POC. These gel-like secretions trap delivered feeds in mucus floc formations and therefore by the clearance rate method, these items would mistakenly appear to be 'ingested' by the coral animal as would prey caught in interstitial spaces. Any heterogeneity in the distribution of delivered prey introduces error. Lastly, this method doesn't account for the prey that escaped or was egested by the coral after capture, which can be a significant proportion of the plankton initially intersected (Wijgerde et al. 2011). In summary, the clearance rate is an approximate, indirect metric which can provide broad insights into prey capture ability. This method is limited by inconsistent normalization metrics (e.g., coral and prey units of measurement), lack of differentiation between prey capture and release and assumptions that all coral polyps within a colony feed uniformly and that prey items are homogeneously dispersed in solution.

Video analysis is a more useful tool to assess the capture and ingestion abilities of corals subjected to different treatments and varying concentrations. The use of video allows for direct

observation of prey behavior, such as ability to evade or escape capture as well as the egestion or release of prey by corals (Table 1.2B) (Heidelberg et al. 1997). A detailed video analysis of single *G. fascicularis* polyps fed *Artemia* nauplii found approximately 98% of *Artemia* were captured though not ingested yet extracoelenteric feeding may have occurred (Wijgerde et al. 2011). Although there are only two documented studies which utilize video recording to visualize prey capture in corals, video analysis elucidated the prey escape behavior of *Artemia* spp. (non-evasive) and copepod (highly-evasive) fractions of meso-zooplankton assemblages by a suspension feeding barnacle*, Nobia grandis* (Trager et al. 1994; Sorochan & Metaxas, 2017). In summary, video analysis offers a more accurate alternative to clearance rate calculations due to the ability to assess not only capture but also release of prey. However, video studies are not well established, require costly equipment and require a random sampling of polyps for accuracy because capture rate may vary between polyps. Whereas the clearance rate approach average feeding across all polyps, video analysis will often be limited to a small subset of polyps.

1.3.3 Internal anatomy and digestion of prey

A digestive system is required for a coral to break down ingested food enzymatically and mechanically. Although relatively little is known about the internal feeding anatomy of corals as compared to external feeding appendages, recent studies do reveal highly evolved digestive tracts (Raz-Bahat et al. 2017). For example, histological studies into the cellular structure of *Stylphora pistillata* polyps suggest that digestive organs may be more specialized and complex than previously thought. Three digestive pathways may be present in some species: (i) extracellular digestion via enzyme secretions (e.g., chymotrypsin) (ii) intercellular digestion through lysosomes and (iii) intercellular digestion on cell wall membranes (Osinga, Schutter, et

al. 2012; Raz-Bahat et al. 2017). Mesentery filaments, longitudinal membranes lining coral gut cavities, are likely the most critical components of the digestive process in corals secreting important enzymes for ingestion, digestion, and nutrient absorption (Raz-Bahat et al. 2017). Despite the lack of detailed documentation of coral digestion systems, there are two established methods for quantifying digestion of feeds in corals: gut dissections and molecular tools (Table 1.2C). Identification of prey present in the gastric cavities (i.e., gut) of corals can provide direct evidence for their consumption and quantitative data on digestion rates. Gut content of wild *G. fascicularis* colonies were used to compare the composition, type and amount of prey consumed by corals occupying habitats with significantly different pH levels (Smith et al. 2016). Gut content analysis of prey consumed by of *P. damicornis*, *A. millepora* and *A. nobilis*, from coral samples fixed at different time points post feed, quantified the time it took to digest *Artemia* nauplii which was estimated at ~2.5 hours (Kuanui et al. 2016). Whereas gut dissections are useful to gain a better understanding of a corals' ability to digest delivered feeds, it cannot be used to assess the importance of factors known to influence capture (e.g., flow speed, swimming speed, nematocyst composition and polyp size). Drawbacks of gut dissections include the difficulties associated with dissecting small polyp corals and correctly distinguishing cryptic, small (e.g., pico -, nano- or mesoplanton and microalgae; 10 – 400 μm) or quickly degraded prey within coral gut cavities. Challenges to detecting prey in gut dissections may be overcome with the addition of non-toxic histological dyes or other visual markers (Lee et al. 2018).

Molecular approaches allow for an alternative direct assessment of the digestibility of a wide range of food sources. Polymerase chain reactions (PCR) using prey-taxa specific primers can amplify target taxonomic genes from extracellular DNA extracted from the gut content of corals,

following a feeding event (Table 1.2D) (Leal & Ferrier-Pages, 2016). Quantitative PCR (qPCR) of rotifers (*Branchionus plicatilis*) and *Artemia* nauplii DNA, extracted from fed *Oculina arbuscular* and *Aiptasia* sp., observed an immediate breakdown of rotifer DNA, as well as longer digestion times than previously demonstrated by gut dissections. Longer digestion times as assessed by molecular methods are to be expected, due the persistence of prey DNA past the post- feeding time point where prey is no longer visible through microscopy (Leal, Nejstgaard, et al. 2014; Kuanui et al. 2016). For example, molecular assessment concluded that digestion of zooplankton prey by *O. arbuscular* and *Aiptasia* sp., takes 1-3 days respectively, whereas microscopic analysis suggested that digestion of zooplankton in *Acropora* spp., *M. mirabilis*, *P. porites* and *M. cavernosa* takes just 6 hours, based on prey detected in gut dissections (Hii et al. 2009; Leal, Nejstgaard, et al. 2014; Kuanui et al. 2016. Furthermore, DNA is rich in phosphorus, which putative endosymbiotic gut microbes may utilize as an important nutrient source and for this reason, corals might benefit from maintaining extracellular DNA rather than digesting it (Leal, Nejstgaard et al. 2013).

Molecular techniques also can provide information on the corals grazing on cryptic microalgal species. Endpoint PCR of algae-specific 18S rRNA gene fragments obtained from purified coral tissue suggested that experimental corals graze on phytoplankton (Leal, Nejstgaard, et al. 2013). Interestingly, the two species in this study to produce high volumes of mucus, the soft coral, *Sinularia flexibilis* and the symbiotic scleractinian coral, *Stylophora pistillata,* were the only species to not graze on any of the five micro-algal species, which supports the claim that algae were ingested, not passively trapped in external mucus secretions (Leal, Ferrier-Pages et al. 2013). Prior to this study, evidence for herbivory on phytoplankton in corals was largely anecdotal yet its discovery has ecologic implications. PCR based detection techniques provide

useful insights into the species-specific degradation, digestion and assimilation of nutrients from varied heterotrophic food sources (e.g., LPM, SPM and DOM). Drawbacks to molecular approaches are however the intensive labor alongside costly reagents required in addition to these approaches not currently being well developed for coral studies.

In conclusion, clearance rate and video analyses can help determine optimum feed densities and histological and molecular studies can evaluate how those feeds are digested. However, these studies do not provide information on the physiological or energetic benefits gained from certain feeds which are variable. Experimental studies suggest that corals can capture and digest a variety of different prey sources. The next step is to assess the importance of diet on physiological, nutritional and microbial health aspects.

Table 1.2 Aquarium based coral feeding studies by experimental approach. Clearance rate and video assess capture (A, B) and gut dissection and molecular techniques assess digestion (C, D).

Reference	Coral Species	Prey species	Experimental factor(s)
A. Clearance Rate Studies			
Tagliafico et al. (2017)	*A. millepora* *H. rigida* *D. axifuga*	*Artemia salina* nauplii	Density* Light
Kuanui et al. (2016)	*A. millepora* *P. damicornis* *A. nobilis*	*Artemia salina* nauplii	Density* Light
Orejas et al. (2016)	*L. pertusa*	Fresh zooplankton and phytoplankton	Flow speeds* Temperature
Gori et al. (2015)	*D. cornigera*	*Artemia salina* nauplii and adults	Flow speeds* Temperature
Hoogenboom et al.(2015)	*Acropora* sp. *G. fascicularis* *P. damicornis* *S. pistillata*	*Artemia salina* nauplii	Species-specific effects*
Osinga et al. (2012)	*G. fascicularis*	*Artemia salina* nauplii	Density*
Ferrier-Pagés et al. (2010)	*S. pistillata* *G. fascicularis* *T. reniformis*	*Artemia salina* nauplii	Temperature*
Hii, Soo and Liew (2009)	*G. fascicularis*	*Artemia salina* nauplii	Density* Light

B. Video Analysis			
Wijgerde et al. (2011)	*G. fascicularis*	*Artemia salina* nauplii	Temporal dynamics of capture and prey release
Heidelberg et al. (1997)	*M. meandrites*	Copepods spp. *Artemia salina* nauplii Chaetognaths	Prey escape behavior Flow speed
C. Gut Dissections			
Hall et al. (2015)	*Dipsastrea pallida*	*Artemia salina* nauplii Microplastics (< 5 mm)	Presence of microplastic in mouth and/or gut
Kuanui et al. (2016)	*Acropora* spp. *P. damicornis*	*Artemia salina* nauplii	Density* Light
Smith et al. (2016)	*G. fascicularis*	Fresh zooplankton	CO_2 levels
Sebens et al. (1998)	*M. mirabilis* *P. porites* *M. cavernosa*	Fresh zooplankton	Comparison of prey capture versus prey availability in a fore-reef habitat
D. Molecular assessment of digestion			
Leal et al. (2013a)	*O. arbuscula*	*B. plicatilis* *Artemia salina* nauplii	Continuous vs. batch feeding Species-specific effects
Leal et al. (2013b)	*O. arbuscula* *P. cactus* *S. pistillata* *T. coccinea*	*R. marina* *I. galbana* *P. globosa* *C. weissflogii* *T. pseudonana.*	Microalgal prey species digestibility

* Indicates significant factor influencing feeding ability.

1.3.4 The impact of Diet on the Physiological and Nutritional Health of Aquarium Corals

Corals that are able to upregulate heterotrophic feeding can benefit physiologically and energetically. Physiological benefits include enhanced tissue growth (Anthony & Fabricius, 2000; Forsman et al. 2012; Conlan, Bay, et al. 2018), symbiont density, chlorophyll content (Hoogenboom et al. 2015) and skeletal growth, observed in fed corals under certain environmental conditions (i.e., light and dark light regimes) (Anthony & Fabricius, 2000; Houlbrèque, Tambutté et al. 2004). Coral nutrition studies focus on the interaction between diet and growth, but the use of other nutritional metrics may also be considered. Assimilation of important biomolecules, such as high energy lipids and beneficial unsaturated fatty acids, could add to the holistic view of holobiont health. To restore localized reef patches with aquarium-reared corals, corals must demonstrate a nutritional competency to withstand stressors prior to reef transplantation. Comparing the nutritional composition of coral tissues sampled before and after long-term feeding trials may provide insights into the importance of diet to coral health. This section summarizes the effects of feeding on coral growth and survival while advocating for the inclusion of nutritional energetic metrics to provide a more holistic view of how feeding can augment holobiont health.

1.3.5 Feeding effects on Growth and Survival

Maximizing growth and survival of corals is vital to the success of coral propagation. For the ornamental aquarium industry, optimizing coral growth at low costs (US\$0.02/mm^3) is essential and dictates the indiscriminate use of *Artemia* nauplii as the standard heterotrophic feed (Toh et al. 2014). *Artemia* nauplii can significantly increase growth in many, but not all, coral

species (Houlbrèque, Tambutté et al. 2004; Conlan, Bay, et al. 2018). For example, *S. pistillata*, *P. acuta* and *P. damicornis* colonies fed *Artemia* nauplii result in 50 -75% faster growth (Houlbrèque, Tambutté et al. 2004) and can grow to twice the weight of unfed corals (Osinga, Schutter, et al. 2012), yet result in insignificant or negative growth in other coral species such as *Acropora millepora* (Conlan, Bay, et al. 2018). Nonetheless, growth is an important physiological metric of health because it confirms that corals can not only meet the energetic costs of maintenance tasks but also assimilate useful nutrients into biomass.

Larger corals tend to exhibit higher survival rates and therefore, feeds which stimulate growth, especially in juvenile corals are particularly important. Size-specific mortality is well documented for *P. damicornis* where larger colonies experience significantly higher chances of surviving one-year post-transplantation (Raymundo & Maypa, 2004; Toh et al. 2013, 2014). Feeding has been shown to improve survivorship with fed *F. fragum* (Petersen et al. 2008) and *P. damicornis* juveniles demonstrating increased post-transplantation survivorship compared to unfed juveniles (Toh et al. 2013, 2014). At this early life stage, a rapid increase of size is important for survival through overcoming competitive interactions, and feeding will stimulate growth and facilitate tissue fusion.

Commercially available, non-living (i.e., artificial) feeds have also been investigated for the application of aquarium coral nutrition (Forsman et al. 2012; Osinga, Schutter, et al. 2012). Artificial feeds (e.g., Nori Micro®, Reef Feast, Reef-Chilli, Reef-Roids) increased the growth and survivorship *P. damicornis* (Forsman et al. 2012). In contrast, the growth and survivorship

of *Montipora captitata* was negligible at lower doses and decreased at high doses of artificial feeds (Forsman et al. 2012). If the goal is to maximize *ex situ* growth rates at the lowest cost then the current wide-spread industry use of *Artemia* may be the best option, despite uncertainties in digestibility (i.e., ability of small polyped corals to ingest *Artemia* nauplii) (Leal, Nejstgaard, et al. 2014), unknown assimilation of nutrients (Osinga et al. 2011; Toh et al. 2013) as well as species-specific physiological responses of corals to *Artemia* feeds (Lim et al. 2017; Conlan, Bay, et al. 2018).

1.4 Feeding effects on energetic parameters

Recent studies suggest that the breakdown of heterotrophic energy sources offer more complete nutrition to corals than autotrophic acquired nutrients, including additional sources of high-energy lipids (Tremblay, Peirano, et al. 2011; Tremblay, Gori, et al. 2016; Tagliafico et al. 2017). Metabolism of lipids from tissue stores may bolster resistance to thermal stress (Imbs & Yakovleva, 2012). Coral species that can utilize lipid stores during thermal stress events, such as *C. caespitosa* (Hoogenboom et al. 2010) and *Turbinaria reniformis* (Tremblay et al. 2016), can better compensate for lower rates of photosynthesis and subsequent energetic losses. Lipid enrichment through feeds can help experimental corals recover from acute bleaching scenarios: however, the causative mechanisms of this are not well described (Tagliafico et al. 2017). Whether manipulating heterotrophic feeds with essential nutrients could also benefit corals that are less reliant on heterotrophy is unknown.

The use of lipid analysis could determine the health effects of different dietary regimes on aquarium corals using the following energetic parameters (i) total lipid stores, (ii) composition of lipids and (iii) fatty acid classes (Conlan, 2017; Conlan, Humphrey, et al. 2018, Brodnicke, 2019). Similar parameters are used to assess baseline measures of health in other organisms (e.g., larval fish) but more research is required in corals. The inclusion of all three parameters could help determine if a feeding regime treatment effect is important for the reasons discussed in this section.

Measuring lipid stores is useful due to the universality of lipids. Lipids are the densest form of energy, with more than twice the caloric value as carbohydrate per gram (Table 1.3). Total lipid content is measured by mg lipid per g of dry coral weight (mg g DW^{-1}) which on average, comprise between 10-40% of ash-free dry tissue weight in tropical corals (Imbs & Yakovleva, 2012). However, total lipid content can vary inter- and intra-specifically as well as within the same colony (Imbs, 2013). For example, total lipid content, within a tissue sample, can fluctuate based on reproductive cycles, rainfall, temperature and photosynthetically active radiation (PAR). PAR affects chlorophyll concentration and is therefore correlated to the biosynthesis of photo-symbiont derived lipids. Lipid content may give relative estimates of the nutritional strategy (e.g., how much a particular coral budgets for reproduction or stress related depletion) but cannot be used to make intra-specific comparisons of overall health. Nonetheless, initial lipid concentrations are good predictors for stress tolerance or susceptibility (Imbs & Yakovleva, 2012)

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Lipid classes can be categorized as energy dense storage lipids or membrane bound structural lipids. Storage lipids (wax esters (WAX) and triacylglycerols (TAG)) offer readily catabolized forms of energy. Membrane-bound, structural lipids, such as sterols (ST) and phospholipids are components of cell membranes and organelles (Imbs, 2013). Whereas lipid class composition provides a more complete picture of coral health, significant differences in the share of WAXs within the same coral are based upon location within the colony (Oku et al. 2002; Imbs, 2013). Regardless, high levels of WAXs within a coral may provide useful indications of health. Bleached colonies tend to have little to no WAXs whereas non-bleached corals tend to have significantly higher levels (Yamashiro et al. 2005). Furthermore, corals with higher storage to structural lipid ratios may be more nutritionally equipped to deal with stressors due to availability of usable energy reserves than corals with lower ratios. However, artificial and *Artemia* feeds delivered to captive *A. millepora* and *P. acuta* corals failed to increase storage to structural lipid ratios as compared to control corals reared in unfiltered seawater (Conlan, Bay, et al. 2018) and *in situ* counterparts (Brodnicke, 2019). Based on these metrics, widely used aquarium feeds may not increase the ratios of storage to structural lipids without enrichment.

Table 1.3 Approximate % of individual lipid classes on total lipid content in tropical Scleractinia. *

Lipid Class	Abbrev.	% Total lipid
Storage		
Wax Ester	WAX	47.7 ± 10.7%
Triacylglycerols	TAG	20.2 to 23.4%
Sterol esters	SE	5.7 to 10.3%
Structural		
Polar lipids	PL	14 to 33%
Phosphatidylethanolamine	PE	
Phosphatidylserine and inosital Phosphatidylcholine	PSPI	
Acetone mobile polar lipids	PC	
	AMPL	
Sterols	ST	5.7 to 10.3 %
Free fatty acids	FFA	1 to 10 %

*Adapted from review by Imbs et al. 2013

Fatty acids (FAs) are lipid constituents accounting for roughly half of lipid biomass. FAs are a direct source of ATP and their synthesis is directly coupled with photosynthesis (Brodnicke, 2019; Conlan, 2017). Lipids extracted from corals contain residues of over 50 types of fatty acids including saturated (SFA) and unsaturated FAs (Imbs & Yakovleva, 2012). Microalgal cultures of *Nanochloropsis oculata* and *Isochyrsisis galbana* contain rich sources of poly unsaturated fatty acids (PUFAs) and are routinely used to enrich PUFA deficient zooplankton

prey cultures (i.e., *Artemia salina*, rotifers and copepods) in finfish hatcheries (Pananghat et al. 2012; Alajmi, 2015). PUFA enrichment, particularly of long-chain PUFAs with three or more double-bonds (i.e., LC-PUFA; DHA), play crucial roles in growth, survival and development of fish larvae (Pananghat et al. 2012; Alajmi, 2015), but have not been well studied in corals. LC-PUFA enrichment of *Artemia* instar II nauplii (using Selco® algal paste) helped mitigate the effects of thermal stress on *A. millepora* and *D. axifuga* in a two-week experiment (Tagliafico et al. 2017). At ambient temperatures (26°C) corals fed enriched feeds had higher levels of PUFAs in their tissues than unfed corals and corals fed unenriched feeds (Tagliafico et al. 2017). However, at elevated temperatures (32°C), feeding was only able to alleviate PUFA reductions in *D. axifuga* whereas in *A. millepora* colonies, feeding had no effect on PUFA levels. The ability to assimilate beneficial fatty acids is likely linked to feeding strategy. Higher PUFA levels in healthy corals (Bachok et al. 2006) suggests beneficial metabolic and stress resistance mechanisms (Imbs & Yakovleva, 2012). Lastly, coral planulae have been found to be deficient in LC-PUFA which must be quickly obtained by auto- or heterotrophic feeding (Sargent et al. 1999). Manipulating levels and compositions of FAs through diets could improve coral health and should be identified as a husbandry goal.

1.4.1 Summary of nutritional optimization in aquaria

Captive coral production systems could benefit from development of cost-effective, optimized feeds to produce robust and resilient corals, but research is required to overcome significant hurdles. Examining the winners and losers of coral bleaching strongly suggest that heterotrophy can help with thermal tolerance by augmenting lipid reserves with energy molecules (Huffmyer et al. 2021). *Artemia* nauplii provide physiological benefits but require

enrichment to fill a lack of PUFAs. PUFA enrichment through microalgal sources is promising yet must be fed to zooplankton larvae with developed mouthparts. *Artemia* instar I nauplii, which is the stage when they are most commonly fed to corals, cannot be enriched (Osinga et al. 2011; Conlan, 2017). Commonly cultured rotifer and copepod species may be suitable for enrichment at earlier development stages, yet are more costly and challenging than *Artemia* to grow, especially in large batches. Lastly, quantitative studies are required to characterize nutrient requirements for corals as well as determine what can be synthesized and translocated from photo-symbionts (e.g., certain PUFAs) and what nutrients must be sourced through diet.

1.5 Overview of Coral microhabitats and Beneficial roles of Microbial partners

The coral holobiont is made up of dynamic and diverse assemblage of microbial partners, including Bacteria, Archaea, protists, Fungi and viruses, all of whom interact with the surrounding environment (Bourne et al. 2016) (Figure 1.2). The relative importance of these different microbial constituents in underpinning coral health is dependent upon their corresponding microhabitat within the coral which includes the surface mucopolysaccharide layer (SML), tissues, skeleton and the gastric cavity regions (Sweet et al. 2011; Bourne et al. 2016; Hernandez-Agreda et al. 2017).

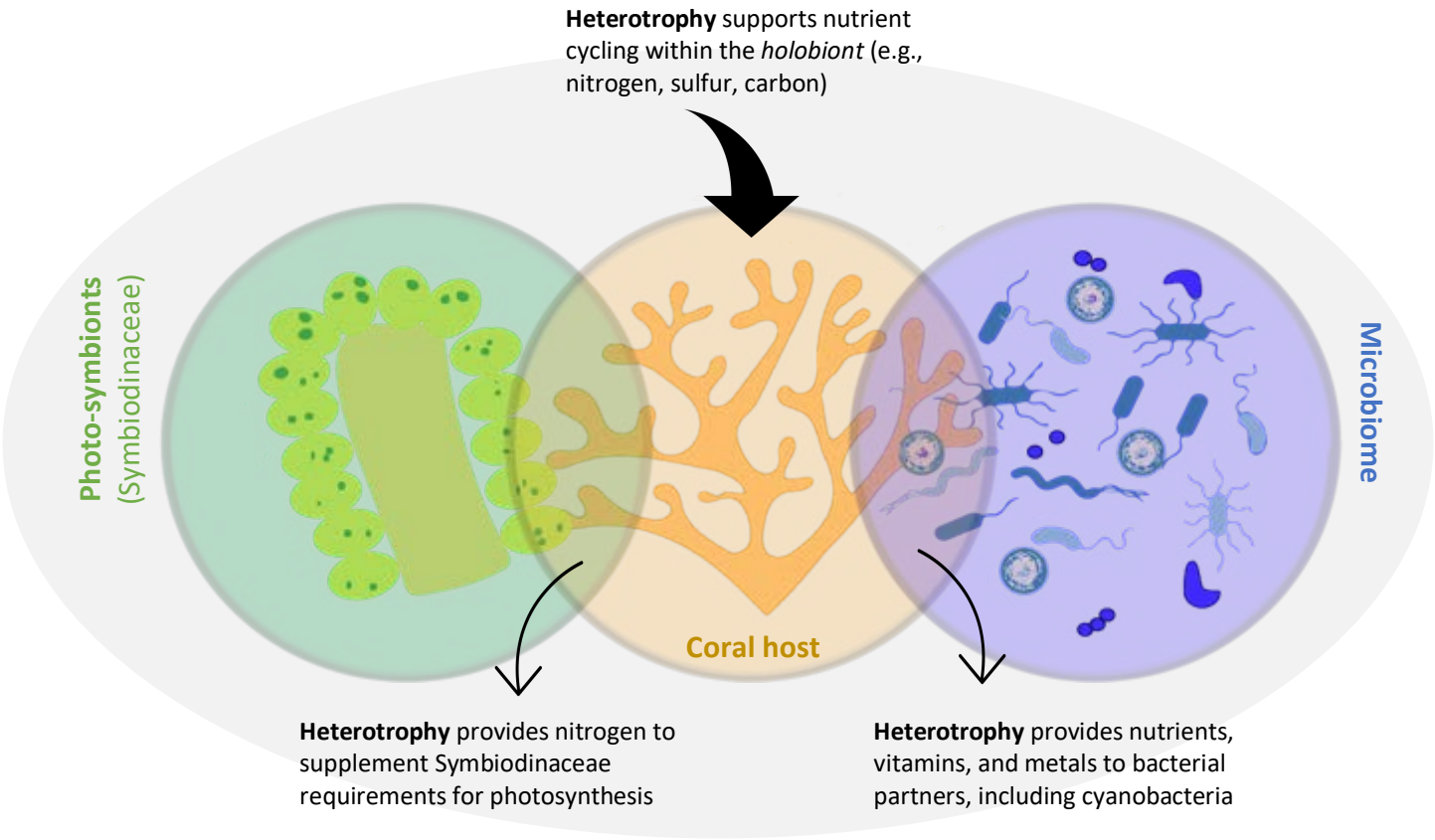


Figure 1.2 The nutritional components of the coral holobiont. Simplified diagram to represent the tri-partite relationship between animal, algal and other microbial assemblages within coral systems. Adapted from Bourne, Morrow and Webster 2016.

The SML is estimated to harbor 10^6-10^8 microbial cells per milliliter, benefiting from mucosal components such as proteins, triglycerides and waxes exudated from the coral host (Wild et al. 2004; Garren & Azam, 2012). Excess translocated carbon is secreted as mucus, providing a nutrient rich source able to stimulate growth of associated microbes and inhibit the growth of pathogens (Ritchie, 2006). In addition to the ecological roles mucosal exudates play in enriching DOC of reef environments, SML associated flora and fauna are a coral's first line of defense against infection and are dynamically shed (Sweet et al. 2011). The SML is regularly shed to rid the coral of pathogens and sediments as well as to enrich the DOC

content of the surrounding reef, providing nutrients for other heterotrophic microorganisms (Wild et al. 2004). The transient nature of mucosal microbiomes and cyclical mucus shedding has been identified as a key microbial mechanism contributing to coral health by controlling the prevalence of potential pathogens (Glasl et al. 2016).

Specialized coral-associated microbial aggregates (CAMAs) within the tissues (epidermis and gastrodermis) are postulated to aid in nutrient exchanges between photo-symbionts and hosts, directly supporting overall holobiont health and homeostasis (Agostini et al. 2012; Bourne et al. 2016; Wada et al. 2019). *Endozoicomonas* spp. have been reported as a dominant and crucial member of the microbiomes of a diverse range of corals (i.e., *S. pistillata*, *P. verrucosa, P. asteroides*) and often aggregate within tissues. *Endozoicomonas* bacteria within tissues potentially contribute to nutrient cycling through metabolism of carbohydrates and proteins (Ding et al. 2016; Pogoreutz et al. 2018). The abundance of *Endozoicomas* cells within CAMAs and the stability of tissue microbiomes is correlated with coral health and heat-tolerance (Ziegler et al. 2017; Pogoreutz et al. 2018). Through nutritional links, these specialized microbial communities likely underpin homeostasis in corals.

Endolithic microbes occupy porous calcium-carbonate skeletal structures. Observed bleaching resistance in *P. lutea* colonies were purportedly due to substantial contributions of endolithic microbiota associated with this species (Sangmanee et al. 2020). Endolithic microbiota, such as microalgae and cyanobacteria species (e.g., *Synechococcus*, *Prochlorococcus*), may aid in nitrogen cycling and by offering additional nutritional support in times of stress. Elevated levels of inorganic nitrogen have been found within water

sampled from coral skeletons suggesting that endolithic communities, consisting of both eukaryotic and prokaryotic microbes, may accumulate and recycle nitrogen (Yang et al. 2019). When bleaching results in the expulsion of photo-symbionts, more light penetrates the coral skeleton stimulating a bloom of photoautotrophic endoliths which can aid the coral host by translocating fixed carbon or be detrimental to the coral by causing skeletal erosion (Pernice et al. 2019)

Lastly, the coral gastric cavity is a semi-closed environment which has distinct chemical characteristics, likely attributed to the bacterial communities it harbors (Agostini et al. 2012; Tang et al. 2019). The gastric cavity houses facultative, anaerobic bacteria aiding in the catabolism of ingested organic matter and recycling nutrients (Agostini et al. 2012). Despite the likely importance of this microhabitat in aiding in the digestion of captured prey, the specific functional roles and identities of coral gut microbiota are largely unknown outside of heterotrophy-dominant *G. fascicularis*. *G. fascicularis* gastric cavities housed twice as many bacteria (dominated by members of α-, γ-proteobacteria, Cyanobacteria, Flavobacteria and Firmicutes) within their gastric cavities, including significantly higher levels of vitamin B_{12}, NO_3, NO_2, NH_4 and PO_4, than bacteria in surrounding seawaters (Agostini et al. 2012; Tang et al. 2019). Gut microbiota may also play a role in mitigating disease. For example, *Pseudoalteromonas* spp. delivered to *G. fascicularis* gastric cavities prevented infection of *V. coralliilyticus* thus illustrating the need to deliver probiotic strains to the microhabitat where their function is required as a measure of disease control (Tang et al. 2019).

1.5.1 Coral Probiotics and Proposed Health Mechanisms

Probiotics have been demonstrated as effective biological tools used widely in aquaculture that support digestive health, benefit immune systems, and promote stress tolerance in culture organisms (Rosenberg et al. 2007; Goulden, Hall, Bourne et al. 2012; Goulden et al. 2013). The development and application of probiotics to corals have been demonstrated recently to increase the resilience of corals to thermal stress and bacterial challenge (Rosado et al. 2019; Peixoto et al. 2021). For example, introducing beneficial bacterial consortia to stressed corals resulted in reduced susceptibility to opportunistic pathogens (Rosado et al. 2019) and bleaching (Damjanovic et al. 2019). However, the mechanisms, either buffering against an acute stressor or maintaining the ongoing health of a coral host, are unclear. Furthermore, observing enhanced resilience in response to delivered inocula could occur without necessarily changing the host microbiome if the inocula serve an indirect function such as supplying additional heterotrophic nutrients (Ferrier-Pages et al. 1998) or initiating a secondary immune response (Palmer, 2018; Dawood et al. 2019). It is important to determine if adding probiotics results in a shift in the coral bacterial community and determine if these shifts contribute to enhanced, long-term coral resilience.

Heterotrophic feeding is proposed as one acquisition pathway to shape the coral-microbial communities through supply of probiotics that establish symbioses within a host microhabitat and support coral holobiont functioning. Balanced heterotrophic diets, supplemented with probiotics, could therefore support coral nutritional and immune competence and buffer against environmental stress and/or disease. However, elucidating the functional roles of bacteria within hosts would help the development of probiotic candidates. This information would also aid the development of probiotic delivery strategies to the niche where its function

is required while also contributing to a better understanding of the chemical and biological characteristics of coral microhabitats. Overall, the interactions between diet, nutritional and microbiome status needs to be resolved to improve aquaculture of a range of coral species. Empirical evidence is required to ascertain that certain inoculum are directly or indirectly able to support holobiont health by establishing and/or maintaining host-bacteria symbioses. To achieve this, we need improved delivery strategies and ways of observing the fate of the delivered inocula.

1.6 Delivery of Coral Probiotics

For probiotics to be the effective, their administration to corals must be optimized based on their functional mechanism. Probiotic bacteria could be successfully delivered to the corals via vector mechanisms, such as water immersion or feeding. *Bacillus* spp. delivered through feeds to the digestive tracts of cultured shrimp (Rengpipat et al. 1998) and finfish (Olmos et al. 2020) can directly control gut pathogens by competitive exclusion or production of anti-microbial compounds both of which mitigate disease (James et al. 2021). Recent studies have demonstrated that live prey vectors are a feasible probiotic delivery strategy to corals (Assis et al. 2020). In the case of delivering probiotics to corals via feeds, understanding what a coral can and cannot consume and how the inocula benefit the host are essential to the selection of a delivery mode as well as the frequency of maintenance inoculations.

1.6.1 Probiotics that Establish Symbiosis within the Coral Holobiont

Probiotics may establish within the targeted niche environment of the coral host, forming a long-term symbiosis providing direct benefits including cycling of key nutrients or protecting the host from pathogen challenge. If probiotics establish within the coral holobiont, the microbiome will display a sustained shift with the putative probiotic detectable as part of the established coral microbiome and the observed health benefit would be facilitated with fewer repeat inoculations. Proposed direct symbiotic mechanisms include the control of opportunistic pathogens through antagonistic inhibitory behaviors, the provision of essential nutrients, and the regulation of nutrient cycles (Peixoto et al. 2021; Rosenberg et al. 2007).

The regulation and production of chemical compounds that protect hosts from opportunistic or pathogenic bacteria and viruses is key to the health and resilience of the coral. Many bacteria commonly associated with the SML can degrade dimethylsufiopropionate (DMSP) and may produce sulfur based microbial compounds (e.g., tropodithietic acid), inhibiting the growth of coral pathogens as well as scavenging reactive oxygen species (Peixoto et al. 2017; Raina et al. 2013). Antagonistic *Pseudoalteromonas* spp. protected the gastric regions of *G. fascicularis* (Tang et al. 2019) and *P. damicornis* (Rosado et al. 2019) from *Vibrio* infection under conditions of heat stress, potentially through the production of anti-microbial compounds. Preserving these anti-microbial functions are critical for the biological control of pathogens in high density aquaculture environments.

In the tissue layers and gastric cavities, certain groups of bacteria are commonly associated with healthy corals and predicted to aid in nutrient cycling (e.g., nitrogen, carbon, and sulfur)

and to produce essential nutrients not able to be synthesized by the host or endosymbiotic photo-symbionts (i.e., Symbiodiniaceae). For example, diazotrophic nitrogen fixing bacteria have been documented to provide additional nitrogen for the photo-symbionts supporting a tri-partite coral-Symbiodiniaceae-bacterial mutualism. Fluorescent *in-situ* hybridization (FISH) studies have enabled visualization of two abundant groups of nitrogen fixing bacteria co-localized with Symbiodiniaceae (Ainsworth et al. 2015) as well as the establishment of diazotrophs in *A. millepora* (Benavides et al. 2017) and *P. damicornis* larvae (Ceh et al. 2013). However, the importance of the nitrogen provision by bacterial partners may depend on nutrient availability in the surrounding seawater and dominant nutrient acquisition mode of the host. Corals more reliant on heterotrophic feeding may benefit less from stable diazotrophic bacteria co-localized with photo-symbionts housed within tissues than those more dependent on autotrophy (Benavides et al. 2017). In addition to nitrogen cycles, non-phototrophic Alphaproteobacterial genes involved in carbon fixation pathways have been identified in *Porites lutea* indicating that certain bacterial members of coral microbiomes contribute to the availability of fixed carbon within the holobiont (Robbins et al. 2019; Assis et al. 2020). Similarly, *Endozoicomonas* bacterial genes indicate its potential role in sulfur cycling and the production of chemicals involved in the acquisition of beneficial bacteria through chemotaxis (Vanwonterghem & Webster, 2020). Lastly, bacterial microbiome members are critical to the synthesis of essential vitamins which neither corals nor Symbiodiniaceae can synthesize, such as vitamin B12, which is critical to normal cell functioning, including amino acid synthesis (Peixoto et al. 2021). Enriched levels of B12, compared to surrounding seawater, have been found in the gastric cavities of corals (Agostini et al. 2012) and recent studies have identified gene pathways in associated microbes encoding for the biosynthesis of B12 as well as other potentially important vitamins (Robbins et al. 2019).

1.6.2 Transient association with addition of probiotics

Observed positive physiological responses of the coral holobiont, following the delivery of probiotics could be the result of an indirect effect such as consumption of bacteria as an additional heterotrophic nutrient source or stimulation of a secondary microbial loop that similarly provides additional nutrients or immune stimulation. If the probiotic association with the coral is transient, more frequent inoculations may be required to observe associated health benefits.

Corals have been documented actively grazing on microscopic plankton, including sources of pico-phytoplankton (single-celled), protozooplankton, and bacterioplankton (Ribes et al. 2003; Houlbrèque, Tambutté, Richard, et al. 2004; Patten et al. 2011). Depleted levels of microbial plankton taxa *Prochlorococcus*, *Synechococcus*, SAR11, SAR116 and *Flavobacteria*, were observed in coral-dominated habitats, as compared to offshore (Nelson et al. 2011) and sandy bottom habitats (Patten et al. 2011). It is also possible that corals farm associated microbiota within mucus sheaths, harvesting them as a sustainable food source (Bourne & Webster, 2012). Adding probiotic bacteria may also stimulate secondary microbial loops that increase abundance of other microbes (Prokarytotes and Eukaryotes) providing additional nutrient sources for host corals. For example, diazotrophic bacteria could indirectly promote health by stimulating nutrient cycling such as supplying ammonium for nitrification or further microbial metabolic activities (McNally et al. 2017).

In summary, the addition of probiotics could provide a range of benefits to the coral host regardless of whether these are established symbioses or transient physiological benefits. The mechanism of the benefit has implications for the mode of delivery of probiotics to corals. Provided the probiotic has a beneficial effect to the coral, it can be repeatedly added to boost physiological capacity as required. In fact, this is the most common use of probiotics in aquaculture settings.

1.7 Visualizing the fate of delivered probiotics

After the characterization of the feeding abilities and preferences of diverse corals, the fate of delivered inocula through live feeds would require confirmation as would the fate of probiotics delivered through other vector mechanisms such as mucus transplantations or water immersion. Fate tracking would not only allow for an ability to compare the best acquisition pathways to produce a benefit but could also illuminate the probiotic mechanism of different inoculum strategies. Approaches that detect the fate of delivered inocula have been established for the purpose of coral pathogen studies but have not yet been applied robustly for coral probiotic studies (Ritchie, 2006; Garren et al. 2014; Pollock et al. 2015). Repeated inoculations and sampling of coral microbiomes with probiotic cocktails have documented the changes in relative abundance of coral-associated bacteria over time (Li et al. 2014; Pogoreutz et al. 2018; Sweet et al. 2021) but metabarcoding techniques using the 16S rRNA gene to assess bacterial communities do not inform about their origin, permanence, or location within the coral. Bacterial profiles based on 16S rRNA gene sequencing over a time series can provide insights into the fate of probiotics though this approach is difficult to sample at the microhabitat resolution, and thus does not provide evidence of a continued symbiosis within a specific niche (Assis et al. 2020). Use of fluorescence *in-situ*

hybridisation (FISH) can visualize the location of bacteria associated with a coral micro-habitat at a given time but cannot provide continuous observations of these communities, nor can it distinguish between probiotics delivered through different pathways. Furthermore, FISH is labor intensive and signals can be difficult to distinguish against coral auto-fluorescence. Fluorescent dyes and stains can help track the delivery of labelled bacteria into a host (Assis et al. 2020; Dezfooli et al. 2021). Use of dyes identified *Brachionus plicatillis* rotifers as an effective probiotic delivery vector for *P. damicornis* and live/dead bacterial staining kit aided in the enumeration of delivered inocula to the digestive tract of cultured abalone, *Haliotis iris,* through various modes (Assis et al. 2020; Dezfooli et al. 2021). One drawback to dyes is that they cannot inform us of the function of the delivered probiotic because these markers will obviously not be passed on to progeny.

Fluorescent proteins (fp) have wide applications in biology due to the ability of the fp genes to be inserted into the genome of many organisms as a marker. Studies into the pathogenicity of *Vibrio* spp. in marine invertebrates demonstrated an ability to successfully insert fluorescent protein genes into bacteria to visualize infection pathways via live feed vectors (Goulden, Hall, Bourne et al. 2012; Goulden et al. 2013; Pollock et al. 2015). Pathogenic *V. owensii* inocula were vectored into live *Artemia*, successfully confirming a prominent infection route and subsequent hepatopancreatic proliferation of bacterial cells in ornate spiny lobster (*Panularis ornatus*) larvae (Goulden, Hall, Bourne et al. 2012; Goulden et al. 2013). Unlike FISH, different colored fp labels could be applied to the same taxa are thus applicable to the comparison of probiotics administered through different acquisition pathways. Not only could fp labelled bacteria be beneficial to select a mode of administration they could also provide invaluable insights into the localization and retention of inocula through

continuous observations of the fate of the delivered inocula and successive generations within a microhabitat.

1.8 Research Aims and Thesis Outline

Cumulative anthropogenic stressors call for effective coral restoration strategies including the captive production of corals. Improved diets and the administration of coral probiotics are one way to promote health and support a larger biomass of corals in controlled environments. Upscaling coral cultivation for commercial and restoration practices requires improved strategies to augment coral health across a diversity of species. A characterization of the inter- and intra-specific differences in feeding strategies and abilities is required for the effective use of exogenous nutrition for morphologically distinct coral species. This thesis establishes direct methods to improve strategies to deliver feeds and beneficial bacteria to aquarium reared corals.

The aims of this thesis are:

(1) To outline what is currently known about the importance of heterotrophic feeding and intersections between heterotrophy and microbiome maintenance in Scleractinia, including an outline of methodologies employed (**Chapter 1**)

(2) To quantify the ability of three Indo-Pacific coral species to consume *Artemia salina* nauplii through existing (clearance rate) and novel techniques (use of fluorescent microbeads

to visualize ingestion) (**Chapter 2**). This objective was achieved via two controlled feeding experiments.

(3) To develop fluorescent strains of coral isolated bacteria to visualize potential acquisition routes of beneficial bacteria through live prey vectors (**Chapter 3**).

The second and third aims were achieved through controlled experimental trials conducted in the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS). Herein, I characterize research findings from three stand-alone experiments in two subsequent data chapters. Supplementary information is provided in the appendix at the end of the general conclusion **(Chapter 4).**

Chapter 2 – Comparing the feeding ability of *Galaxea fascicularis*, *Pocillopora acuta* and *Acropora millepora* fed *Artemia salina* nauplii through dissection and capture rate methods.

2 Chapter 2

2.1 Introduction

Heterotrophic feeding is essential to the health of all symbiotic, reef-building corals (Brafield & Llewellyn, 1982; Anthony & Fabricius, 2000; Houlbrèque et al. 2004). Symbiotic corals are mixotrophs, obtaining energy through autotrophic assimilation of photosynthates derived from their algal symbiotic partners (*Symbiodiniaceae*) and through heterotrophic feeding (Houlbrèque & Ferrier-Pagès, 2009; Ferrier-Pagès et al. 2011). Although some corals can acquire most of their energy needs from photosynthates, all corals require heterotrophic inputs to survive. Energy budgets of symbiotic corals are improved by increased heterotrophy which affords corals with supplementary nutrition and an increased capacity to withstand external stressors (Ezzat et al. 2016; Hughes et al. 2010; Palardy et al. 2008).

On the Great Barrier Reef (GBR), there are hundreds of documented coral species that vary in morphology across and within species (Hutchings et al. 2019). Like in most animals, morphological characteristics can provide useful insights into a coral species' feeding ability. For example, fast-growing branching or tabulate corals, such as an *Acropora* sp., have small polyps (< 1 mm diameter) and the high surface to volume ratios of colonies maximizes light-acquisition but can impede capture of live prey. In contrast, a slow-growing massive coral colony, such as a *Favites* sp., has larger polyps (> 5 mm diameter), which may have evolved to maximize plankton-capture (Porter, 1976; Houlbrèque et al. 2004; Houlbrèque et al. 2009 Conti-Jerpe et al. 2020).

Chapter 2

Coral aquaculture is expanding rapidly to supply the ornamental trade but also to replenish reefs that have been degraded globally due to anthropogenic impacts (Osinga et al. 2011; Leal et al. 2016). Across the ornamental and hobbyist industry there are over 100 coral species propagated in relatively small-scale aquarium systems (Borneman, 2009; Tagliafico et al. 2018a). However, as the demand for coral increases, the scale that corals are cultured in captivity must also expand. Aquaculture of high value species is always challenging with factors such as sub-optimal nutrition and disease impacting productivity (Goulden et al. 2012; Sheridan et al. 2013). For coral aquaculture specifically, there is currently a limited understanding of the broad nutritional requirements of corals and importantly of the species-specific requirements that may impact successful aquaculture.

Among a range of diets employed by aquarium facilities, *Artemia* spp. nauplii is a commonly utilized live feed, offered to an estimated 85% of all marine organisms in aquaculture (Trager et al. 1994; Sebens et al. 1998; Hii et al. 2009; Kumar & Babu, 2015; Kuanui et al. 2016). *Artemia* is a cost-effective, commercially available live feed option that has been demonstrated to stimulate growth and improve survivability of a range of captive corals (Osinga et al. 2012; Tagliafico et al. 2018). The growth and survival of juvenile and adult aquarium reared *Pocillopora acuta* (Toh et al. 2013; Huang et al. 2020), *Pocillopora damicornis* (Conlan, Bay et al. 2018), *Acropora tenuis, Favia fragum* (Petersen et al. 2008) and *Duncanopsammia axifuga* (Tagliafico et al. 2018b) improved significantly when fed *Artemia* nauplii as compared to unfed corals. *Artemia* nauplii, upon developing of mouthparts following their first naupliar molt, are routinely enriched with beneficial poly-unsaturated fatty acids (PUFAs) to further enhance the health of aquaculture species such as ornamental fish (Vagelli, 2004). Initial findings from corals fed nutritionally enriched

Artemia indicate that these diets may similarly enhance the health of captive corals by reducing bleaching mortality rates (Tagliafico et al. 2017), yet these diets require further development. Previous findings highlight that scaling up the delivery of live feeds to aquaculture corals could result in higher yields and more resilient corals.

A range of indirect and direct approaches have assessed coral feeding abilities, each with strengths and limitations. The capture rate approach is an indirect method of assessment commonly used to select optimal feeding densities for aquarium reared corals (Osinga et al. 2008, 2012; Kuanui et al. 2016). Determination of mean capture rates involves counting prey in a fixed volume of water before and after a feeding period, and then normalizing to a unit coral such as surface area or individual polyp number. The capture rate approach is a low-tech, cost-effective way to assess coral feeding and has been utilized to investigate effects of environmental factors, such as initial prey density, pH and light regime on coral feeding as well as the effects of coral feeding on various physiological metrics such as photosynthesis and carbon intake (Hoogenboom et al. 2010; 2015). The capture of *Artemia* by a range of corals, including small polyp *Acropora* species (Hoogenboom et al. 2015; Kuanui et al. 2016; Tagliafico et al. 2018a), *P. damicornis* (Hoogenboom et al. 2015; Kuanui et al. 2016) and relatively large polyp *Galaxea fascicularis* (Hii et al. 2009; Osinga et al. 2012; Hoogenboom et al. 2015) and *D. axifuga* (Tagliafico et al. 2018a) have been measured through this indirect approach. However, such indirect methods, rely on assumptions that prey captured equals prey consumed and do not account for the dynamics of prey capture, digestion, or release (Osinga, Van Delft, et al. 2012). The assumptions are problematic because prey can be passively caught, for example by mucus secretions, but not ingested. Additionally, this approach calculates average capture rates per polyp based on whole fragment measurements,

although there is likely a non-uniform distribution of ingested prey across polyps within a fragment or larger colony.

To quantify and assess the dynamics of prey capture, ingestion and digestion by individual coral polyps within a fragment, direct approaches must be utilized. Direct approaches employ molecular, video and histological analyses to more accurately assess various facets of heterotrophic nutrient acquisition by captive corals (Kuanui et al. 2016; Osinga, Van Delft, et al. 2012; Smith et al. 2016; Tagliafico et al. 2018a). Molecular assessments have investigated digestion rates of corals fed *Artemia* and rotifers through the breakdown of extracellular DNA in coral gut cavities, through qPCR (Leal, Nejstgaard, et al. 2014), and the relative contribution of autotrophy versus heterotrophy through stable isotope analysis (Leal et al. 2014; Conti-Jerpe et al. 2020). Video analysis of the feeding behavior of *G. fascicularis* fed *Artemia* found that 98% of "captured prey" was not ingested but rather released or digested externally (Osinga, Van Delft, et al. 2012). Video footage has also investigated the prey escape dynamics of zooplankters delivered to *Meandrina meandrites* and found that 67% of copepods evaded predation after initial contact with a coral tentacle (Wijgerde et al. 2011). These studies led to an improved understanding of feeding behavior, but video analyses are limited to the study of one polyp per feeding event.

Polyp dissections combined with histological analysis have investigated the digestion of fed *Artemia* nauplii in *P. damicornis*, *A. millepora* and *A. nobilis* (Kuanui et al. 2016; Axworthy & Padilla-Gamino, 2019). Histological approaches have also been applied to determine the composition of plankton in *G. fascicularis* gastric cavities across reef habitats (Kuanui et al. 2016; Smith et al. 2016). However, coral dissections require expensive equipment (e.g., an

advanced stereomicroscope) and are further complicated by small coral polyps and often cryptic prey (Trager et al. 1994; Houlbrèque et al. 2004). One approach to overcome these limitations is to utilize visual aids (e.g., dyes, fluorescent markers, advanced microscopy equipment) to better detect ingested prey. Importantly, though polyp dissections are an important tool to understand the amount and types of prey ingested and digested by corals, it is not always feasible to differentiate degraded prey from coral tissue.

The addition of fluorescent markers, such as polystyrene microbeads to live prey, could extend our understanding of feeding by facilitating the documentation of uptake and increasing contrast between partially digested prey against coral tissue. Visualization methods using fluorescent polystyrene microbeads first emerged as a biomedical diagnostic tool (Popielarski et al. 2005; Madden et al. 2013; Bott, 2014; Xu et al. 2018). Use of this technology has since been applied in marine studies to investigate the bioaccumulation of microplastics in marine organisms (Setälä et al. 2014; Axworthy & Padilla-Gamino, 2019; Miller et al. 2020) and to investigate prey preference in ornamental marine fish (Lee et al. 2018). Many zooplankton species indiscriminately graze on particulate matter and microplastics are no exception. Therefore, zooplankton can be readily incubated with fluorescent microbeads (Setälä et al. 2014; Horn et al. 2019; Miller et al. 2020). Cultured copepods, rotifers, ciliates and *Artemia,* for example, readily ingested different colors of microbeads thereby differentiating prey types with results illustrating species specific prey preferences for captive marine fish (Lee et al. 2018). The use of fluorescent markers has not yet been used to aid coral feeding protocols and is thus a critical component of this study.

Chapter 2

This chapter compares the capture and ingestion abilities of three scleractinian coral species (*Galaxea fascicularis*, *Pocillopora acuta* and *Acropora millepora*) collected from the central Great Barrier Reef, Queensland, Australia and brought into experimental facilities at the National Sea Simulator, at the Australian Institute of Marine Science (Cape Cleveland, Queensland, Australia). These species represent three distinct polyp sizes, are popular in the ornamental industry (*G. fascicularis*), reef restoration operations (*Acropora* spp.) and all three species are the focus of previous comparable studies (Hii et al. 2009; Osinga, Schutter, et al. 2012; Osinga, Van Delft, et al. 2012; Kuanui et al. 2016). In this chapter, the ability of these corals to capture and ingest food items was investigated through two experiments. The first experiment, referred to as "the capture rate experiment," compared the ability of replicates from each of the three experimental species to capture *Artemia salina* Instar I nauplii supplied at different initial densities, as measured by the number of prey items cleared from a fixed volume of water normalized to the number of polyps on the fragment in capture rate calculations. The second experiment, referred to as "the ingestion experiment", directly quantified the number of prey items ingested by coral polyps via fluorescence microscopy detection of *Artemia* instar II nauplii and *Brachionus plicatillis* rotifers enriched with fluorescent microbeads followed by image analysis. The aim of this work was to quantify feeding rates of morphologically distinct corals fed *Artemia* nauplii and to compare and further develop methods that can accurately inform feeding regimes for corals in aquaculture. Results from this study characterises the heterotrophic feeding abilities of captive corals and improves strategies to visualize the fate of exogenous feeds in the gut cavities of individual coral polyps.

2.2 Materials and methods

2.2.1 Coral collection and maintenance

Adult colonies of *A. millepora*, *P. acuta* and *G. fascicularis* (n = 3 distinct genotypes per species), were collected from Davies Reef (lat.: 18°49'31" S, long.: 147°38'50" E) located in the central region of the Great Barrier Reef, Queensland, Australia in July 2019 and July 2020 (AIMS General Permit G12/35236.1). Following collection, corals were transported to the National Sea Simulator located at the Australian Institute of Marine Science (AIMS, Cape Cleveland, Australia, lat.: 16°177'S, long.: 145°271'E) and acclimated to aquarium conditions in outdoor quarantine tanks for two weeks, with the temperature set to emulate reef conditions (24 °C) at the time of collection.

Adult colonies were cut into small fragments (experiment 1: ~ 5 cm length, experiment II: ~ 2.5 cm length) using a small band saw (Gryphon Coral Saw) then adhered using super glue to either aragonite plugs (experiment I) or ceramic plugs with a wax coating to prevent biofouling (experiment II). Plugs were labelled to track genotypes and placed on elevated coral plug trays. Trays were moved to indoor holding tanks (3 x 50 L tanks and 1 x 250 L tank in experiment I and II, respectively) with filtered (0.04 μm) flow-through seawater. Coral fragments were acclimated in indoor holding tanks for 4 weeks prior to experimental trials. Each holding tank contained a circulation pump (Tunze® Turnbelle® nanostream®) to assist water circulation with water exchange set to 1 turnover per hour (50 L h^{-1} and 250 L h^{-1}). Herbivorous snails (*Turbo* spp. and *Thalotia strigata*) and an orange-shoulder surgeonfish (*Acanthurus olivaceus*) grazed on algae to prevent overgrowth. Holding tanks were manually cleaned by scrubbing acrylic surfaces, siphoning waste, and manually removing algae from plugs weekly. Following cleaning, trays were relocated to another location within the tank to

account for slight variations in lighting intensity. *G. fascicularis* fragments were segregated from other genotypes and species to prevent competition via sweeping tentacles. Corals in holding tanks were routinely fed low concentrations of *Artemia* instar I nauplii (~ 1 ind./mL) or *Brachionus plicatilis* rotifers (2 ind./mL) daily. The temperature of the holding tanks was maintained at 27 ± 0.02 °C and the light regime was programmed to match the natural day and night cycle (12 hours light, 12 hours dark) and oscillations in intensity. Intensity was measured in photosynthetically active radiation (PAR; µmol photons m^{-2} s^{-1}) and periodically checked using a PAR meter. Overhead lights (AIMS custom built) were adjusted periodically by lowering or raising the lighting units to adjust for intensity differences between the light bulbs and to ensure the maximum light intensity averaged 200-250 µmol photons m^{-2} s^{-1}. Lights were ramped up to 200-250 µmol photons m^{-2} s^{-1} for 4 hours, then held for 4 hours before ramping down for another 4 hours.

2.2.2 Experimental set-up

Prior to feeding trials, coral fragments were moved from the holding tanks to cylindrical acrylic feeding chambers (AIMS custom made; 2.5 L volume) to acclimate for 24-hours (Figure 2.1). Each feeding chamber received flow-through filtered seawater with water exchange set to 1 turnover per hour (2.5 L/h). The temperature of the feeding chambers was held constant (27 ± 0.02°C) and regulated by a fresh-water bath tank fitted with a temperature probe. Water powered, magnetic stirring plates were added to the fresh-water bath tanks and a magnetic stir bar was added to the bottom compartment of each feeding chamber to homogenize delivered prey and provide water movement.

2.2.3 Prey preparation

Artemia salina cysts (Sep-Art magnetic GSL *Artemia* cysts, INVE, Belgium) were hatched overnight and harvested daily by SeaSim personnel (2.5 g cysts in 1L seawater; pH:8, salinity 25-35 ppt). A magnetized collector tube (Sep-Art^{TM}, INVE, Belgium) separated cysts from unhatched nauplii. Newly harvested *Artemia* nauplii stock were stored at 4 ºC with aeration. *Artemia salina* Instar I nauplii were then taken from this stock and diluted with seawater from the experimental room and gently aerated (~1:4 *Artemia* to seawater ratio; ~600 ind./mL). The diluted solution was kept at 27 ± 0.02 °C until used for the feeding trials. Replicate counts (n =5 or until the standard error was < 10% of the mean) were conducted in a Bogrov counting chamber to determine the delivered prey densities.

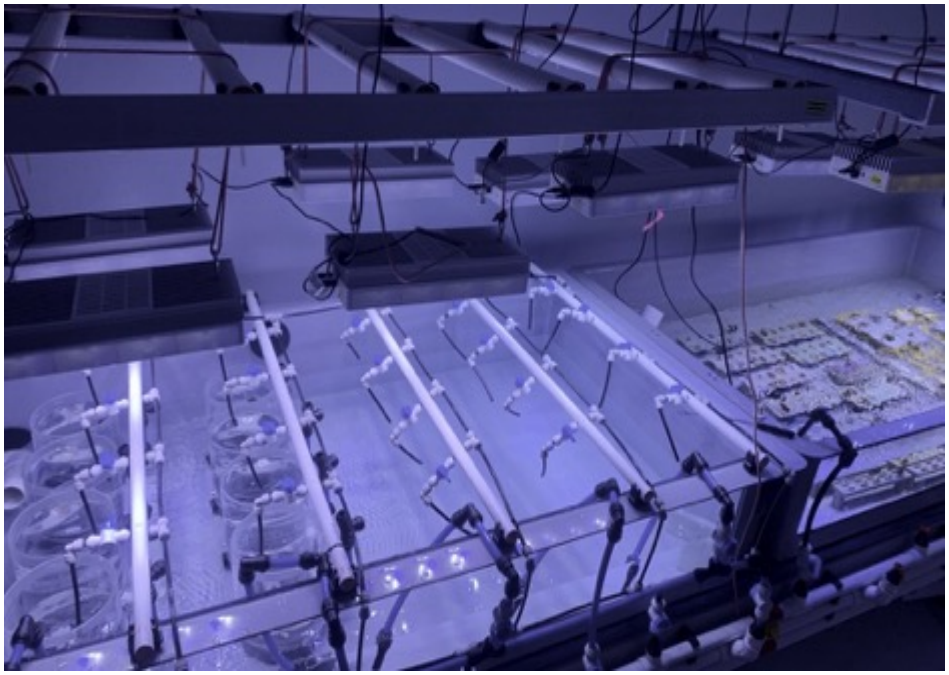


Figure 2.1 Tank set-up. (Left) Cylindrical feeding chambers, with coral plug trays, a magnetic stirrer, seawater inlet and outlets, were placed on water driven stir plates to homogenize prey in individual chambers. Overhead lights emulated day and night oscillations in light intensity on the reef where colonies were collected. (Right) A 250 L bath tank held fragmented corals on plug trays to acclimate to aquarium conditions.

For *Artemia* incubations with microbeads, nauplii measurements (n = 30) were taken at 8 fixed time points between harvest and feeding time to assist in determining when delivery to corals should occur. Lengths were measured from the eye to the end of the tail (see Ekonomou et al. 2019) in Leica Application Suite X (LAS-X) microscope software. Nauplii were incubated with fluorescent microbeads (ThermoFisher FluoSpheres® yellow-green carboxylate-modified 1 μm microspheres, 505/515 nm excitation and emission maxima, Lot Number 2161865), then mounted onto glass cavity slides with mounting gel (Fluoro-gel with Tris buffer) with the addition of a viscous solution (1% methocel) to slow down prey movement for optimal visualization. Visualization was conducted using a fully automated inverted fluorescent microscope (LEICA DMI6000B, LAS-X software) with fluorescent filters (ET-GFP, 450/490 nm) and images were taken on an in-built camera (AxioCam MRc Rev. 3). Larval developmental stage was determined by detection of fluorescence beads in the guts of the *Artemia* (Figure 2.4A). Additionally, copepods (*Parvocalanus crassirostris*) and rotifers (*Brachionus plicatilis*) were incubated similarly with yellow-green and blue (350/440 nm excitation/emission maxima) fluorescent microbeads (Figure S2-3). Incubated prey were carefully mounted, as previously described, onto glass cavity slides using the same inverted microscope and corresponding software as detailed above.

The FluoSpheres® (herein referred to as microbeads) stock concentration was 3.8×10^{10} particles/mL, in a 2% solids solution, and were stored at 4 ºC, protected from light, in line with the manufacturers' recommendation. Prior to use, small volumes of the microbead solution were dispensed in Eppendorf tubes and diluted with ultra-filtered seawater (0.22 μm) at 1:1 ratio. The diluted solution was sonicated briefly to prevent aggregation and 10 μl of the diluted microbead solution was added to 25 mL suspension culture flasks with vented

caps (Sarstedt product no. 83.3910.500) with 7 mL of *Artemia* nauplii (~ 600 ind./mL). Flasks were placed on an orbital shaker (50 rpm) for 1 hour (0630-0730). Incubation success was confirmed via fluorescent microscopy and an *Artemia* aliquot was fixed in 4% neutral buffered formaldehyde (NBF) in seawater solution, for reference. Incubated instar II nauplii were rinsed thoroughly with seawater using a 250 µm mesh net for 5 minutes. A second aliquot was fixed in 4% NBF to check that microbeads had been removed from nauplii surfaces and the surrounding solution. A third sample was similarly fixed after feeding trials to confirm gut retention of microbeads for downstream detection.

2.2.4 Feeding trials

Prior to delivery of *Artemia*, seawater inlets to the feeding chambers were closed and the water volume in each chamber was carefully adjusted by siphoning to 2 L (for experiment I; the capture rate experiment) or 1 L (for experiment II; the ingestion experiment). Checks were conducted to ensure that the magnetic stir bars were spinning at the approximate rate of 80 revolutions per minute, which was determined to be an appropriate rate to effectively distribute prey without stratification. Pre-measured feeds were then delivered to the feeding chambers via syringe. Observations on feeding response indicators (e.g., polyp extension, tentacle movement, formation of mucus-prey agglomerations) were taken at 10 minutes post-feed delivery as well as throughout each trial.

2.2.4.1 Capture Rate Experiment. A small drop of suspended *Artemia* was added to each chamber to elicit a feeding response prior to delivering feeds. For *P. acuta* and *A. millepora* trials, instar I nauplii were delivered to chambers (9 replicates per species), each housing one coral fragment, per prey density treatment (3 treatments: Low (1 ind./mL),

Medium (2 ind./mL) and High (4 ind./mL)) (Figure 2.2). For *G. fascicularis* trials, an additional prey density treatment (8 ind./mL) was added due to the higher feeding rates previously documented in *G. fascicularis* (*see* Osinga et al. 2008; 2012). For each experimental chamber with coral, there was a corresponding control chamber without coral. Trials commenced at 10:00 (during peak light intensity) on three consecutive mornings (Figure 2.2A).

Initial (C_0) and final (C_t) prey concentrations were determined by counting 20 mL seawater aliquots collected 5 minutes after feed delivery and again one hour later. Upon conclusion of the third day of trials, polyp counts were obtained for each fragment. *G. fascicularis* polyps were counted manually. *A. millepora* and *P. acuta* polyp counts were derived from surface area measurements, calculated using a wax dipping protocol, as previously described (Veal et al. 2010). Briefly, wax dipping of cylindrical objects of known surface area were used to build a surface area calibration curve. *A. millepora* and *P. acuta* fragments were rinsed with seawater and placed in a tray of diluted sodium hypochlorite (i.e., bleach) overnight. Once dry, fragments were dipped in molten paraffin wax once and *P. acuta* samples were dipped twice (Veal et al. 2010). Coral surface area was multiplied by the average number of polyps per square centimeter, for each species, estimated via stereoscope imaging (Software: Toup-view). Mean capture of *Artemia per* coral polyp was calculated by the following equation (2.1):

$$\textbf{Capture rate: } (C_o - C_t) \times V_{water}/N \qquad \textbf{(2.1)}$$

Capture rate is a concentration dependent measurement where C_o is the initial and C_t is the final number of nauplii in a sample volume of water at the end of an allocated feeding time (1 hour). V_{water} is the volume of water in the feeding chamber. N is the number of coral polyps (See Osinga, Schutter, et al. 2012).

2.2.4.2 Ingestion Experiment. *Artemia* instar II nauplii, incubated with fluorescent microbeads, were delivered at a fixed density (3 ind./mL) to four replicate feeding chambers per species. Each chamber housed 3 fragments of different genotypes (~ 2 cm length, n = 12 fragments per species) (Figure 2.2B). Initial and final prey counts were taken from 20 mL seawater aliquots collected immediately after feed delivery and again 70 minutes later, allowing 10-minutes for corals to elicit a feeding response to added prey.

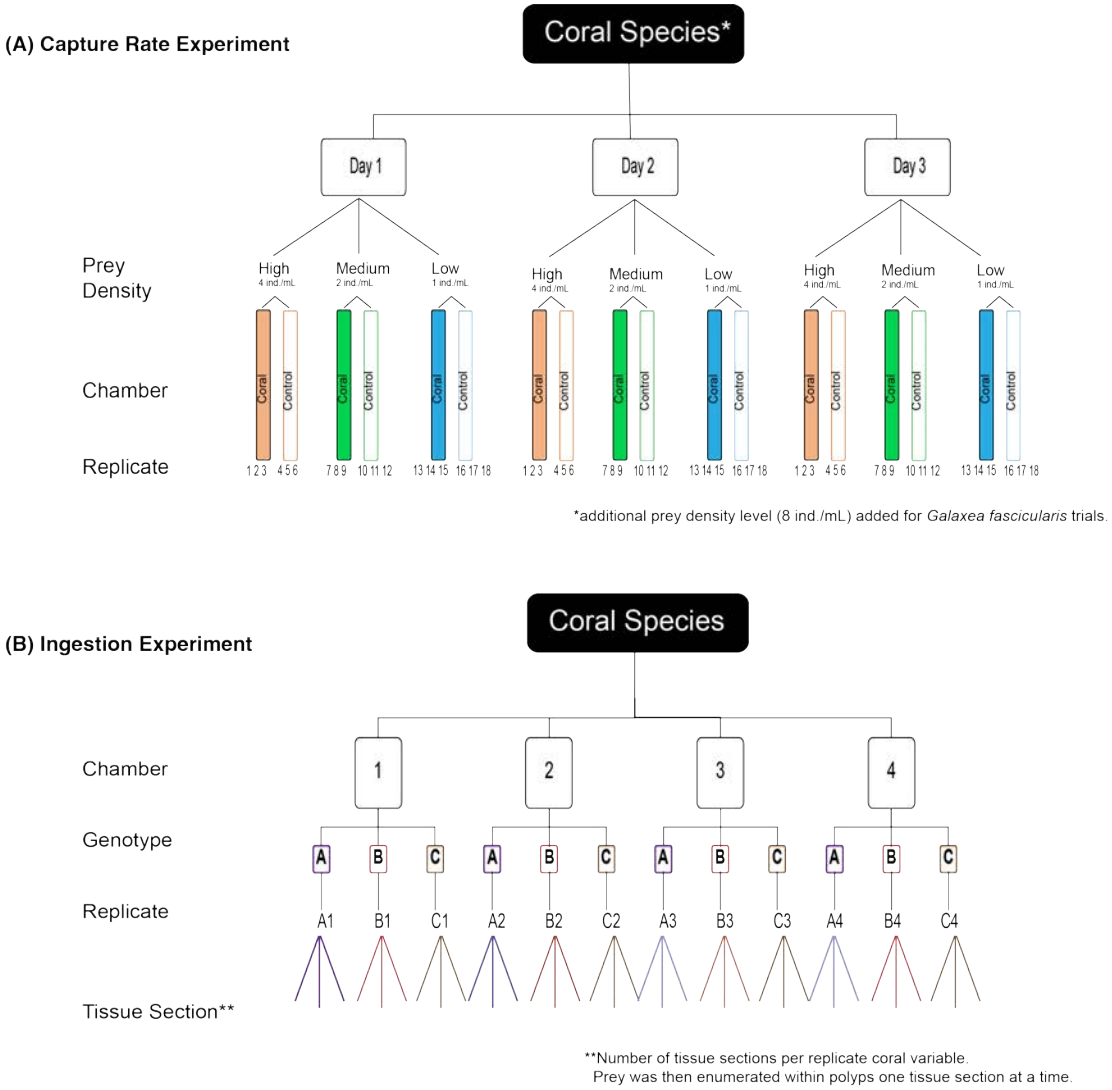


Figure 2.2 Capture Rate Experiment and Ingestion Experiment design. (A) This figure represents the sampling design for *A. millepora* and *P. acuta* species. For each chamber (n = 18; 9 with corals and 9 without) initial (C_o) and final (C_t) prey concentrations were measured daily, before and after the one hour feeding trial for capture rate calculations. (B) The feeding trials were conducted in four replicate chambers with three fragments, one from each of the three coral genotypes ('A', 'B' and 'C') collected, per species. Corals were cut into sections and ingested prey were enumerated within a subset of intact polyps per each section.

At the end of the 70 minutes feeding time, coral fragments were fixed for 24-hours in a 4% NBF solution at 4 °C. The following day, replicates were rinsed in ultra-filtered seawater (0.22 µm) prior to placement in 4% formic acid solution for decalcification. Samples were agitated at 50 RPM on an orbital shaker, placed under a benchtop extraction fan to remove fumes. The decalcifying solution was replenished after 24 - 48 hours as needed until the hard skeletal matter was dissolved. Decalcified fragments were rinsed again in ultra-filtered seawater to remove traces of the acid, and then placed in 2 x PBS in 50% ethanol (0.44 µm syringe filtered) for storage at -20 °C until dissected.

Decalcified corals were pinned to wax dissecting plates using 5 mm long, small, headless, stainless-steel pins (Cat no.: E185, Australian Entomological Supplies$^{PTY\ LTD}$). Samples were dissected under a dissecting stereoscope (LEICA MZ109) with an in-built GFP long-pass filter (LEICA ET-GFP LP FLUO filter; excitation filter: 480/40 nm, barrier filter: 254/511 nm) using forceps, scalpels, and fine tip dissecting probes (0.25 mm, product 10140-Fine Science Tools, Inc.). Samples were cut into sections and imaged one section at a time (Software: Leica Application Suite V4, Camera: Leica DFC450 C Digital Camera). After imaging, sections were discarded to avoid double counting.

The 12 *A. millepora* fragments (L: ~2 cm, W: ~ 0.5 cm) were incised longitudinally with the axial corallites bisected. The 12 *P. acuta* fragments (L: ~ 2 cm, W: ~ 2 cm) were 'unfolded' and flattened using small dissecting scissors and scalpels and each fragment was then cut into 6 ± 2 sections. The *G. fascicularis* fragments (L: ~2 cm W: ~2 cm) had 4 to 5 polyps which

were dissected separately. First, the mouth of each polyp was probed to separate clumps of *Artemia* (characteristic of partially digested prey consumed by this species) from the tentacles, which auto-fluoresce. Each probed polyp was longitudinally incised, flattened and pinned to the wax dish. The flattened sections were further divided to avoid double counting. Each section was probed to ensure that prey could be easily captured via fluorescent imaging and detected by eye. A series of images were taken for all dissected samples and imported into Image J for analysis and annotation. In each section, polyps were outlined and numbered with prey enumerated using the multi-point tool in Image J. Correct detection of ingested *Artemia* was ensured by comparison with control images generated in pilot studies where corals were delivered (i) *Artemia* without beads; (ii) unwashed *Artemia* incubated with beads; or ((iii) beads only.

2.2.5 Data analysis

All statistical analyses were carried out in R-studio (version 1.4.1106, R Core Development Team, 2009) using a significance level of $P < 0.05$. Values are reported as means ± standard error. Models that best explained the trends in the feeding rate data, outlined below, were selected using Akaike Information Criterion (AIC). For the capture rate experiment, maximum-likelihood models were used to evaluate the relationships between two fixed factors, day and initial prey densities (individuals/mL), on the response variable, capture rates (individuals/polyp/hour) (car, lme4 R-packages). Two sets of models were fitted. One examined the effects on prey capture rate of coral species, day, and prey density treatment, and all interactions between them. This model was then reduced to the simplest model, using AIC to compare alternative models. To clarify the significant interactions identified in the final model, each species was then analyzed separately. Additionally, the

difference in initial and final *Artemia* nauplii concentrations between control chambers (*Artemia* only) and experimental chambers (*Artemia* + corals) were assessed by paired t-tests.

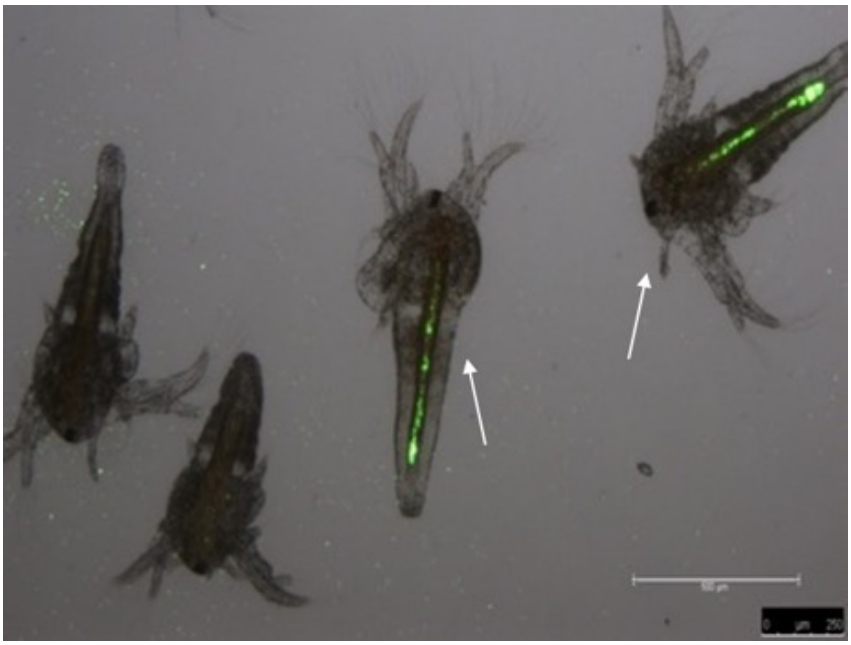
For the ingestion study, count regression models assessed the effect of genotype and feeding chamber on the number of *Artemia* counted within polyps of each dissected section of a particular species. Dissected sections within samples were included as random effects, and this was found to improve the model. Overdispersion in the nauplii counts was handled by using the negative binomial as the family for the count regression. Analyses of deviance (car R-package) determined whether the two main effects (genotype, chamber) influenced the number of *Artemia* consumed by each species. Paired t-tests assessed differences in feeding rates as calculated by capture rate and ingestion rate values for a given chamber. Lastly, pairwise two-sample t-tests, allowing for unequal mean variances, were used to analyze the mean feeding rates from both experiments where initial prey densities were comparable to 3 ind./mL ± 1.5 to compare results derived from three calculations.

2.3 Results

2.3.1 Artemia developmental stages

The size of *Artemia* nauplii at the time of delivery to corals was measured with the average length of instar I nauplii being 508 ± 45 µm (capture rate experiment) and average length of instar II nauplii being 735 ± 94 µm (ingestion experiment). The average length of *Artemia* instar II nauplii at the time of molting was 680 ± 118 µm. Instar II nauplii were easily identified as they immediately commenced feeding on the fluorescent microbeads in the solution following the development of mouthparts after molting (Figure 2.3A). Nauplii length at the time of molting was highly variable and therefore *Artemia* were added to feeding chambers in the ingestion experiment once 100% of nauplii had molted and taken up the microbeads, which occurred at approximately 22 hours post-harvest (Figure 2.3B). Further analysis of prey samples, via fluorescence microscopy confirmed that the *Artemia* retained their gut contents until 2 hours post-rinsing. This confirmed that the fluorescent markers were retained in the *Artemia* gut and not lost through excretion during the feeding trials which were conducted over 1 hour.

(A)



(B)

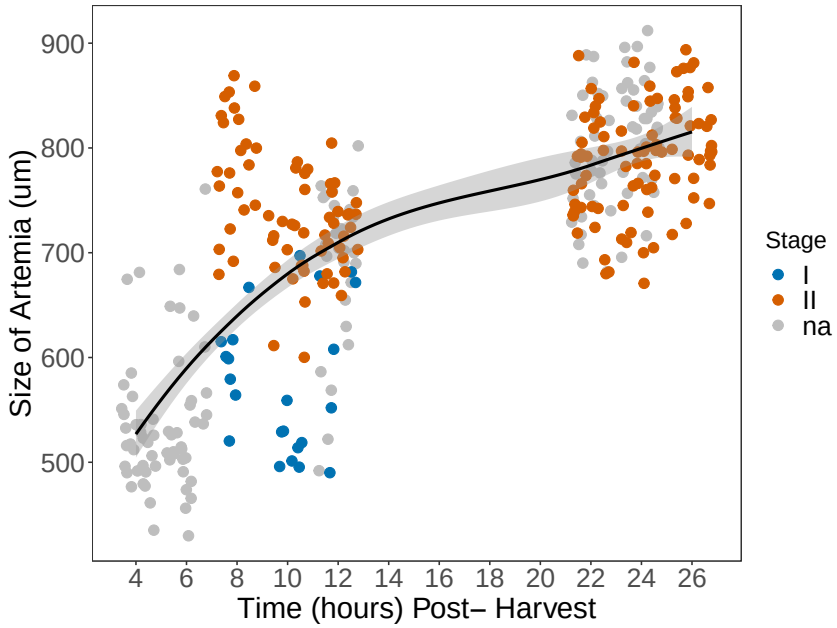


Figure 2.3 Measuring and tracking naupliar development of *Artemia salina* with fluorescent microbeads. (A) Lengths of nauplii harvested from the same batch measured 10 h post-harvest, from right to left, 705 µm, 535 µm, 774 µm, 765 µm. Molted nauplii were detected via elongated guts lined with 1 µm yellow-green, fluorescent polystyrene microbeads (see arrows). (B) measurements of *Artemia salina* nauplii size (n = 30) up to 26 hours post-harvest. Assessment of Instar I:II stage ratios were carried out at the 8 h and 10 h post-harvest time points. NA refers to nauplii simply measured but not incubated with microbeads (see legend).

2.3.2 Experiment I: Capture Rate Study Results

The average surface area of *A. millepora* fragments (n = 9) was 84 ± 4.5 cm^2 and the average number of *A. millepora* polyps per square centimeter was 46 ± 2.4 polyps/cm^2 (n = 10), which equated to approximately 3,844 ± 219 polyps per fragment (Table 2.1A). There was no significant difference in initial *Artemia* prey counts between control and experimental chambers (t = - 0.75, df = 52, *P* = 0.5) and no significant difference between the initial and final *Artemia* counts in control chambers without corals (t = - 0.04, df =52, *P* = 0.96) (Figure S2-1). However, there was a significant difference between initial and final *Artemia* counts for chambers with corals present, with final counts being lower (t = 6.39, df = 52, *P* = < 0.0005). Hourly *Artemia* capture rates for *A. millepora* ranged from less than 1 to 2 individual *Artemia* nauplii per polyp (individuals/polyp/hour) across the 9 individual replicates in separate feeding chambers across three days. Specifically, the capture rates when delivered *Artemia* at three pre-measured prey levels of 1, 2 and 4 ind./mL were 0.28 ± 0.08, 0.57 ± 0.08 and 1.22 ± 0.13 ind./polyp/h (± SE) respectively. There was a strong linear relationship between capture rate as prey density increased (capture rate ~ initial ind./polyp, r 2 = 0.88) (Figure 2.5A). Capture rates calculated from *Artemia* counts before and after the trial, normalized to polyps across chambers on three consecutive days and delivered prey levels (1, 2, and 4 ind./mL) did not differ significantly (Analysis of Deviance, x2 = 1.33, P = 0.25 and x2 = 3.17, P = 0.20, respectively). However, coral mucus secretions were observed trapping delivered *Artemia,* particularly in chambers delivered 2 or 4 ind./mL (Figure 2.4). There were no significant interactions between fixed factors (Analysis of Deviance, x2 = 3.25, *P* = 0.19).

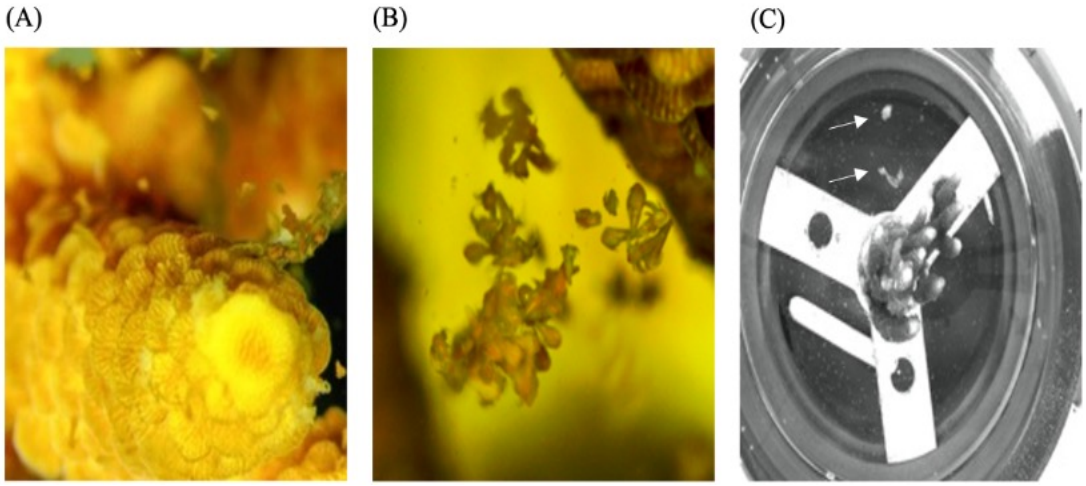


Figure 2.4 Mucus production by *A. millepora* in response to delivered *Artemia* in medium and high-density treatments. (A*) A. millepora* replicates in high density *Artemia* treatments observed producing large volumes of mucus. (B) Mucus-prey agglomerate detaches from coral (C) Agglomerates cause stratification of prey in feeding chambers (see white arrows).

The average surface area of *P. acuta* fragments (n = 9) was 153 ± 10 cm^2 and the average number of polyps per square centimeter was 70 ± 2 polyps/cm^2 (n = 11) resulting in an average polyp count per fragment estimated at 10,762 ± 748 polyps (Table 2.1B). Mean *P. acuta* capture rates ranged from 0 to 0.29 ind./polyp/h based on daily calculations from individual chambers (Figure 2.5B). Initial and final *Artemia* counts in control chamber were not significantly different (t = -0.06, df= 34, $P = 0.949$) but initial counts from control chambers were significantly higher than initial *Artemia* counts taken from chambers with corals, five minutes after feed delivery (t = 2.73, df=30, $P < 0.005$). There were significantly fewer *Artemia* counted from the final as compared to the initial samples from experimental chambers containing *P. acuta* fragments (t = 3.69, df = 22, $P < 0.005$). Mean capture rates when delivered *Artemia* at three pre-measured prey levels of 1, 2 and 4 ind./mL were 0.06 ± 0.02, 0.06 ± 0.01 and 0.14 ± 0.09 ind./polyp/h (± SE). Mean *Artemia* capture rates across

chambers on consecutive days and delivered prey levels did not differ significantly (Analysis of Deviance, x^2 =1.73, P = 0.22 and x^2 = 1.33, P = 0.30, respectively).

The number of *G. fascicularis* polyps per fragment ranged from 68 to 133 polyps, with an average of 103 ± 5 polyps (Table 2.1C). Mean daily *G. fascicularis* capture rates ranged from 1 to 110 ind./polyp/h. (Figure 2.5C) The initial and final *Artemia* counts in control chamber as well as initial *Artemia* counts in control and experimental chambers did not differ significantly (t = - 0.75, df = 52, P = 0.5 and t = - 0.04, df =52, P = 0.96, respectively). There were significantly fewer *Artemia* counted in the final compared to the initial samples taken from experimental feeding chambers containing *G. fascicularis* (t = 2.95, df = 67, $P < 0.005$) (Figure S2-1). Capture rates when delivered *Artemia* at four pre-measured prey levels of 1, 2, 4 and 8 ind./mL were 16.15 ± 2.2, 26.16 ± 1.69, 43.67 ± 5.01 and 47.87 ± 9.56 ind./polyp/h (± SE). Delivered prey density had a significant effect on capture rates (Analysis of Deviance, x^2 = 25.04, P = < 0.001). Capture rates were not influenced by day (Analysis of Deviance, x^2 = 1.58, P = 0.21) but there was a significant interaction between day and delivered prey level indicating effects of an outlier (Analysis of Deviance, x^2 = 20.46, P = < 0.001). Capture rates from a single *G. fascicularis* fragment in one chamber equated to 110 on day one and just 13.5 prey captured per polyp on day three (see * on Figure 2.5 C).

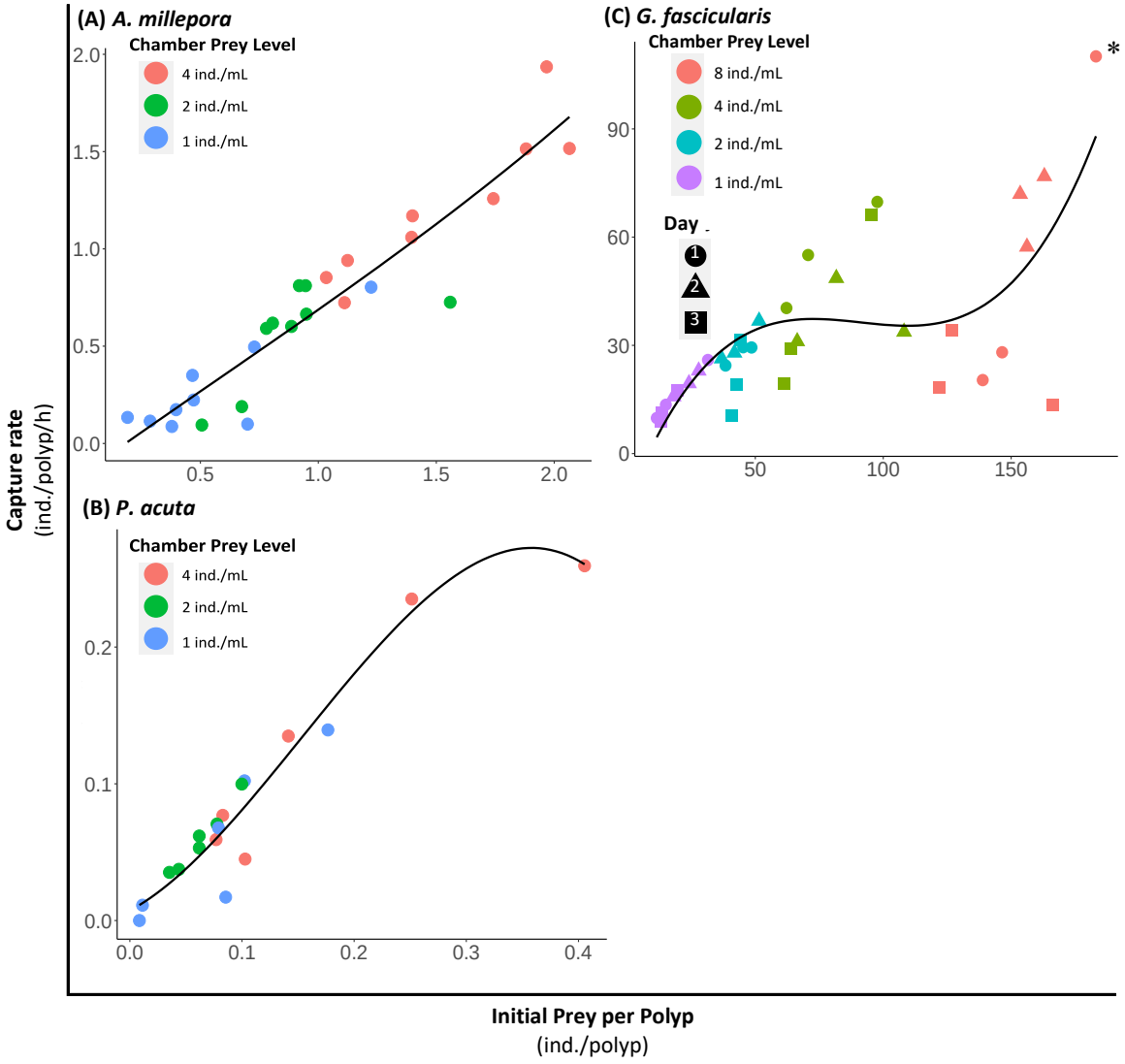


Figure 2.5 Relationship between capture rate and initial prey per polyp in corals fed *Artemia* Instar I nauplii. The x- axis shows number of prey items normalized to polyps per coral fragment (ind./polyp) and the y-axis is the number of prey caught per polyp over an hour from a coral within a chamber on any given day (ind./polyp/h). In *A. millepora* and *P. acuta*, effects of day were insignificant and not shown (A,B). Chambers receiving pre-measured prey densities of 1, 2, and 4 ind./mL are signified by blue, green and red circles, respectively (A, B) and 1,2,4, and 8 ind./mL signified by red, green, blue and purple shapes, for *G. fascicularis* chambers (C). In *G. fascicularis*, there was an interaction between day and level on capture rates. Shapes are included to signify the day of the feeding trial. The (*) signify results from an outlier where the day 1 capture rate was 110 and day 3 capture rate was 13.5 from the same coral in the same chamber.

Table 2.1 Summary of mean surface area, polyp number and capture rates of corals fed *Artemia* instar I nauplii. *

Species	Surface area (cm^2)	No. Polyps	Concentration of nauplii (ind./ml)	Mean capture rate (ind./polyp/h)
(A) *A. millepora*	84 ± 4.5	3,844 ± 219	1	0.28 ± 0.08
			2	0.57 ± 0.08
			4	1.22 ± 0.13
(B) *P. acuta*	153 ± 10	10,762 ± 748	1	0.06 ± 0.02
			2	0.06 ±0.01
			4	0.14 ± 0.09
(C) *G. fascicularis*	n/a	103 ± 5.36	1	16.15 ± 1.70
			2	26.16 ± 2.20
			4	43.67 ± 5.01
			8	47.87 ± 9.56

* Individual corals were places in triplicate chambers per prey concentration level (ind./mL) and fed over three successive days. Capture rates are expressed as Mean ± S.E.

2.3.3 Experiment II: Ingestion Study

Dissection of coral polyps fed instar II *Artemia* nauplii with fluorescent beads identified significant variation in prey ingestion between coral species. Few instar II *Artemia* were detected within the decalcified *A. millepora* fragments. The total number of polyps assessed over the 12 replicate fragments was 477 with an average of 40 replicate polyps per fragment. Only 8 of 165 polyps (5% of total polyps) from genotype A fragments had ingested between 1 to 3 *Artemia*. Only 2 of 150 polyps (1.3% of total polyps) from genotype B fragments were detected to have ingested 2 *Artemia*. In contrast, 45 out of 162 polyps across all four genotype C fragments ingested *Artemia*, with the number of *Artemia* ranging from 1 to 4 per polyp. The fragments from genotype C were observed to have full polyp extensions, at 10 minutes post-feed delivery, and were noticeably paler than the fragments sourced from the other adult colonies.

A count regression model fit with a negative binomial distribution, corrected for over-dispersed *Artemia* count data per individual polyp. The model was not improved by including tissue sections as a random effect, or by including chamber as a fixed effect. No significant interaction between tank and genotype was observed. Genotype significantly influenced the counts of *Artemia* in dissected *A. millepora* polyps (Analysis of Deviance, $x^2 = 15.6$, $P < 0.0005$). There was also a significant interaction between genotype and chamber (Analysis of Deviance, $x^2 = 29.8$, $P < 0.0005$) (Table 2.2) (Figure 2.7A).

An average of 3.3 ± 0.1 *Artemia* instar II nauplii were present in *P. acuta* polyps. Data was collected from 1,443 polyps within 72 sections of 12 *P. acuta* fragments. A negative binomial mixed-effects model with genotype and chamber as fixed effects was selected to analyze count data on the number of *Artemia* ingested by *P. acuta*. Effects of chamber and genotype did not improve the model however adding in section as a random effect did (Car package R) (Table 2.2) (Figure 2.7B). This is consistent with the observation that *Artemia* within polyps were clumped and that polyps closer together consumed similar numbers of *Artemia* prey. *Artemia* clusters were present in polyps furthest away from the fragment's point of attachment to the ceramic plug (Figure 2.6).

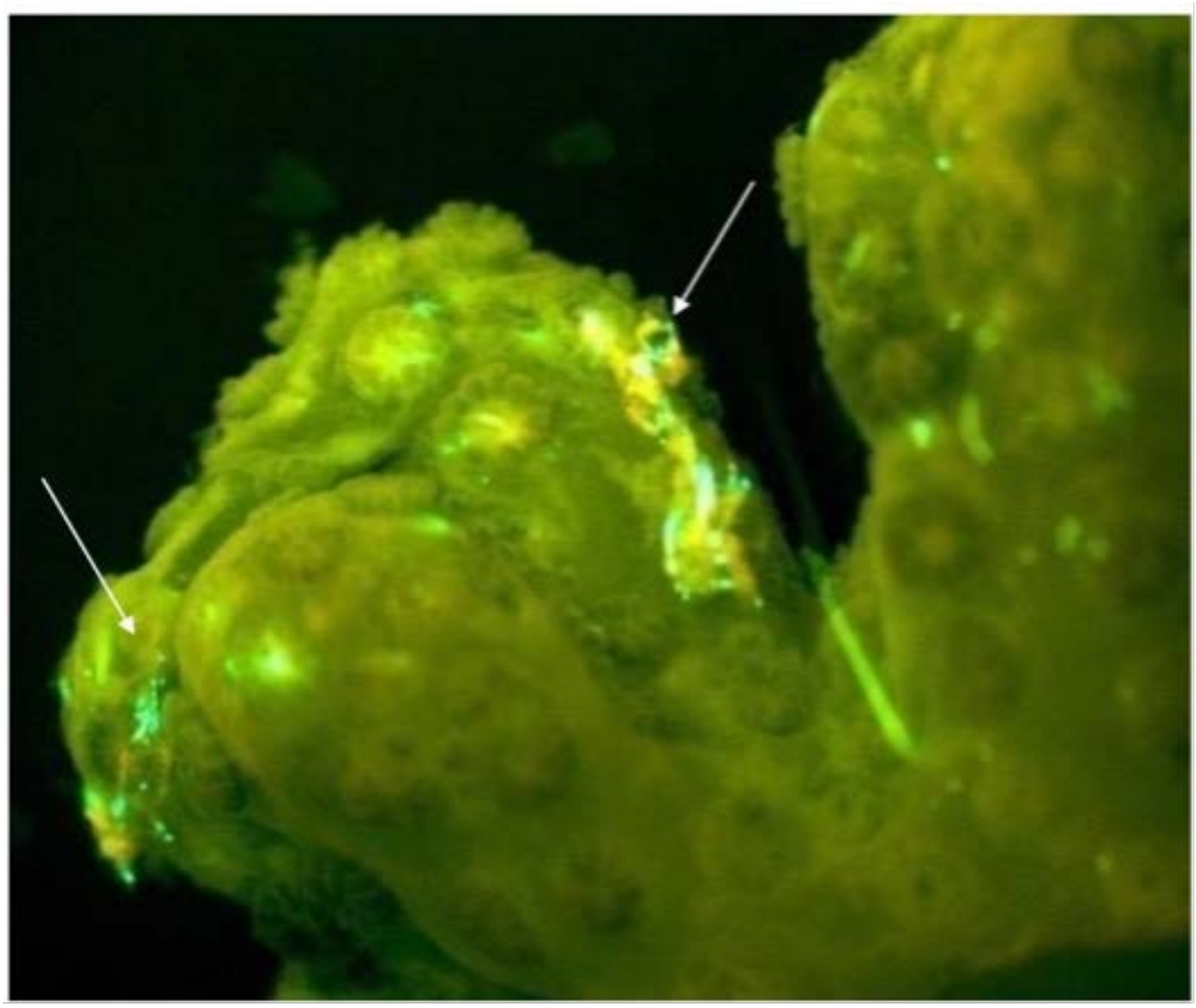


Figure 2.6 Artemia clusters in *P. acuta* tips. White arrows point to clusters of ingested *Artemia* Instar II nauplii by groups of polyps in the tips of fragments. Polyps closest to the point of attachment to ceramic plugs had fewer ingested prey.

Table 2.2 Analysis of Deviance table for the linear mixed and fixed effect models of the number of *Artemia salina* instar II nauplii ingested by *A. millepora* and *P. acuta*.

	x^2	df	P-value
P. acuta			
Genotype	4.54	2	0.1
Chamber	2.83	3	0.4
Genotype x chamber	7.29	6	0.3
A. millepora			
Genotype	15.6	2	< 0.0005 ***
Chamber	2.05	3	0.56
Genotype x chamber	29.8	6	<0.0005***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

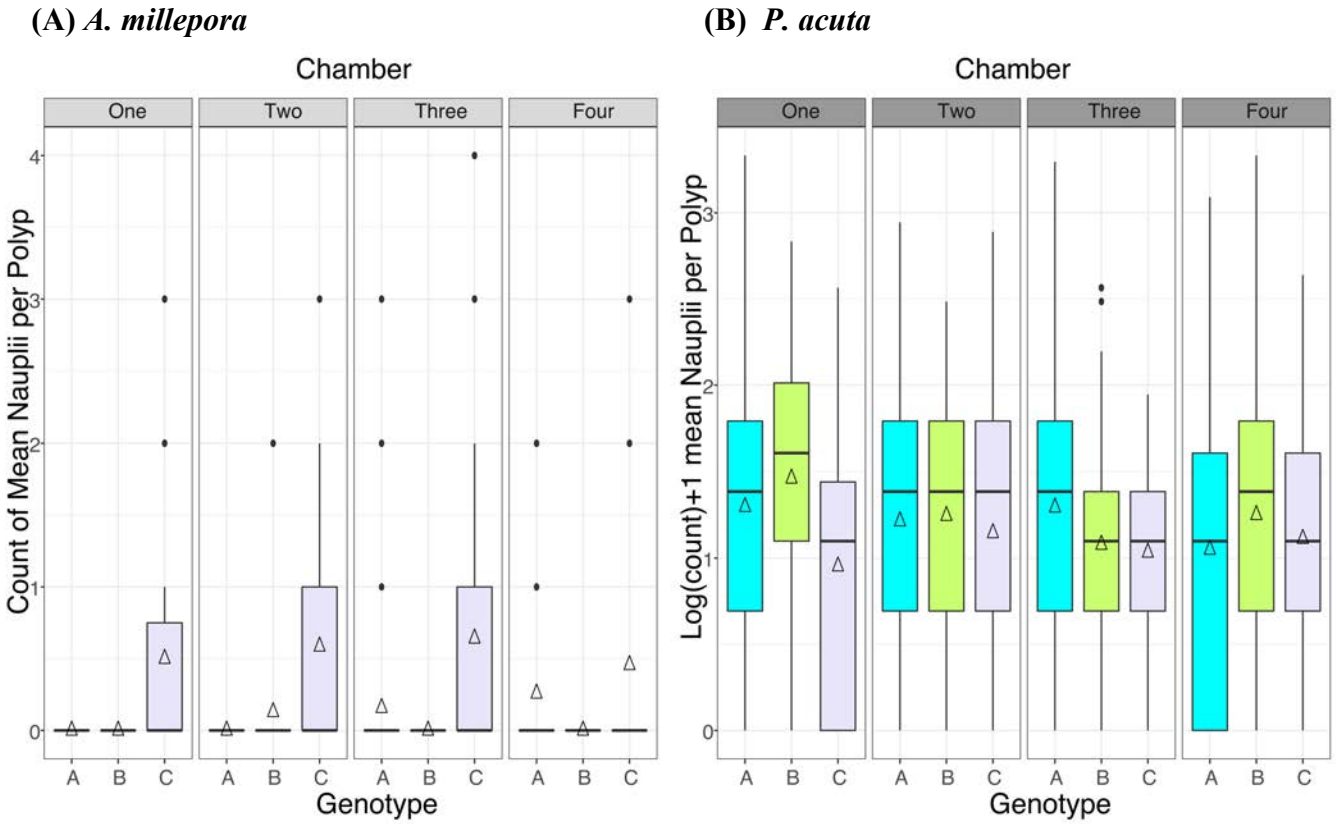


Figure 2.7 Mean *Artemia* instar II nauplii per (A) *A. millepora* (n =477) and (B) *P. acuta* (n=1,443) polyps in 12 replicates from 4 feeding chambers and 3 genotypes (A, B, C). Significant effects of genotype and chambers ("One", "Two", "Three", "Four") on the response variable, prey nauplii counts, were analyzed using a negative binomial generalized linear mixed-effects model in R (car-package) with tissue section as the mixed effect. D = mean no. nauplii in *A. millepora* or log (mean no. nauplii) +1 in *P. acuta*.

Auto-fluorescence in the tissues of the eight replicates belonging to two *G. fascicularis* genotypes prevented enumeration of ingested prey in these replicates. Auto-fluorescence was not an issue in replicates of the other two species. *Artemia* enumerated from dissected polyps within four replicates sourced from the same adult *G. fascicularis* colony averaged 75 ± 13 ind./polyp/h (n = 20).

2.3.4 Differences in feeding rates on Artemia Instar II nauplii as calculated by capture rate and ingestion rate values per a given chamber

Mean feeding rates averaged across corals from within the same feeding chamber (4 chambers per species), from experiment 2, quantified by capture rate and ingestion rate calculations were significantly different for *A. millepora* and *P. acuta* but not *G. fascicularis* (Figure 2.8). Ingestion rates refer to the mean number of *Artemia* enumerated from dissected polyps of the triplicate fragments per each feeding chamber, over one hour. Capture rates refer to the mean number of *Artemia* "cleared" from the same feeding chamber housing the triplicate fragments and enumerated by changes in the number of *Artemia* counted in 20 mL aliquots before and after one hour, normalized to the number of polyps present. Significantly higher capture rates than ingestion rates were recorded for *A. millepora* ($t_{\text{one-tail}} = 2.99$, df = 4, $P < 0.05$) (Table S2-2). In contrast, *P. acuta* ingestion rates were significantly higher than capture rates ($t_{\text{one-tail}} = -14.84$, df = 4, $P < 0.0005$). For *G. fascicularis*, capture and ingestion rates were similar ($t_{\text{one-tail}} = -0.83$, df = 6, $P = 0.21$). The ingestion rate averages for *G. fascicularis* are based on counts from polyps within only one of the three fragments within each chamber, due to interference of auto-fluorescent tissue obfuscating counts in the other two genotypes.

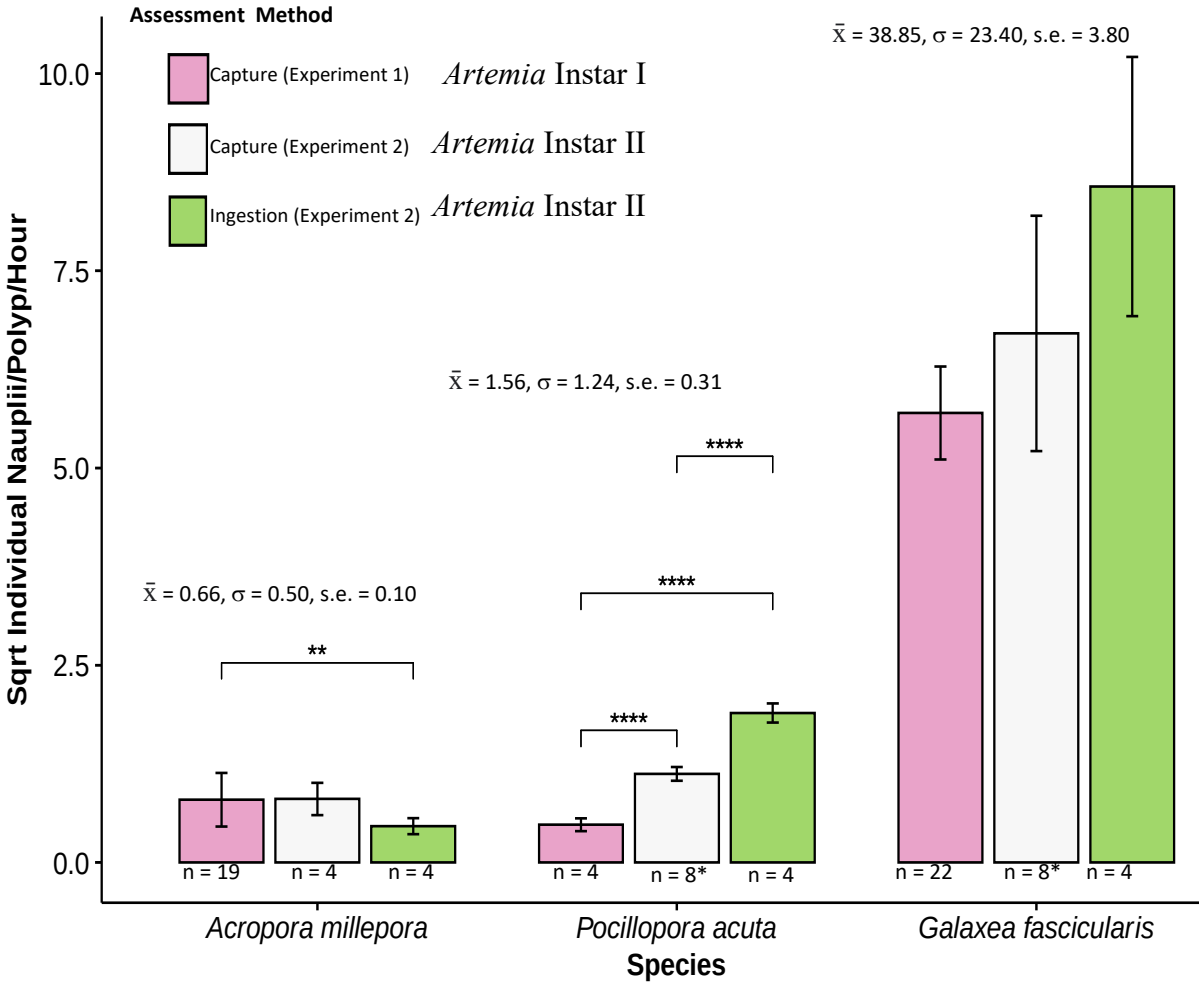


Figure 2.8 Cross-study and cross-species Comparison of mean feeding rates (capture and Ingestion) in corals derived from feeding chambers: Mean capture rates (pink) from chambers with initial Instar I nauplii prey counts of 3 ± 1.5 ind./mL are included from experiment 1. Mean capture rates (white) and mean ingestion rates (green) from chambers delivered instar II nauplii and 3 ind./mL are included from experiment 2 chambers. Significant values adjusted from pair-wise Welch two-sample t-tests to account for unequal variance of mean feeding rates per chamber. For *P. acuta* and *G. fascicularis* an addition 4 chambers from experiment 2 pilot studies (*) were included for this analysis.

2.4 Discussion

Symbiotic corals, like all animals, require heterotrophic nutrients to survive. A well-fed coral can grow faster (Toh et al. 2014), assimilate useful nutrients more efficiently (Tagliafico et al. 2017), yield higher symbiont densities and chlorophyll levels (Anthony & Fabricius, 2000; Tremblay et al. 2016), and exhibit greater resilience to stress than an unfed counterpart (Borell & Bischof, 2008; Tagliafico et al. 2017). The potential health benefit that can be derived from any given feed item depends on the ability of a coral to capture and ingest it. The differences in feeding abilities documented between the three coral species investigated in this study highlight the complexity of optimizing diets for the hundreds of coral species currently reared in *ex situ* aquaculture systems with diverse taxonomies, morphologies, and nutrient acquisition strategies (Leal et al. 2016).

Characterizing the ability of corals sharing similar traits, such as polyp size, to feed on various prey items is imperative to create scalable feeding regimes suitable to accommodate different coral species. To date, most aquarium-based studies have quantified coral feeding abilities using indirect capture rate approaches, with observed linear correlations between capture rates and prey densities suggesting that corals feed opportunistically on *Artemia* nauplii until satiated (Petersen et al. 2008; Osinga, Van Delft, et al. 2012; Tagliafico et al. 2018a). Direct ingestion rate approaches do however, as seen in the results from this chapter, produce more accurate feeding rates and provide greater insight into how prey is ingested across the entire coral fragment. Improving the nutritional vigor of aquaculture-produced corals requires a better understanding of what happens to captured prey. Ingested prey is often difficult to differentiate from coral tissue, and visual markers are one way of mitigating this constraint. Here, visualization of ingested prey was achieved by the inclusion of fluorescent

microbeads in the coral feeding protocols developed in this chapter (summarized in Figure 2.9).

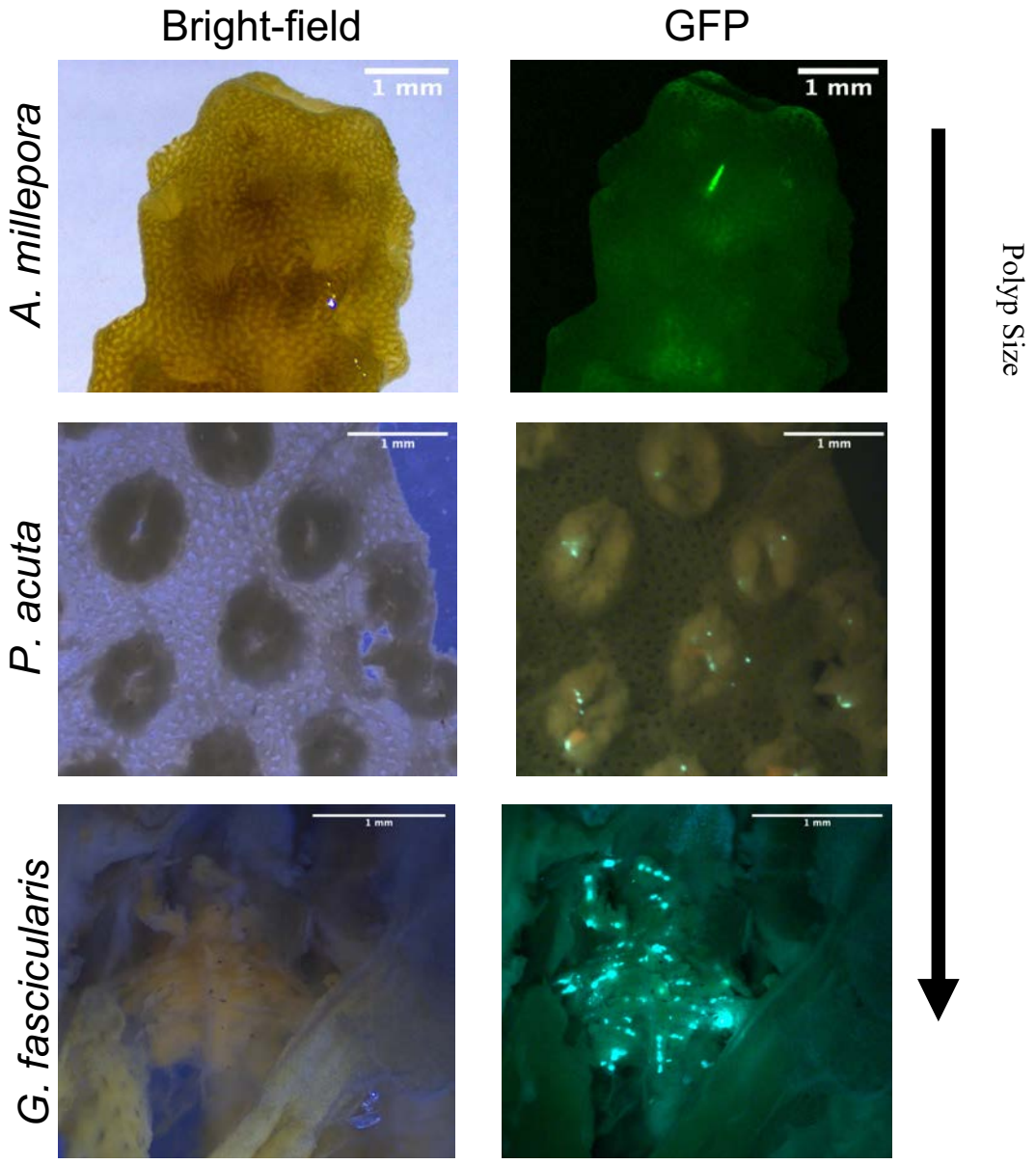


Figure 2.9 Visual summary of ingestion experiment. *Artemia salina* instar II nauplii shown within dissected corals tissue sections. Side-by-side microscope images of tissue sections visualized using (L) Brightfield and (R) a GFP filter. Polyp size correlated with mean prey ingested per polyp.

2.4.1 Capture rate calculations: Standardization required for cross-study and cross-species comparisons

The results from the first experiment (capture rate experiment) presented in this chapter confirmed the hypothesis that different species differ considerably in their response to delivered *Artemia salina* nauplii (Instar I). The capture rates of *Artemia* nauplii by coral fragments generally increased with higher prey density normalized to polyps per fragment (Initial no. ind./polyp). However, when initial prey delivered to feeding chambers was not normalized to individual polyps but rather fixed to a set number of prey items per millilitre of seawater delivered to "high", "medium" and "low" density treatment chambers, these fixed prey density levels were not a significant factor for capture rates of *A. millepora* and *P. acuta*. This is likely because the fixed prey density levels were too low and insufficient to produce observable differences between levels. Furthermore, given the large number of polyps per *A. millepora (*x = 3,844 polyps) and *P. acuta* (x = 10,762 polyps) fragments there were too many mouths to influence satiation. Iteratively, this led to a decrease in both the size of the sample fragments (i.e., from ~ 5 cm length to 2.5 cm length) and the amount of water in the feeding chambers (2 L in the capture rate experiment and 1L in the subsequent ingestion experiment) for subsequent experiments.

G. fascicularis have larger polyp diameters and lower surface area to volume ratios. Replicates in this study had a mean of 103 polyps per fragment. Prey density was a significant factor of *Artemia* capture rate by *G. fascicularis* corals, likely due to having fewer individual polyps per fragment and therefore fewer mouths to feed as well as less variability in the number of polyps per each fragment. This was also explicitly observed in the initial prey density per polyp calculations which were much higher in the experimental chambers containing *G. fascicularis* compared to the chambers hosting *A. millepora* and *P. acuta*

fragments. *G. fascicularis* is a fast-feeding coral which requires high numbers of *Artemia* for satiation (Osinga, Schutter, et al. 2012; Tagliafico et al. 2018a) and with the possible exception of one outlier fragment that consumed a mean 110 ind./polyp on day 1 and 13.5 ind./polyp on day 3, satiation was not observed here (refer to Figure 2.5C). The differences in *Artemia* capture rates across the three species investigated in this study is consistent with other aquarium-based studies. For example, the heterotrophy dominant, larger polyp corals, such as *G. fascicularis* and *D. axifuga* have been observed to consume over one hundred *Artemia* nauplii per polyp compared to autotrophic dominant, smaller polyp corals, such as *A. millepora*, or *H. rigida* which may consume one nauplii per every 10 polyps at the same prey densities (Kuanui et al. 2016; Tagliafico et al. 2018a).

Pocillopora spp. have been documented to consume significantly more *Artemia* Instar I nauplii than *Acropora spp.* (Latyshev et al. 1991; Toh et al. 2013; Hoogenboom et al. 2015; Conlan, Bay, et al. 2018). As such, the lower mean capture rates per delivered prey treatments (1, 2 and 4 ind./mL), observed here in *P. acuta* trials as compared to *A. millepora* trials, may seem unexpected. Given the significantly higher mean polyp counts per replicate in the *P. acuta* fragments than *A. millepora,* calculated capture rates may not be the most suitable measure for the comparison. A study by Hoogenboom et al. (2015) estimated 9.6 nauplii cm^{-2} h^{-1} (approx. 0.14 ind./polyp/h^{-1}) consumed by an *Acropora* sp. fragment and 188 nauplii cm^{-2} h^{-1} consumed by *P.acuta* (approx. 18 ind./polyp/h^{-1} based on average polyp density per cm^2 calculated here) at 1 ind./mL which is not consistent with the results seen here at the same delivered prey density (Hoogenboom et al. 2015). However, the results of this study are similar to those reported by Kuanui et al. (2016) which found that, at low *Artemia* densities (< 1 ind./mL), *P. damicornis* corals captured 0.05 ind./polyp/h and *A. millepora*

corals caught 0.13 ind./polyp/h versus 0.06 ind./polyp/h and 0.3 ind./polyp/h (Kuanui et al. 2016). Variable capture rates have been observed in *P. damicornis* with those from Thailand capturing significantly more *Artemia* than those from the Federated States Micronesia when brough into aquarium conditions (Kuanui et al. 2016) indicating intra-specific variations in feeding rates, possibly affected by external variables such as reef habitat. However, the literature suggests that *Pocillopora* spp. readily feed and derive benefits from *Artemia* diets such as significant changes in growth and survivorship in fed *Pocilliopora* spp. as compared to unfed counterparts (Raymundo & Maypa, 2004; Toh et al. 2013, 2014; Kuanui et al. 2016; Huang et al. 2020).

Capture rates estimated using the initial prey density per millilitre of seawater results in inconsistent measures across studies but normalizing the initial prey densities to the individual unit of the polyp can generate robust capture rate estimates. For example, capture rate calculations of *G. fasicularis* delivered *Artemia* instar I nauplii at 2 ind./mL produced rates of 9 ind./polyp/h (Osinga et al. 2008), 26 ind./polyp/h (this study) and 51 ind./polyp/h (Ferrier-Pages et al. 2010). Similarly, when prey was delivered at 4 ind./mL, capture rates recorded in this study were 44 ind./polyp/h compared to 93 ind./polyp/h in a study by (Wijgerde et al. 2011). In contrast, when initial prey per polyp is considered, results are more consistent across studies. For example, the capture rate calculated for *G. fasicularis* delivered *Artemia* nauplii (instar I) at a density of between 40 and 100 ind./polyp, fell between 10 and 70 ind./polyp/h in this study, which is consistent with capture rates of 15 and 75 ind./polyp/h by *G. fascicularis* in a different study, using the same initial *Artemia* prey range (Osinga, Van Delft, et al. 2012). Even though delivered prey density treatment significantly affected capture rates of *G. fascicularis* in this study, normalizing prey densities to individuals per polyp still

produced more consistent results for cross-study analyses and is likely to produce more comparable results across studies looking at multiple species (Table 2.3).

Table 2.3 Comparison of capture rate (ind./polyp/hr) studies on Aquarium based corals fed *Artemia salina* Instar I nauplii.

Species	Artemia density (ind./mL)	Capture rate (ind./polyp/h)	Source
(A) *G. fascicularis*			
	1	16	This study
	2	51	Osinga et al. (2008)
	2	9	Ferrier-Pages et al. (2010)
	2	26	This study
	4	93	Wijgerde et al. (2010)
	4	44	This study
	8	48	This study
(B) *A. millepora*			
	0.3	0.13	Kuanui et al. (2016)
	1	0.14	Hoogenboom et al. (2015)
	1	0.3	This study
	2	0.6	This study
	4	1.2	This study
(C) *Pocillopora* spp.			
	0.3	0.05	Kuanui et al. (2016)
	0.3	0.14	Kuanui et al. (2016)
	1	17.46	Hoogenboom et al. (2015)
	1	0.06	This study
	2	0.06	This study
	4	0.14	This study

2.4.2 Ingestion rate calculations: Use of visual tools to assess how and how much prey is ingested

Capture rate calculations cannot track the fate of delivered prey nor can we assume that the difference in initial and final prey counts are due to successfully captured and ingested prey. The results from the second experiment presented in this chapter (ingestion experiment) provided more direct information on the fate of delivered *Artemia* nauplii (instar II) through gut dissections facilitated by fluorescent markers. Determining the ingestion rate of *Artemia* therefore provides a more accurate assessment of feeding ability. Understanding how and how much prey corals are ingesting is critically important for the development of improved feeding regimes. Calculation of coral ingestion rates demonstrated that not only do corals have different feeding rates on the same prey (*A. millepora,* 0.2; *P. acuta* 3.5; *G. fascicularis*, 75 ind./polyp/h at 3 ind./mL) but they also display species-specific patterns in how prey is ingested across the fragment. Factors such as genotype or habitat may explain differences in feeding rates between fragments of the same species, such as observed in *A. millepora*. Lastly, the ingestion rate study enabled observations into how ingested prey is distributed across a fragment, with non-homogenous distribution between polyps directly observable, as seen in this study with *P. acuta* fragments.

In *A. millepora*, interestingly, the genotype had a significant influence on the feeding response and calculated ingestion rates. Specifically, coral fragments from two of the three genotypes did not display any obvious feeding response such as extended tentacles. Furthermore, in these two genotypes, few dissected polyps (10 out of 315) contained ingested prey. In contrast, 45 out of 162 polyps, from the third genotype contained ingested prey and fully extended tentacles were observed at 10 minutes post-delivery. Notably, this genotype contained fragments that were visually paler, indicating potentially some level of bleaching

stress. Stressed corals may compensate for nutrients when stressed and in the event of bleaching, can survive if able to utilize lipid reserves or heterotrophic feeding to buffer against energetic losses (Hughes & Grottoli, 2013). For example, increased assimilation of heterotrophic carbon has been observed in bleached *Montipora capitata* and *Porities lobata* compared to unbleached controls (Hughes & Grottoli, 2013). These results indicate that although it is possible for *A. millepora* to consume *Artemia* instar II nauplii, this may only occur in the event of dysbiosis with their algal photo-symbionts which can occur during stress.

P. acuta fragments had clusters of ingested prey in polyps located in the branch tips and fewer clusters were found in polyps near the point of adhesion to the coral plug, although the mean number of prey ingested per polyp did not differ across replicates. While a similar distribution pattern was not observed for *A. millepora*, previous studies have demonstrated that polyps in different colony branch positions exhibit significantly different nutritional profiles with high-energy nutrients catabolized more readily at the edges of colonies, such as branch tips (Conlan, Humphrey, et al. 2018). A similar mechanism could be at play in the *P. acuta* fragments, where the polyps at the tips need to consume more prey to meet the higher nutritional demands of proliferating cells. Alternatively, polyps within the tips may reap the benefits of higher surface area exposed to prey items, increasing their chances of an encounter. This clumped distribution pattern of prey ingestion cannot be observed by capture rate methods, which assume an even consumption of prey by polyps across a fragment, yet the observation has important implications. For example, it may be important to maximize the number of branch tips that have unrestricted exposure to the water column and maximize

the vertical height of each fragment when arranging and attaching *P. acuta* fragments to a substrate in aquaculture.

2.4.3 Development of methods

Although capture rate studies have been the most used measure of coral feeding rates, our knowledge into how corals feed and which prey they prefer could benefit from strategies which detect prey ingested. Whereas the capture rate approach is a quick and efficient metric, a coral's feeding behaviour may influence the efficacy of this assessment method. In the second experiment of this chapter, both capture and ingestion rates were calculated from the same feeding event, which enabled a side-by-side comparison of the values calculated through each approach. The method of assessment used (capture versus ingestion rates) produced significantly different mean values for *Artemia* (Instar II) nauplii consumed by *A. millepora* and *P. acuta* fragments, with estimates from the capture rate method being higher for the former and lower for the latter. One explanation for this result is that *Acropora* corals secrete high volumes of mucus in response to nutrients (Huettel et al. 2006; Naumann et al. 2009) whereas *G. fascicularis* and *P. acuta* do not. Since capture rate methods assume homogenous prey distribution, corals such as *Acropora* spp. that secrete mucus cause mucus-prey agglomeration, leading to stratification. This method of procuring prey clearly influences the accuracy of the capture rate approach.

Although the difference in average prey consumed per *G. fascicularis* polyp was not significantly different between capture rate and ingestion rate calculations, with a larger sample size, it is expected that the ingestion rate approach would yield higher numbers. The sample size of *G. fasciscularis* was limited due to background interference between the

corals' tissues and the signal emitted from the microbeads. Whereas the green fluroescent visual markers were highly effective in detecting prey in *A. millepora* and *P. acuta,* the auto-fluorescent pigments in tissues of *G. fascicularis* interfered with the microbead fluorescent signal. In two out of the three *G. fascicularis* genotypes the autofluorescence signal was so strong that it prevented differentiation between prey ingested and coral tissue, hence obfuscating analysis of ingestion rates in these genotypes. This emphasizes the need to select fluorescent markers and detection methods that maximize contrast with the auto-fluorescent pigments present in the coral species and genotypes under study.

A recent study found rotifers to be an effective vehicle for delivering putative probiotics to captive *P. acuta* fragments by using a fluorescent dye to track bacteria (Assis et al. 2020). However, it was found that auto-fluorescent pigments in the internal structures of the rotifers interfered with the used LIVE/DEAD staining unless the rotifer was thoroughly starved prior to the experiment (Assis et al. 2020). This illustrates the need for optimized protocols to ensure correct detection of ingested prey carrying a visual marker. The distinct patterns that fluorescent microbeads made inside incubated zooplankton prey, in addition to colour, assisted detection and enumeration in larval finfish tissues (Lee et al. 2018). With the right fluorescent markers and filters, elucidating prey preference with fluorescent microbeads can help identify otherwise cryptic prey types (e.g., ciliates, copepods) through microscopy-based approaches. Although, in this study, other zooplankton prey species (rotifers and copepods) were also successfully incubated with the fluorescent polystyrene microbeads, they were ultimately excluded from analysis due to the delicacy of soft bodied rotifers, which complicate enumeration in gut cavities, and issues with a reliable copepod supply at high densities. In contrast, *Artemia* are hardy, can be reared in high densities and are ubiquitously

used in aquaculture settings, thus they are ideal for the development of feeding protocols. However, it will be important for future research to investigate the inclusion of other live prey items and fluorescent microbeads or other dyes that can help discern preferential feeding.

Gut dissections alone can provide useful information, but the inclusion of visual aids expands its applicability to a wider range of prey types. The drawbacks to the use of histological approaches are that dissections are tedious and it is challenging to dissect small polyps and to detect cryptic, small (e.g., pico -, nano- or mesoplanton and microalgae; 10 – 400 μm) or degraded prey within coral gut cavities (Agostini, Suzuki et al. 2012). The use of fluorescence proved to be a promising and effective way to overcome these challenges if the fluorophore can be discriminated from autofluorescence from the prey item itself or the coral. Future coral feeding studies should utilize visual markers for the assessment of feeding rates in corals fed other live prey items that are also suitable for nutritional enrichment.

2.4.4 Chapter conclusion

The potential health benefit derived from an enriched diet depends on the ability of a coral to capture, ingest, digest and furthermore, assimilate nutrients from the feed. Here, we were able to confirm and compare the capture and ingestion rates of *Artemia salina* nauplii using fluorescent microbeads, for the first time in a coral feeding study. This allowed for the direct detection of prey within individual polyps which produced results easier to standardize across studies, regardless of the number of polyps present on a given fragment. Not only do other zooplankton species easily uptake microbeads, as demonstrated here as well as in previous studies (Setälä et al. 2014; Lee et al. 2018), these fluorescent markers can be used as

a proxy for other enriched media, such as probiotic bacteria to further develop nutritional supplementation strategies for corals.

Chapter 3 -

Labelling coral bacteria with fluorescent protein genes

3 Chapter 3

3.1 Introduction

Anthropogenic pressures have resulted in concerning declines in coral reef health globally (Hoegh-Guldberg, 2004; T. P. Hughes et al. 2015; Morrison et al. 2019) and shifted reef management strategies towards exploring active interventions to enhance coral resilience (Spurgeon & Lindahl, 2000; Bongiorni et al. 2011; Hein et al. 2017). Numerous strategies have been proposed to aid reef recovery (Anthony et al. 2020), including the application of probiotics to corals (Peixoto et al. 2017; Rosado et al. 2019). Probiotics are defined by the U.N Food and Agriculture Organization as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" (Araya et al. 2001). This has been demonstrated in corals where the addition of putative probiotic bacteria consortia to *Pocillopora* sp. nubbins mitigated the effects of bleaching and pathogen challenge (Rosado et al. 2019). How the added microorganisms benefitted the coral was not investigated; however, this could be through direct association with the corals or indirectly through the stimulation of secondary nutrient acquisition, thereby buffering the host from bleaching and pathogenic effects. Though probiotics offer potential to improve coral resilience, to date the mechanisms by which the added probiotics improve coral resilience have not been elucidated. Importantly, studies have not demonstrated if the added probiotics are retained by the coral. Therefore, to establish how probiotics benefit coral, approaches need to be developed to visualize and track added probiotic strains.

Chapter 3

The microbiome of corals is diverse and partitioned across several micro-habitats including inner and outer mucosal layers, gut cavities, skeleton and within tissue layers (Bourne et al. 2016). While still relatively poorly understood, the importance of bacterial functions within the coral holobiont depends on their corresponding niche, and therefore, the putative functional benefits provided by added coral probiotics depend on delivery to the appropriate microhabitat within the coral. For example, exclusion of opportunistic pathogens may require the addition and maintenance of antagonistic probiotics to the mucosal layers, while putative reactive oxygen species (ROS) scavengers may need delivery and maintenance in coral tissues where oxygen free radicals are generated and drive the cellular mechanisms leading to the bleaching response (Reshef et al. 2006; Nielsen et al. 2018; Rosado et al. 2019). In all these cases, understanding the efficacy of the added probiotic and determining their direct or indirect benefits depends on tracking the added strains to determine if they are retained, active and carrying out a function of interest.

Approaches that can vector probiotics into corals are under development and could involve addition to the water surrounding corals, directly onto corals or via delivered feeds. Probiotic enriched feeds have had demonstrable success in other aquaculture organisms. For example, aquaculture fish species fed live diets supplemented with a mixture of beneficial bacteria, such as *Bacilllus* spp., which effectively colonize digestive tracts, resulted in improved assimilation of nutrients and higher growth rates (Gomez-Gil et al. 2000; Merrifield et al. 2010; Allameh et al. 2017). Similarly, aquaculture western white shrimp larvae fed rotifers enriched with *Lactobacillus* spp. resulted in higher growth, survival and disease resistance (Najmi et al. 2018). Recently, rotifers were also used to track the delivery of coral probiotic candidates (e.g., *Pseudoalteromonas* spp.) to gut cavities of *Pocillopora* sp. but the viability

of the bacteria was not investigated (Assis et al. 2020). Nonetheless, these studies support the hypothesis that live diets could effectively administer probiotics to corals to improve their health status (Assis et al. 2020).

Prior to scaling up the delivery of probiotic treatments to aquaculture corals, an understanding of key host-microbiome symbioses is needed and requires ways to visualize the fate of delivered inocula over time. Visualization would aid in investigations of the mechanisms underlying the acquisition and establishment of beneficial microbial consortia in a coral microhabitat, which has important implications for delivery strategies in large-scale aquaculture settings. Fluorescent tagging is one potential approach to track delivered inocula and determine if they are retained within a coral microhabitat.

Fluorescent protein genes, first isolated from the jellyfish *Aequorea victoria* (Prasher, 1992), are commonly used to observe biological processes, including pathogenic and symbiotic interactions between species (Goulden, Hall, Bourne, et al. 2012; Goulden et al. 2013; Pollock et al. 2015). Fluorescent tagging involves gene transfer from an organism, whose genome contains a fluorescent protein (fp) gene, into an unrelated organism (Feiss, 1996; Lambertsen et al. 2004a; Bott, 2014). Gene transfer commonly occurs through conjugation, which is a mode of genetic transfer between bacteria that involves direct cell-to-cell contact (Feiss, 1996; Bott, 2014). Conjugation is common in Gram-negative bacteria due to the ubiquitous presence of pili, long hair like surface appendages, which help form a mating pair. The genetic material transferred from a donor to a recipient cell via conjugative pili is typically in the form of a plasmid, defined as circular extrachromosomal DNA that can replicate independently (Feiss, 1996; Bott, 2014; Madigan et al. 2015).

Plasmids broadly fall into two categories: conjugative and non-conjugative. Conjugative plasmids are typically over 30 kb, exist in one or two copies per host cell, and are able to promote their own transfer into recipient cells (Feiss, 1996; Bott, 2014). Non-conjugative plasmids (e.g., the ColE1 plasmid) are smaller, usually present in multiple copies per host cell and are unable to promote their own transfer into recipient cells (Feiss, 1996; Bott, 2014). In the case of conjugation in Gram-negative bacteria, conjugative plasmids are transferred via cell-to-cell contact, facilitated by pili, using the steps of (i) retraction, (ii) cell-wall stabilization and (iii) transfer of genetic material followed by (iv) separation of the cells (Feiss, 1996). However, plasmids are often unstable and easily lost from a population at a high frequency due the metabolic costs on a recipient bacterium. Artificially constructed plasmids are especially unstable and, when present in high copy numbers, can interfere with normal gene functioning (Feiss, 1996; Lambertsen et al. 2004). Genetic material from a plasmid can also become stably integrated into a recipient bacterial cell's chromosome if it contains genes encoding transposition enzymes (transposases) in addition to advantageous genes (e.g., encoding antibiotic resistance) (Bott, 2014). Recombinant cells, called transconjugants, contain DNA with certain plasmid encoded genes and, in some cases, these mobile DNA elements may become integrated into the bacterial chromosome. Plasmid-mediated conjugation, therefore, is often used to create transgenic species by transferring chromosomal genes or plasmid-encoded genes, from one species to another (Feiss, 1996; Richards et al. 2003; Bott, 2014).

Lambertsen and co-workers (2004) optimized a procedure for chromosomally tagging Gram-negative bacteria with fluorescence marker genes at one specific, neutral, highly conserved

bacterial *glmS* (L-glucosamine-6-phosphate synthase) gene region. This was accomplished using a transposon; a genetic element that moves from one DNA molecule to another. Transposases (*tns*) are encoded by the transposon and are critical for activating a transposition pathway which results in the movement of genes from the transposon onto the host's DNA. Many transposons insert themselves randomly into a bacterial genome and they may interfere with normal gene functioning. In contrast, the Tn7 transposon, originally discovered in an *Escheria coli* plasmid, has a tightly controlled, site-specific, transposition pathway which results in insertion within the *glmS* gene region of a recipient's genome and does not interfere with the expression of other phenotypes. For transposition to occur at this site, there are specific sequences that must be recognized by the Tn7 transposon's target-site selector (tnsD). Successful transposition of the Tn7 elements into this insertion site can be confirmed by use of primers or probe combinations that target the insertion site (Lambertsen et al. 2004; Parks & Peters, 2007). However, a different Tn7 transposition pathway may target other conjugative plasmids, the mechanisms of which are less well-studied than the site-specific transposition pathway (Wolkow et al. 1996; Kuduvalli et al. 2001).

Two *Vibrio alginolyticus* strains isolated from healthy corals were selected as the recipient strains in this study. While some *Vibrio* species are commensal (Austin et al. 1995) or mutualistic (Ceh et al. 2013) members of the community, others are opportunistically pathogenic, especially under conditions of host stress (Bourne et al. 2008; Garren et al. 2014; Pollock et al. 2015). Here, *V. alginolyticus* strains were chosen as a proof of concept due to (i) the availability of previous isolates from healthy corals, (ii) an ability to be cultured on a highly specific medium (Sweet et al. 2021), thereby aiding the selection of transconjugants and (iii) the success of similar fluorescent gene tagging protocols in other *Vibrio* species

(Goulden, Hall, Pereg, et al. 2012; Pollock et al. 2015; Dubert et al. 2016). Previous studies observed in real time the infection pathways of pathogenic *Vibrio* spp. in lobster larvae (Goulden, Hall, Bourne, et al. 2012; Goulden et al. 2013), larval clams (Dubert et al. 2016), and juvenile corals (Pollock et al. 2015; Wada et al. 2016) using similar fluorescent tagging protocols. It was demonstrated that many *Vibrio* species are associated with a host's digestive system, increasing the likelihood that labelled *V. alginolyticus* could be observed proliferating and establishing within a coral's gut cavity if delivered through live feeds.

This study aimed to develop fluorescent coral isolated bacteria, through bacterial conjugation, to deliver and detect the acquisition of coral bacteria into a host through live feeds and observe subsequently downstream localization. A four-parental conjugation procedure to tag *V. alginolyticus* strains, isolated from healthy corals, with transposon encoded green, red, cyan and yellow fluorescent protein genes was conducted. The development of a method to visualize beneficial host-microbe symbioses in a range of coral hosts is critically important for the expansion of coral aquaculture and has implications for large-scale reef restoration.

3.2 Materials and Methods

3.2.1 Bacterial recipient strains

Recipient *V. alginolyticus* strains were made available from the Australian Institute of Marine Science (AIMS) culture collection. *Vibrio alginolyticus* 090405_12 (Accession no. KF158809-10) was isolated from *Pocillopora acuta* larvae (Ceh et al. 2013) and *Vibrio alginolyticus* AMM-P-12 (Accession no. MW828406) was sourced from mucus of healthy adult *Acropora millepora* coral. These strains were reported in the study of Sweet et al (2021) that documents the bacterial strains isolated from coral and available for research

purposes. *V. alginolyticus* 090405_12 was previously isolated on marine agar and *V. alginolyticus* AMM-P-12 was previously isolated on a modified minimal basal medium. Both strains were grown overnight in marine broth at 28°C at 170 rpm prior to storage in glycerol stock. All bacterial strains used in this study were stored in the PC2 Laboratory at AIMS at – 78 °C and subsequently taken from 20% - 30% glycerol stocks prior to use.

3.2.2 Mobilization by Four-Parental Conjugation Procedure

Gene mobilization was carried out by a 4-parental conjugation using a mini-Tn7 gene tagging system using a combination of *E. coli* strains (donated by the Department of Zoology, Oxford University) including a helper (plasmid; pUX-BF13, *E.coli;*AKN68), mobilization (plasmid; pRK600, *E.coli* strain; AKN98) and a donor strain to mobilize a delivery plasmid (pUC19 *E. coli* derivatives) into a recipient (Figure 3.1A) (Lambertsen et al. 2004). Genes of interest (fp genes and chloramphenicol resistance genes) were located between the transposon ends (Tn7L and Tn7R), on each of the four delivery plasmids used. The four delivery plasmids contained a Tn7 element encoding a green (fp gene; *egfp-a*, *E. coli* strain; AKN35), yellow (fp gene; *eyfp-a*, *E. coli* strain; AKN36), cyan (fp gene; *ecfp-a*, *E. coli* strain; AKN35) or red (fp gene; *DsRedExpress-a*, *E.coli* strain; AKN131) fluorescent protein gene. Differently colored fp genes were trialed to improve downstream detection of bacterial cells against a wide range of auto-fluorescence found across coral species. The transcription and expression of these Tn7 transposon elements, including fp genes and antibiotic resistance genes for Chloramphenicol and Gentamycin (Cm^R, Gm^R), are regulated by a constitutive growth-independent promoter ($P_{A1/063/04}$) (Koch et al. 2001; Teal et al. 2006). In addition to Tn7 elements, the delivery plasmids (Figure 3.1B) carry an origin of replication site (Ori_{ColE1}), genes required for conjugation (i.e., those encoding the synthesis of sex pilus).

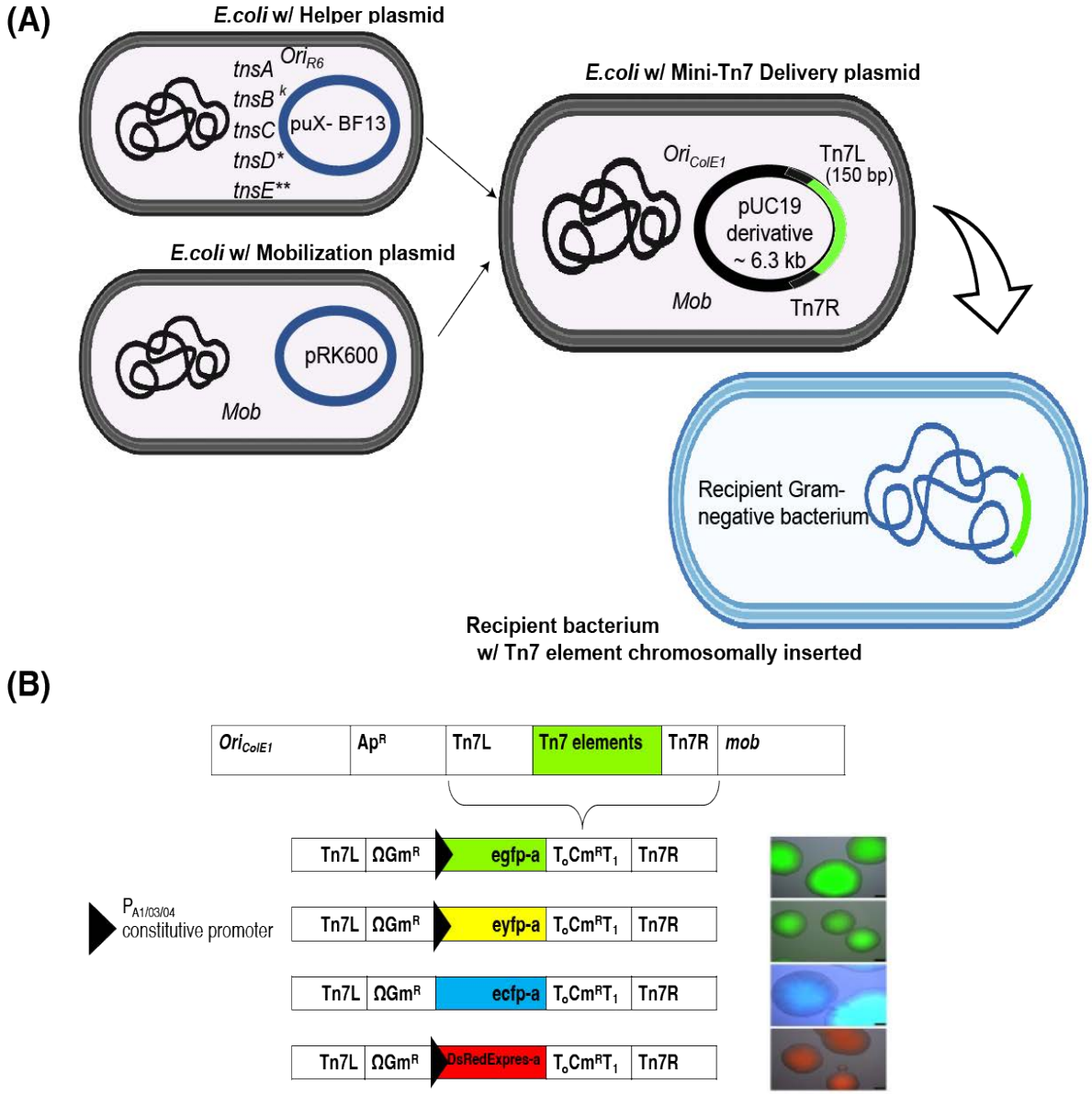


Figure 3.1 Adapted from "Mini-Tn7 Delivery plasmids, ver.1" by Lambertsen 2004. *E. coli* helper, mobilization and delivery plasmids and recipient Vibrio alginolyticus with transposable (Tn7) element. (A) The mobilization plasmid (pRK600) contains a *mob* gene which enables the transfer of genes onto a recipient's DNA. The helper plasmid (pUX-BF13) contains 5 transposase genes (tnsA+B+C+D+E) to insert mobile DNA elements (Tn7 elements) including a fluorescent protein gene, into a recipient's chromosome. *TnsD* is a site-specific target site selectors and *tnsE* is an alternative target site selector. The delivery plasmid contains the Tn7 element and the *mob* and transposase genes from the mobilization and helper plasmids. The genes of interest are then mobilized into a recipient's chromosome, in this case, *V. alginolyticus*, isolated from corals. (B) The mini-Tn7 delivery plasmids and elements are presented. Ap^R, Gm^R and Cm^R represent antibiotic resistance genes to ampicillin, gentamycin and chloramphenicol.

The donor and helper strains were revived from frozen glycerol stocks by streaking onto Luria-Bertani (LB) agar with the appropriate antibiotics (*E. coli* pRK600 with 15 μg/mL chloramphenicol; *E. coli* pUXBF13 with 50 μg/mL ampicillin; *E. coli* pUC19 derivatives with 5 μg/mL gentamycin and 15 μg/mL chloramphenicol). Glycerol stocks of the wild type, recipient *V. alginolyticus* strains were streaked onto TCBS agar without antibiotics. Single colonies from overnight plates were used to inoculate cultures in LB20 broth with the appropriate antibiotics (30 °C, 170 rpm), and to inoculate the selective medium (TCBS, 15 μg /mL Cm) to confirm no growth. Overnight cultures (1-1.5 mL depending on growth) were harvested, washed in LB broth (4650 rpm, 5 min) and pellets resuspended in LB broth (500 μL).

The *E. coli* helper, mobilization and donor strains were combined (400 μL of each) and incubated at 30 °C for 30 minutes, without shaking, to allow growth of the pilus. *V. alginolyticus* cells were incubated in a 37 °C water bath for 15 minutes to make them more susceptible for DNA uptake (heat shock). Immediately following the incubations, 25 μL of *V. alginolyticus* cells and 75 μL of the helper/donor mix were carefully deposited together onto LB plates (spot plate method) or onto 0.2 μm polycarbonate filters (filter method) and incubated overnight (30 °C). Successful transconjugants, defined as colonies which grew on the selective media, were purified by the streak plate method then grown in LB broth with antibiotics (15 μg/mL chloramphenicol and 5 μg/mL gentamycin) overnight (28 °C, 170 rpm) and stored in glycerol stocks.

3.2.3 Plasmid stability assessment and fluorescence detection

Stability of acquired chloramphenicol resistance was assessed by continuous subculture with re-inoculation of fresh LB broth (as described above) every 24 hours. At the start of the procedure, three colonies were selected from selective growth plates containing successful GFP-labelled strains and YFP-labelled transconjugants for growth in LB broth without antibiotics. Twice daily inoculation of triplicate TCBS plates per liquid culture (18 plates per strain per dilution level) with and without chloramphenicol were carried out at five dilution levels through spread plating, to determine presence/absence of growth on antibiotics and calculation of CFU/ml with and without antibiotics. The wild-type recipient *V. alginolyticus* strain and uninoculated LB broth were included as controls.

To detect fluorescence, plates were viewed and imaged using a blue light transilluminator, a UV transilluminator (GelDoc Imaging system, Bio-Rad) with an ethidium bromide emission filter. Additionally, plates and liquid culture samples were assessed for fluorescence using an inverted multi-channel fluorescent microscope (LEICA DMI 6000 B). The following fluorescent channels with respective excitation/emission ranges (nm) were used: channel A, bandpass (BP) excitation 340 – 380, longpass (LP) suppression 425; A4, excitation BP 360/40, suppression BP470/40; N3, excitation BP 546/12, suppression BP 600/40; I3, excitation BP 450-490, suppression LP515; YFP, excitation BP 500/20, suppression BP 535/30. All microscopy images were taken with a digital color camera (Leica DFC310 FX) and processed in Leica Application Suite X (LAS-X). Images were captured at 1s exposures at lower magnification (1.5X and 5X) or 0.5s exposures at higher magnification (10X and 20X). A bright-field channel was always set to auto-exposure and images from each channel were overlayed using the LAX software.

3.2.4 Enumeration and fluorescence assessment of transconjugants in liquid culture and zooplankton hosts

Two green and three yellow stable transconjugants (demonstrated maintenance of chloramphenicol resistance) were grown overnight as described above. Cell counts were determined using a hemocytometer and a microscope (Zeiss AxioImager.M2) so that the inoculum concentration could be adjusted to a suitable range for enrichment of *Artemia* (between 1 x 10^7 and 1 x 10^8 CFU/mL) (Goulden, Hall, Bourne, et al. 2012). Vented, suspension- culture flasks (Sarstedt product no. 83.3910.500) were filled with 5 mL *Artemia salina* instar II nauplii (~ 500 ind./mL) or 5 mL *Brachionus plicatilis* rotifers (~ 1000 ind./mL) and 2 mL of 0.2 µm filtered seawater (FSW) and inoculated with one of the transconjugants. Following room temperature incubations (40 minutes, 50 rpm), small amounts of *Artemia* and rotifers from each flask (2 replicates x 4 strains x 2 species) were visualized using 5X and 10X magnification to check for fluorescence.

3.2.5 Analysis of chromosomal insertion through PCR

Insertion of the Tn7 elements onto chromosomal *V. alginolyticus* DNA was assessed via end-point PCR using forward and reverse primers targeting the *glmS* gene in *V. alginolyticus* and the Tn7 element. The forward primer was designed based on the 3' end sequences of the *glmS* gene, retrieved from the annotated full genome reference sequences of type strains of *V. alginolyticus* (ATCC 17749; Accession NC_022349) and related species (*Vibrio campbellii, Vibrio tubiashii, Vibrio nigripulchritudo*). The designed forward primer had the following sequence: 5'-GATCTCTTACATCCACGCAGA-3'. The reverse PCR primer anneals to the right end of the transposon (Tn7R) and had the following sequence: 5'-CAGCATAACTGGACTGATTTCAG-3' (Lambertsen et al. 2004). The primer pair is

expected to amplify a 510 bp product, containing sequences upstream of and within the expected insertion site, which would confirm chromosomal integration of fluorescent protein genes.

Ten transconjugants, representative of the different conjugation methods (spot versus filter method) and from each fp donor, were randomly selected for PCR assessment. Template DNA was prepared by selecting a single transconjugant colony, using a 5 µL inoculation loop, placed in 50 µL of autoclaved water and boiled for 5 minutes. Replicate template DNA tubes were prepared at 4 dilution levels (1:1, 1:10, $1:10^2$ and $1:10^3$). The PCR mixtures (total volume, 25 µL) contained 10 pmol of each primer, 5 µL of 5 x PCR buffer (Meridian Bioscience®, Cincinnati, Ohio, USA), 5 µL of template DNA, and 5U of MyTaq™ DNA polymerase (Meridian Bioscience®). Three annealing temperatures were tested individually and, on a gradient (55°C, 56°C and 57°C). The final magnesium concentrations in PCR mixtures were adjusted to 3.5 mM, 4 mM, 4.5 mM and 5 mM. The PCR amplification cycles were as follows: 95°C for 5 min; 30 cycles each at 95°C for 30 sec, 55- 57°C for 30 sec, 72°C for 30 sec; and a final elongation at 72°C for 5 min or 10 min. PCR products were visualized on a 2 % agarose gel with EtBr in TBE run at 80V. A positive control was not available because the forward primer was designed specifically for use in *V. alginolyticus* strains.

3.2.6 Genomic DNA extraction and Illumina sequencing.

Genomic DNA from one green (egfp-*V. alginolyticus*_AMMP_12) and one yellow (eyfp-*V. alginolyticus*_090405_12) transconjugant were extracted from cultures grown overnight. Genomic DNA was extracted using the Promega Wizard Genomic DNA kit for isolating genomic DNA with the protocol for Gram-negative bacteria. DNA quantity and quality (using A260/280 and A260/A230 values) were obtained through spectrophotometry (Thermo Scientific NanoDrop^{TM} 1000). Additionally, gel electrophoresis (1.5% agarose with EtBr in TBE) assessed yield (with 1 kbp and 100 bp ladders) and visually assessed quality. Genomic DNA was sent to the Australian Centre for Ecogenomics (ACE, University of Queensland, Brisbane) for Illumina Nextera® sequencing. Sequencing was performed on the NextSeq500/550 running 300 cycles on a 2 x 150 bp run with 1 Gb of coverage per sample.

3.2.7 Bioinformatics analysis

Genomic sequence data were de-multiplexed and imported as paired read (FASTQ) files into Qiagen's CLC Genomic Workbench (Version 9) for next generation sequence analysis. Quality control of raw sequence data was performed using CLC Genomics Workbench quality scores of 0.01 and 0.001, corresponding to Phred scores of 30 and 40 (probability of a base being wrong $1{:}10^2$ and $1{:}10^4$, respectively) to remove or trim low-quality reads and adaptor contamination. High quality reads were used for de novo assembly using CLC Genomics workbench. The parameters evaluated were the number of contigs ≥ 500 bp, the average contig length, the length of the longest contig and the value of the N50 (a weighted median statistic describing the length of the shortest contig at 50% of the length of the genome).

Assembled contigs were extracted and used to create basic local alignment databases as a search tool (BLASTn: DNA sequence and database program) in CLC Genomics Workbench, for each strain. Next, the ~700 bp eyfp-a and egfp-a fluorescent protein gene sequences (sourced from the Addgene Vector Database) and the 1833 bp *glmS* gene sequence (sourced from the annotated *V. alginolyticus* type strain) were input as search queries to find homologous sequence within the respective databases. High scoring Segment Pairs (HSPs) from each contig databases were further evaluated. HSPs were assessed by e-values, which is a quality metric of the match with higher values indicating less homologous sequences, and by the number of identical residues (an identity value) from the multi-BLAST output tables.

Lastly, contigs containing the conserved *glmS* gene sequences were extracted from the de novo assembly and aligned to the *V. alginolyticus* type strain, from which the forward PCR primers were designed. The consensus *V. alginolyticus* sequence was then aligned to the Tn7 site-specific attachment site *E. coli* sequences (attTn7; 68 bp) containing 36 overlapping nucleotide critical sequences within the *glmS* open reading frame (ORF) for the initiation of the site-specific transposition pathway (DNA target-site selector tnsD *E.coli* binding site; 36 bp). Linear alignments were conducted in Geneious Prime ® using a Clustal Omega algorithm.

3.3 Results

3.3.1 Production of Transconjugants

The 4-parental conjugation protocol using mini Tn7-transposon delivery plasmids with *V. alginolyticus* recipients, produced 22 transconjugant *Vibio alginolyticus* strains; 10 yellow (*eyfp-a*), 6 cyan (*ecfp-a*), 3 red (*dsRed-express-a*), 2 green (*egfp-a*), purified from initial growth on selective TCBS Cm15 agar plates. Eleven of these transconjugant strains originated from filter plates and ten originated from spot plates. However, over successive re-inoculations from glycerol stock, transconjugants originating from filter plates were observed to grow faster and to produce larger colonies on TCBS Cm15 plates than transconjugants originating from spot plates.

3.3.2 Antibiotic resistance and fluorescence levels

By the end of the nine successive days of continuous subculturing without antibiotic pressure, 1 (out of 4) green transconjugant strain and 6 (out of 8) yellow transconjugant strains continued to grow on the selective TCBS Cm15 plates. Cell counts were unable to be obtained due to an inability to predict a suitable dilution level at which to inoculate selective media given the loss of resistance by transconjugants to chloramphenicol over time. The 7 transconjugants that maintained resistance for 9 days or more were all originally isolated from the filter conjugation plates. Despite their antibiotic resistance, the colonies did not emit fluorescence that could be detected by eye, camera or fluorescence microscopy. When visualized by blue-light and UV transillumination and multi-channel fluorescence microscopy, the transconjugant colonies appeared the same as for wild-type recipient *V. alginolyticus* colonies. In contrast, fluorescence was observed for each *E. coli* donor strain, containing the red, cyan, green and yellow fp gene.

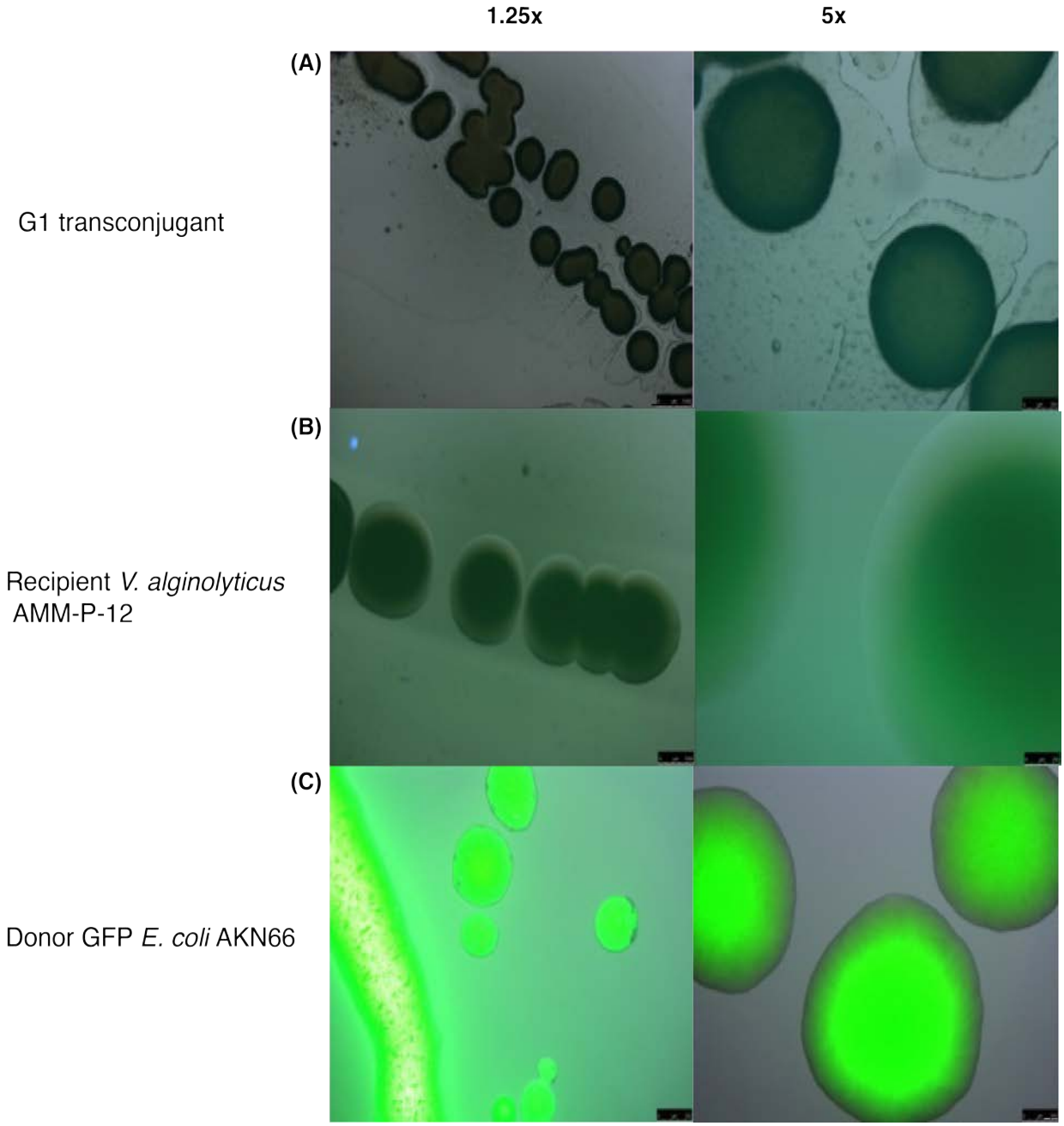


Figure 3.2 Comparison of fluorescence in (A) a *gfp* transconjugant *Vibrio alginolyticus* (G1), (B) the wild type recipient *Vibrio alginolyticus* strain and (C) the donor *E.coli* strain containing a green fluorescent protein gene. Overlay images taken using an inverted multi-channel fluorescent scope (LEICA DMI 600B) with exposure set to 1s for 5 fluorescent channels (only 1 used in bottom right image) and automatic for bright-field channel.

The *E. coli* strains containing the donor plasmids were used a positive control here although they contain a high copy number of the fp genes so it is expected that their fluorescence level would be much brighter than the transconjugant strains. Nonetheless, this result still indicated that detection methods (e.g., microscope settings and filter combinations) should be sufficient to detect fluorescence in the transconjugant strains, even though we would expect a lower level of fluorescence. Similarly, fluorescence was not detected in enriched zooplankton, equally inspected.

3.3.3 PCR and sequencing results

PCR amplification using primers targeting the *glmS*-Tn7R insert region diagnostic of successful chromosomal insertion (see section 3.2.4) failed to produce amplified products from any of the ten transconjugant strains selected randomly for analysis. Since a positive control could not be included in the PCR assay due to the novel use of *V. alginolyticus* as a recipient, it was not possible to ascertain if the lack of successful PCR amplification was because the Tn7 transposon did not integrate onto the chromosome of the *V. alginolyticus* strains or because the PCR conditions were suboptimal, despite extensive optimization. Therefore, genomic sequencing was conducted on two *V. alginolyticus* transconjugants (G1 and Y3) to investigate this further. High quality genomic DNA was extracted from both strains (G1: 426.4 ng/µL and Y3: 476.9 ng/µL). Sequencing yielded 1.23 Gb for G1 and 1.41 Gb for Y3. De novo assembly of paired-end sequence reads yielded 120 contigs with a N50 contig length of 407,136 nucleotides for G1 and 210 contigs with a N50 contig length of 351,911 for Y3 (Table 3.1).

Table 3.1 De novo assembly and summary of Y3 and G1 sequences.

ID	N50	Avg.	Min.	Max.	No. contig	Total contig length
G1	407,146	42,949	503	871,396	120	5,153,928
Y3	351,911	24,920	500	871,395	210	5,233,107

When the fp genes were Blasted against the locally constructed and assembled contigs of the two *V. alginolyticus* strains, low sequence identity was observed in both alignments. A maximum of 12 bp out of 717 bp matches between the egfp-a sequence and G1 contigs and 3 bp out of 707 bp matches between the eyfp -a sequence and Y3 contigs were found. The minimum e-values were 0.86 for both alignments (Figure 3.3 A,B) suggesting that these sequence identities were random. In contrast, the *glmS* gene sequence retrieved from both *V. alginolyticus* conjugants displayed high sequence identity against the annotated type strain *V. alginolyticus* sequence (1803 sequence identity over 1833 bp; e-value 0) (Figure 3.3 C,D).

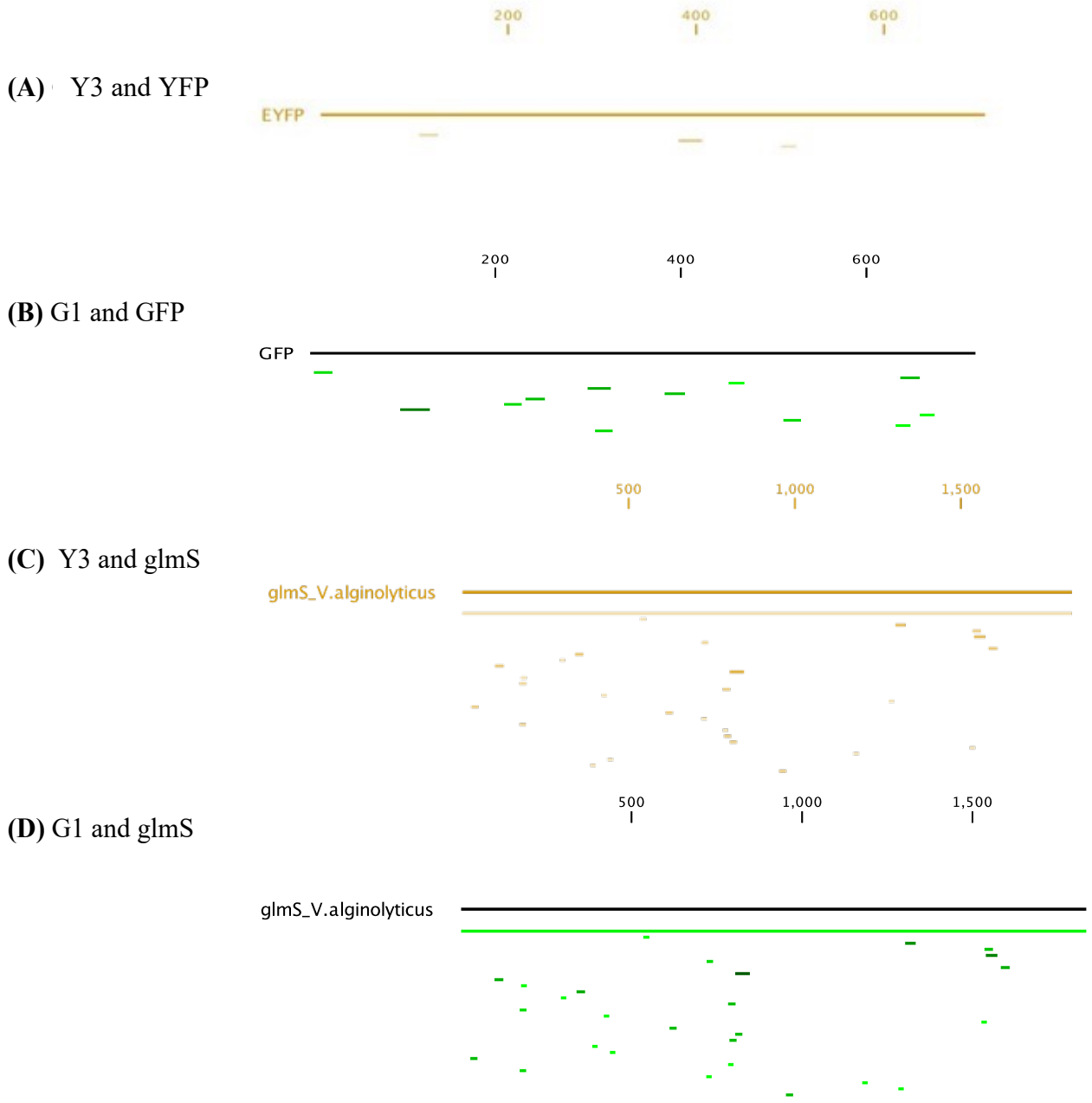


Figure 3.3 Multi-Blast query results of locally constructed contig databases for G1 and Y3 *V. alginolyticus* transconjugants to search for *eyfp-a* (A), *egfp-a* (B), and *glmS* (C,D) sequences within their genomic DNA. In each alignment, the first track represents the reference fp (A,B) or *V. alginolyticus glmS* (C,D) gene sequence in full with the subsequent track represented by contig sequences, with those more similar at the top, from the de novo assembly.

Further analysis of key sequences allowed for a better understanding of the lack of chromosomal insertion of the Tn7 transposon in the two sequenced *V. alginolyticus* transconjugant strains, used in this study. When the extracted contigs containing *glmS* sequences in the G1 and Y3 *de novo* assemblies were aligned to the corresponding region of the *V. alginolyticus* type strain, it was found that the 68 bp attTn7 sequences, contained within the *glmS* ORF, were 100% conserved. When this consensus *V. alginolyticus* sequence was aligned to the reference TnsD *E. coli* binding site, one missense mutation (Serine → Alanine) in the 11 amino acid sequence was found (Figure 3.4).

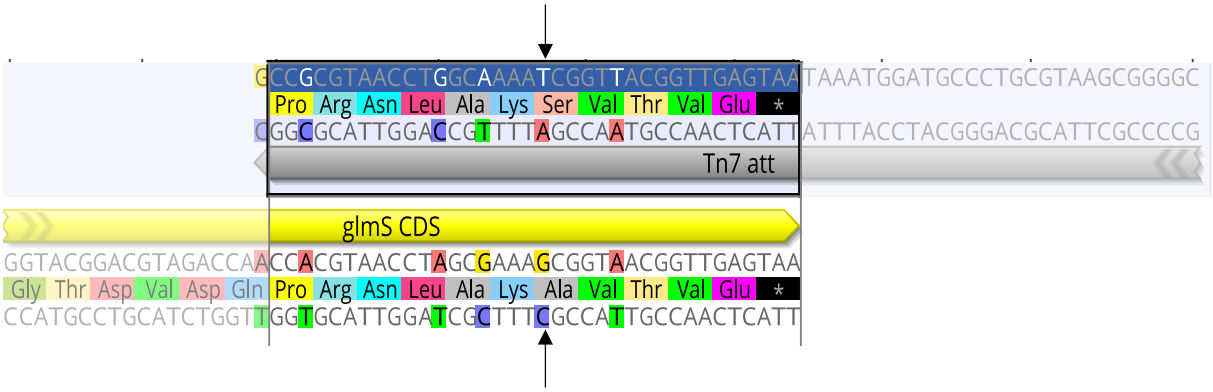


Figure 3.4 TnsD binding site alignment visualized by (top) attTn7 transposon attachment site, from *E. coli* type strain and (bottom) glmS gene region from *V. alginoltyicus* strains. The overlapping 36 bp region is where the DNA binding protein (TnsD) binds. Arrows point to location of missense mutation.

Lastly, to inform future molecular analyses in these *V. alginolyticus* strains, extracted contig sequences were aligned to the forward primer sequences which were found to start at the +1470 position on the 1833 bp *V. alginolyticus glmS* gene (approx. 300 bp upstream for the attTn7 site) and had 21/21 nucleotide identities. The reverse Tn7-109R primer sequence, containing sequences present on the Tn7 transposon, were absent on these genomic transconjugant sequences further confirming the lack of chromosomal insertion and reason behind lack of PCR amplification.

3.4 Discussion

Development of approaches to visualize and track bacteria are essential to elucidate if bacterial probiotics added to corals are retained and ultimately perform beneficial functions when associated with the coral host. While *V. alginolyticus* strains have previously been used as an aquaculture probiotic (Ceh et al. 2013; Paopradit et al. 2021) they are unlikely to be chosen for probiotic addition to corals due to the importance of horizontal gene transfer in dissemination of virulence genes in *Vibrio* spp (Sweet and Bulling 2017). Nevertheless, they were expected to be good model organism to test the feasibility of chromosomally labelling bacterial strains and visualizing this strain when added to corals

In this study a mini-Tn7 transposon delivery system was used to create transconjugant *Vibrio alginolyticus* strains with inserted green (GFP), yellow (YFP), cyan (CFP) and red (dsRed-Express) fluorescent proteins. Growth of 22 transconjugant strains on chloramphenicol selective media was observed, indicating that the Tn7 transposon could replicate in the *V. alginolyticus* host. However, PCR assays targeting the transposon insertion site and genomic sequencing results showed that the transposon encoded genes were not chromosomally inserted in these *V. alginolyticus* strains and suggested that the Tn7 element was potentially located on an autonomously replicating plasmid. The transconjugant strains lacked visible fluorescence indicating little fp gene expression in the *V. alginolyticus* recipient strains even if located on mobile plasmids.

3.4.1 Site-specific Tn7 insertion in V. alginolyticus

Genome sequencing of two transconjugant strains successfully allowed assembly of large sequence contigs (210 and 120 contigs for G1 and Y3, respectively). Although the Tn7 elements containing the *fp* gene required for fluorescence and the antibiotic resistance marker were not detected in assembled contigs, the growth of the transconjugants on selective antibiotic media suggested that the transposon was present on a mobile DNA element. Chromosomal insertion was therefore unsuccessful. While the Tn7 transposon is routinely used for its ability to insert itself specifically into a recipient chromosome, which assists in its detection through molecular assessment (e.g., PCR) and does not interfere with normal cell functioning, these results suggest that this site-specific, transposition pathway, facilitated by TnsD, was not utilized. The TnsD transposition pathway inserts exactly one copy of the transposon at a high-frequency into a very specific intergenic site, called *attTn7* (Kuduvalliet al. 2005; Mitra et al. 2010). The site of insertion within *attTn7* is located downstream of the *glmS* open-reading-frame (Kuduvalliet al. 2005; Mitra et al. 2010) a highly conserved housekeeping gene found in a wide range of organisms (Parks & Peters, 2007). The lack of chromosomal insertion is potentially a result of the TnsD not recognizing and binding to the insertion site on the *V. alginolyticus* chromosome (Bainton et al. 1993; Teal et al. 2006; Mitra et al. 2010). To initiate transposition, TnsD must bind to a 36 bp site on the 3' end of the *glmS* gene, which encodes a part of the TnsD protein. If binding does not occur, this sequence would prevent the recruitment of the rest of the transposon machinery, including three transposases, that are also required for the insertion of the *fp* gene into a DNA target (Craig, 1991; Skelding et al. 2002; Mitra et al. 2010).

Genome sequencing demonstrated that the *V. alginolyticus* transconjugants strains, in comparison to the *E. coli* type strain, have nucleotide wobbles at the *attTn7* positions +35, +40, +44, +47 and +56 (Figure 3.5). The +40 wobble in the fifth amino acid of the eleven amino acid sequence results in a missense mutation (Ser → Ala). However, the +40 position is the least conserved in this otherwise highly conserved region in many bacterial species (Choi et al. 2005; Kuduvalliet al. 2005; Mitra et al. 2010). Furthermore, a study by Mitra et al. (2010), reviewed important mutations in this region, which resulted in significantly decreased TnsD binding (Mitra et al. 2010). Seven nucleotides extending from the *attTn7* region (+23 → +58) were identified as crucial for TnsD-*glmS* binding (+31, +33, +42, +43, +45, +51 and +54). All of these seven nucleotides were conserved in the *V. alginolyticus* recipient strains and therefore we would expect the TnsD to recognize the target site in these transconjugants (Mitra et al. 2010). Still, without 100% conservation of this amino acid sequence, it is possible that the TnsD-mediated transposition pathway would have impaired protein-to-protein interactions between transposases or that the alternative transposition pathway would have been more favorable.

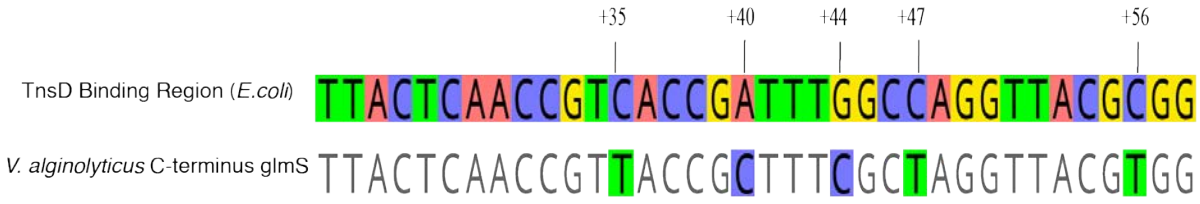


Figure 3.5 TnsD binding region within the C-terminus of the glmS amino acid sequence (*E.coli*). Adapted from Figure 1 Mitra et al. 2010. In *E. coli*, 36 bp in the 3' end of the conserved *glmS* gene is critical for TnsD binding and synthesis. The wobbles in the *V. aliginolyticus* sequence as compared to the *E. coli glmS* protein were not identified as key positions for the TnsD binding site within attTn7, according to Mitra et al. 2010.

Genome sequencing confirmed the transposon was not chromosomally inserted though the transconjugants displayed antibiotic resistance when growing on selective chloramphenicol media. One possibility is that delivery plasmid, a derivative of the *E. coli* originated pUC19, is maintained and replicating in host *V. alginolyticus* cells, though it is also possible that the transposon has transpositioned onto a conjugative plasmid. An increasing number of plasmids have been described from *Vibrio* hosts, likely underlying the high frequency of horizontal gene transfer between members of this bacterial family (Pan et al. 2010; Sobecky et al. 1998). Interestingly, a family of Marine RNA-based (MRB) conjugative plasmids was found in 6 *Vibrio* species with a similar replication mechanism previously only described in *E.coli* ColE1 plasmids, such as pUC19 (Le Roux et al. 2011; Pan et al. 2010). The pUC19 plasmid, and constructed derivatives, have been known to replicate in *E. coli* and other *Enterobacteriaceae* but not in other Gram-negative bacteria (Jeffrey & Joachim, 1991; Lambertsen et al. 2004; Parke, 1990). Unlike in most plasmids, whose replication is governed by a Rep protein adjacent to the origin of replication site (*ori*), ColE1 and MRB plasmids replicate from two RNAs, transcribed into complementary sequences from opposite strands of plasmid DNA within these *ori* regions (Jeffrey & Joachim, 1991; Le Roux et al. 2011; Myers & Myers, 1997). Incompatibility of delivery plasmids in recipient cells (i.e., a suicide vector) is critical for chromosomal insertion of transposon encoded genes because it creates a selection pressure for the transposition of important genes into the host's chromosome (Parke, 1990). If pUC19 is unexpectedly capable of autonomous replication within *V. alginolyticus*, then there would be no selective pressure to transpose. Future research into compatibility groups, host ranges and replication systems of endemic plasmids in marine bacteria may provide insight into effective gene vector systems.

The alternative possible location of the mini Tn7 transposon in the *V. alginolyticus* recipient strains is on a naturally occurring conjugative plasmid. In a less studied non-specific transposition pathway for Tn7, TnsE is the target site selector, initiating the transposition event (i.e., recruitment of TnsA, TnsB and TnsC). Unlike TnsD, TnsE preferentially targets conjugative plasmids within a recipient (Bainton et al. 1993; Finn et al. 2007; Peters, 2014; Waddell & Craig, 1988; Wolkow et al. 1996). In this pathway, the transposon will insert itself, often in multiple copies, into the *tra* operon of conjugative plasmids such as the pOX-G and R388 or other mobile DNA elements (e.g., filamentous bacteriophages) (Bao et al. 1991; Peters, 2019; Wolkow et al. 1996). If the transposon did transpose onto another plasmid in the *V. alginolyticus* recipients, it is unclear whether the mismatched nucleotides in the TnsD binding site prevented chromosomal integration or whether host factors favored execution of the TnsE transposition pathway. A better understanding of the mechanisms involved in determining which transposition pathway is initiated in a given host could help predict which coral isolated bacteria would be suitable for chromosomal labelling with Tn7 elements. Extracting plasmid DNA for sequencing could determine whether or not the transposon went into a conjugative plasmid and help understand this less studied Tn7 transposition pathway.

3.4.2 Levels of fluorescent gene expression

Sub-cultured transconjugant strains, without antibiotic pressure, maintained resistance to chloramphenicol for 9 consecutive days. Failure to detect fluorescent cultures on agar plates and when added to enriched live feeds could be due to initial low levels or rapid degradation of the protein product. Regulation of gene expression is a complex process involving transcription factors and translational and post-translational effects (Feiss, 1996). Transcriptional control of gene expression is largely regulated by gene copy number, the

nature of the promoter, and the stability of resultant mRNA (Feiss, 1996). If the results were explained solely by gene copy number, chromosomal insertion would not be expected to produce different expression result because the TnsD-mediated pathway results in exactly one gene copy (Waddell & Craig, 1988), hence it seems likely that the promoter may be the culprit. In favorable conditions, one gene copy has been demonstrated to be enough to detect fluorescence in transconjugants created with this system (Bao et al. 1991; Pollock et al. 2015). The nature of the promoter is a powerful determinant of gene expression. Promoters regulate the amount of mRNA produced and the interaction of the RNA polymerase with a bacterial promoter is governed by the ability of the σ (sigma) protein subunit of the RNA polymerase to recognize the initiation site (Bott, 2014; Feiss, 1996). The strength of this interaction can be governed by host-encoded factors called transcription factors. One possibility is that the *V. alginoltyicus* host does not produce certain transcription factors that are required for expression (Stretton et al. 1998) or that the plasmid DNA is missing important sequences resulting in weak promoter strength thus yielding low levels of mRNA from which the Tn7 genes are transcribed into protein. The mini-Tn7 delivery plasmids constructed by Lambertsen and co-workers (2004) have one of two possible promoters: a growth dependent ribosomal promoter ($P_{rrnB\ p1}$) or a constitutive growth-independent promoter ($P_{A1/063/04}$). In either case, the fp gene and the chloramphenicol resistance gene sit on the same operon and, by definition, are transcribed into a single mRNA regulated by the same promoter. However, genes (e.g., antibiotic resistance genes) which can be expressed from lower product yields may produce sufficient protein to mediate antibiotic resistance, even with weak promoters. Less useful genes, such as those encoding a fluorescent protein, may require higher expression levels. It is possible that the alternative growth-dependent ribosomal promoter would have resulted in higher mRNA yields. High rates of protein synthesis in fast-growing *V. natriegens* are attributed to strong rrn P1 promoters leading to a

high capacity of rRNA transcription (Bao et al. 1991). Importantly, the rrn P1 promoter in *V. natriegens* is activated by UP elements similar to those present in *E.coli* (Aiyar et al. 2002; Bao et al. 1991). This promoter was used previously to successfully GFP-tag *V. coralliilyticus* using the mini-Tn7 delivery system (Pollock et al. 2015). The lac-derived promoter was used in this study due to its availability in a range of colors, which is important due to varying auto-fluorescence ranges of corals and reasonable expectation that it would work in a *Vibrio*, based on previously successful fluorescent labelling (Dong et al. 2019; Pollock et al. 2015). Furthermore, the use of the growth-independent promoter ($P_{A1/063/04}$) resulted in higher fp expression levels than the growth-dependent ribosomal promoter in a *Pseudomonas* sp., highlighting the additional influence of host transcription factors in gene expression (Lambertsen et al. 2004).

Lastly, the lack of detectable fluorescence signal could be attributed to a post-transcription effect such as stability of the mRNA or degradation of the fluorescent protein. Wild-type *gfp* is a very stable protein yet unstable variants are often used (i.e., *gfpASV, gfpAGA, gfpAAV*) in gene expression studies (Andersen et al. 1998; Lambertsen et al. 2004; Prasher, 1992). Proteins encoded from variant *gfp* genes degrade quickly due to intracellular mechanisms described by Andersen et al. 1998). For the purposes of this experiment, stable versions of fluorescent protein genes (*eyfp-a*, *gfp2*) were selected to enable real-time observations over consecutive days without losing fluorescent signals. However, regardless of the gene version, natural proteases within bacterial species may convert a stable protein product into an unstable one (Andersen et al. 1998).

3.5 Conclusion

Fluorescent labelling techniques have the potential to uncover important dynamics in the establishment and maintenance of host-microbe relationships within corals yet developing techniques for different bacterial species is time and resource intensive. Plasmid inserted fluorescent protein genes have been sufficient for short-term studies (Dubert et al. 2016; Goulden, Hall, Bourne, et al. 2012; Wada et al. 2016), which are suitable to understand infection cycles in larval aquaculture species only requiring stability for a few days. However, to visualize the establishment and retention of beneficial bacteria within a coral host, real-time observations over several days may not be sufficient to fully understand the underlying mechanisms or to observe a beneficial function. A better understanding of host factors that could limit efficacy of such protocols and pre-screening genomic DNA for sequences known to interfere with the insertion of various transposable elements could improve success of chromosomal tagging. Vibrios are good for a proof-of-concept due to their ubiquitous presence in marine environments, and ability to be cultured and isolated on selective TCBS media. However, the high levels of mobile DNA and an ability of broad host range plasmids to replicate within *Vibrio* cells may reduce the selective pressure required for the transposition of Tn7 onto to a *Vibrio* host chromosome. Although the *V. alginolyticus* transconjugants described here cannot be used to visualize acquisition pathways, the results from the study illustrate the importance of using different delivery systems to increase chances of successful fluorescent labelling. The transconjugants developed here can still be useful in selecting a vehicle of administration for putative probiotics. The transconjugant *Vibrio alginolyticus* strains could be used to incubate live zooplankton feeds (e.g., *Artemia*, rotifers, copepods) to help develop coral probiotic delivery strategies using the chloramphenicol resistance phenotype as a marker.

Chapter 4 -

General Discussion and Conclusion

4 Chapter 4

4.1 Introduction

The research within this thesis develops methods to advance nutritional strategies and probiotic deployment for aquaculture corals. This work extended upon previous coral feeding experiments which showed variation in the ability of corals to capture, ingest, digest and assimilate useful nutrients from feeds (Ferrier-Pagès et al. 2011; Hoogenboom et al. 2015; Osinga, Schutter, et al. 2012). Specifically, this thesis improved assessment methods which directly detect and measure captured and ingested prey items using visual aids. The prey incubation, delivery and fluorescence microscopy procedures developed in chapter 2 to track *Artemia* into coral gastric cavities can be applied to fate-tracking coral probiotic candidates into a specific niche within a coral microhabitat. Overall, this research furthered our understanding of feeding in diverse aquarium reared corals which will help address the nutritional hurdles limiting the expansion of coral aquaculture for the purpose of reef restoration and more sustainable practices for the marine ornamental industry.

Corals possess diverse feeding strategies, physiologies, and varying degrees of reliance on autotrophic and heterotrophic nutrient acquisition pathways (summarised in Chapter 1). Subsequently, chapter 2 demonstrated the diverse feeding strategies and heterotrophy rates for corals with different physiologies and macro-morphologies. Taking this diversity into consideration suggests that a one-size-fits all approach to nutritional supplementation will not fulfill the needs of all aquaculture coral species. Different corals will vary in their ability to capture prey or show proclivities to one prey over another. *Artemia salina* nauplii are offered to an estimated 85% of cultured marine organisms (Kumar & Babu, 2015), are commercially available and are readily harvested from cysts at high stock concentrations (200,000 to

300,000 nauplii per 1 gram of cysts). Furthermore, they are hardy and thus easy to fix and preserve (Wiebe et al. 2017). For these reasons, *Artemia* was the logical starting point for coral feeding studies.

Artemia instar I nauplii are non-feeding but possess protein and lipid packed yolk-sacs whereas *Artemia* instar II nauplii, are feeding, and able to be enriched with beneficial nutrients (Kumar & Babu, 2015). *Artemia* are promising delivery vehicles for the administration of beneficial bacteria to corals due to their palatability by many coral species, as demonstrated in the results from chapter 2. Enriched *Artemia* could deliver both beneficial bacteria and nutrients to corals imparting microbial and energetic health benefits including increased lipid stores and constituent fatty acids. Despite the characteristics which make *Artemia* an attractive option for supplementing coral diets, *Artemia* are not found in coral reef environments and therefore not a natural food source for corals, which implies that their nutritional profiles may not be sufficient to supplement a corals heterotrophic diet (Houlbrèque, Tambutté, Allemand, et al. 2004). Studies highlight that the lipid and fatty acid profiles of coral held in aquarium conditions and fed *Artemia salina* are different to the profiles of corals in natural systems (Brodnicke et al. 2019; Conlan et al. 2017). Alternate formulated diets should therefore also be explored in future studies.

Alternative, culturable live prey options to augment coral health include copepods and rotifers. Copepods make up a significant proportion of zooplankton assemblages (80%) and may even be preferentially consumed by certain reef corals (Smith et al. 2016). In other aquaculture systems such as ornamental reef fish species, cultured copepod species like

Parvocalanus crassirotris, are important for the nutritional supplementation of larva stages and have been shown to be preferentially consumed by wild *G. fascicularis* corals (Alajmi, 2015; Costa et al. 2016; Evjemo & Olsen, 1997; Smith et al. 2016). However, copepods easily evade the coral species that are poorly adapted predators, such as *Acropora* spp. (Heidelberg et al. 1997) and are difficult to culture or harvest in high densities as compared to *Artemia* (2 to 5 ind./mL versus > 500 ind./mL for *P. crassirotris* and *Artemia salina,* respectively) (Alajmi, 2015). Cultured rotifer species, *Brachionus plicatilis*, can be harvested in high densities and are able to be enriched at a smaller size than *Artemia salina*. Assessing the ingestion ability of different groupings of corals on these additional live feed species could further improve feeding regimes for captive corals.

Grouping species together by shared physiological characteristics, such as polyp size ranges, would be an appropriate place to start for catering to the needs of different corals and developing appropriate nutritional supplementation regimes. In this study, polyp size served as an effective predictor of relative feeding ability but is certainly not the only physiological trait to be considered. Future studies could expand the protocols established herein to assess feeding abilities and prey preferences using different diets marked with fluorescent beads of different colors. Selecting a trait could help cater to the specific needs of different corals in terms of the types of feeds utilized and delivered feed densities. In this way, aquaculture facilities may need to organize tanks and therefore species assemblages based upon similar nutritional requirements.

4.2 Visualization strategies for development of coral probiotics delivery

Fluorescent labelling of putatively beneficial bacteria, delivered to corals through feeds, could yield useful insights into the mechanisms of an observed health benefit. In chapter 3, *V. alginolyticus*, previously isolated from coral, was used as a proof of concept to develop fluorescence to track their potential delivery through live feeds. *Vibrio* species have previous known gastrovascular associations in marine invertebrates and hence represented a good model species to develop these approaches (Dubert et al. 2016). Fluorescent tagging has helped uncover important mechanisms of infectious bacteria in aquaculture larval clams (Dubert et al. 2016), catfish (Evenhuis et al. 2013) and lobster larvae (Goulden, Hall, Bourne, et al. 2012). Real-time visualization allowed for the identification of *Artemia* feeds as the proximate cause of gastrovascular infection in larval lobsters and larval clams (Dubert et al. 2016). Given the role of live feeds as vectors, enriching the feeds with beneficial bacteria represents one viable way to administer bacteria and improve host health.

Fluorescent labelling techniques have the potential to uncover important dynamics in the establishment and maintenance of host-microbe relationships within corals, yet developing techniques for different bacterial species is time and resource intensive. In chapter 3 a novel approach to fluorescently tag coral isolated *V. alginolyticus* strains was taken to utilize different colored fluorescent gene markers to mitigate potential auto-fluorescent interference between the bacteria and coral tissue as well as to compare the efficacy of delivery pathways (e.g., different zooplankton vectors and water immersion). However, fluorescence was not observed in the transconjugant strains, despite resistance to the antibiotic chloramphenicol carried on a delivery plasmid that also contained the fp genes. A better understanding of host

factors that could limit efficacy of such protocols and pre-screening genomic DNA for sequences known to interfere with the insertion of transposable elements, could improve success of chromosomal tagging.

To develop fluorescent transconjugants of coral isolated bacteria, I propose two future strategies to accomplish chromosomal labelling. For labelling with the mini-Tn7 delivery system, probiotic candidates should be screened for changes in the *attTn7* insertion site to gain a better understanding of any conjugative plasmids commonly associated with that species which may interfere with chromosomal integration. For labelling *Vibrio* species, plasmids specifically isolated from or able to replicate in *Vibrio* species should be used and this may include the pVSV102 derived delivery plasmids. For example, the approach used to GFP-tag pathogenic *V. harveyi* (Travers et al. 2008) and *V. owensii* (Goulden, Hall, Bourne, et al. 2012) to study the infection cycles in host marine invertebrates represents a good potential way forward. The use of a growth rate dependent promoter with the mini-Tn7 conjugation protocol led to visible fluorescence in GFP-labelled *V. coralliilyticus* cells, isolated from *Pocillopora damicornis* (Pollock et al. 2015). Trialing different chromosomal tagging systems such as the suicide plasmid delivery system is another option. Plasmids carrying a bacteriophage Mu property, such as the RP4 plasmid have successfully delivered tn7 elements into a *Vibrio* species as well as other non-enteric bacteria (Akhverdyan et al. 2011; Parke, 1990; Van Gijsegem, 2018).

Vibrios are good for proof-of-concept for labeling efficacy due to their ubiquitous presence in marine environments, and ability to be cultured and isolated on selective TCBS media.

However, many *Vibrios* are opportunistic pathogens and therefore not suitable probiotic candidates. Furthermore, the high levels of mobile DNA and an ability of broad host range plasmids to replicate within *Vibrio* cells may reduce the selective pressure required for the transposition of tn7 onto a *Vibrio* host chromosome. Although the *V.alginolyticus* transconjugants described in chapter 3 cannot be used to visualize acquisition pathways, the results illustrate the importance of using different delivery systems to increase chances of successful fluorescent labelling.

The protocols used in chapters 2 and 3 of this thesis developed techniques which will streamline the application of fluorescent labelled coral probiotic candidates into real time observations of the acquisition and establishment of beneficial microbial communities associated with coral. Furthermore, tracking bacteria can help determine if these probiotics confer a beneficial function, purportedly linked to a specific coral microhabitat where their activity is needed and that they are maintained long enough to provide a health benefit to a host coral (Bourneet al. 2016; Lema et al. 2012).

4.3 Potential of diets to support coral health in aquaculture

Improving the health of coral in aquaculture through diet could significantly increase production and up-scale propagation to levels required for reef restoration and is one proposed intervention by the Reef Restoration and Adaptation Program (Anthony et al. 2020). This research furthered our understanding of feeding in diverse aquarium reared corals and utilized a novel, more direct approach to quantify live prey ingestion rates.

Dissecting corals is a tedious task but the results tell us more about feeding than simple capture rate calculations. Direct assessment methods are required to develop enriched coral diets that could deliver supplemental nutrients and probiotic bacteria to aquaculture corals. The protocols established in this study can be used for future probiotic delivery work to see if we can inoculate a specific coral microhabitat where their benefit is most useful. The methods developed here can provide useful insights for application of live feeds and probiotics on a larger scale as a tool for reef restoration.

Appendix A

Chapter 2 - Supplemental Methods and Results

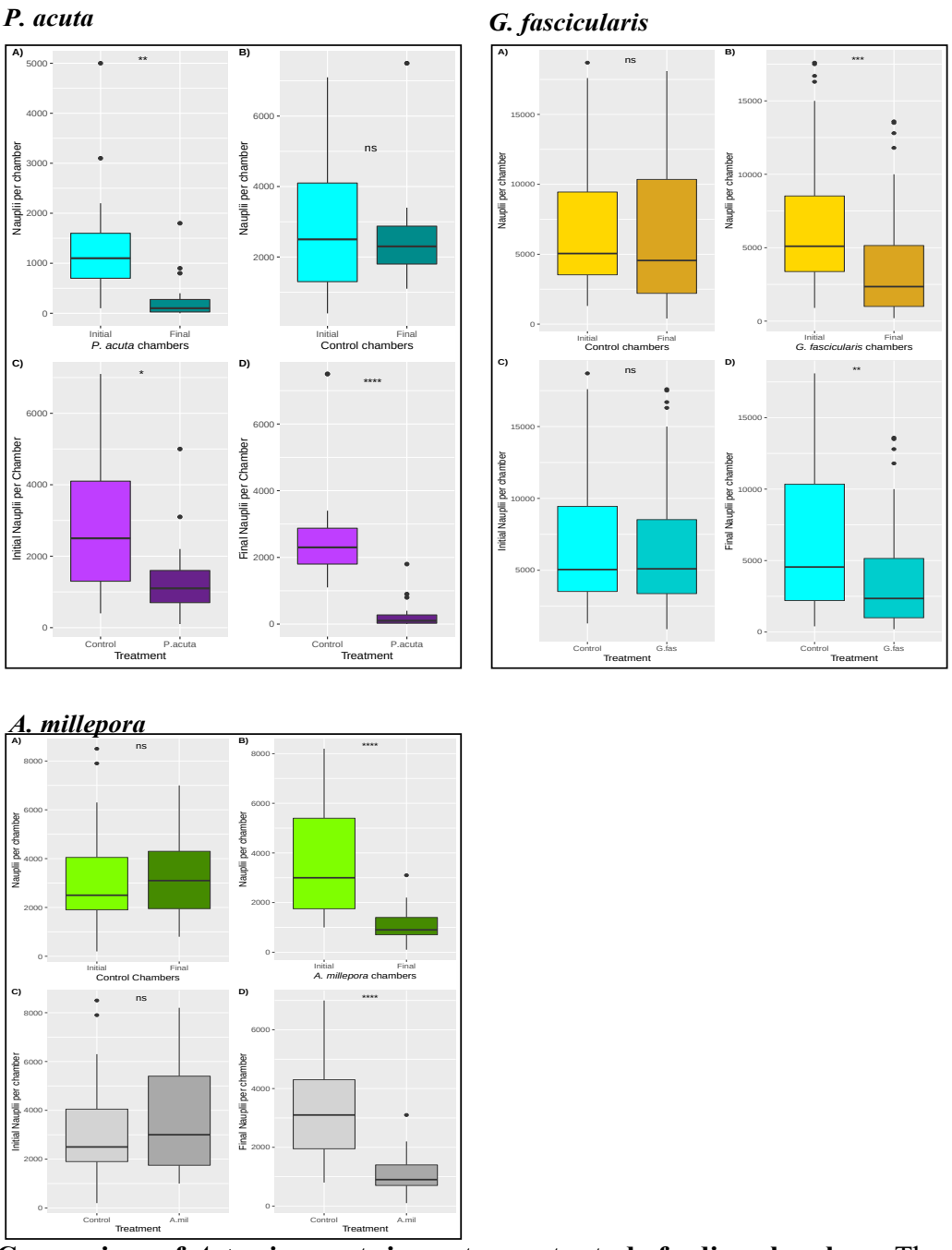


Figure S2-1 Comparison of *Artemia* counts in capture rate study feeding chambers. The box plots compare the initial and final number of *Artemia* nauplii (Instar I) per experimental feeding chambers (A) and per control feeding chambers (B) as well as compare the initial number of *Artemia* enumerated in control and experimental chambers (C) and the final number in control and experimental chambers (D) per each species.

Table S2-1 Best fit model equations per species for each experiment to best explain relationship between response variable (# of *Artemia* ingested by experimental corals) and explanatory variables.

	Description	Best-fit model equation
Capture Rate Experiment –		
A. millepora	linear model	capture rate ~ initial prey/polyp
P. acuta	generalized least squares model allowing for mean variance to increase with random effect:	gls(capture rate ~ initial prey/polyp)
G. fascicularis	generalized least squares model with a quadratic fit requiring heterogenous mean variances	gls(capture rate ~polyp(initial prey/polyp,3)+day*lev)
Ingestion Experiment – count regression models		
A. millepora	negative binomial generalized linear model	glm.nb(count ~ gen*chamber, data=AM)
P. acuta	generalized linear mixed-effects with negative binomial distribution	glmmTMB(prey count ~ genotype*chamber + (1|sample/tissue section), family=nbinom2, data=PA)

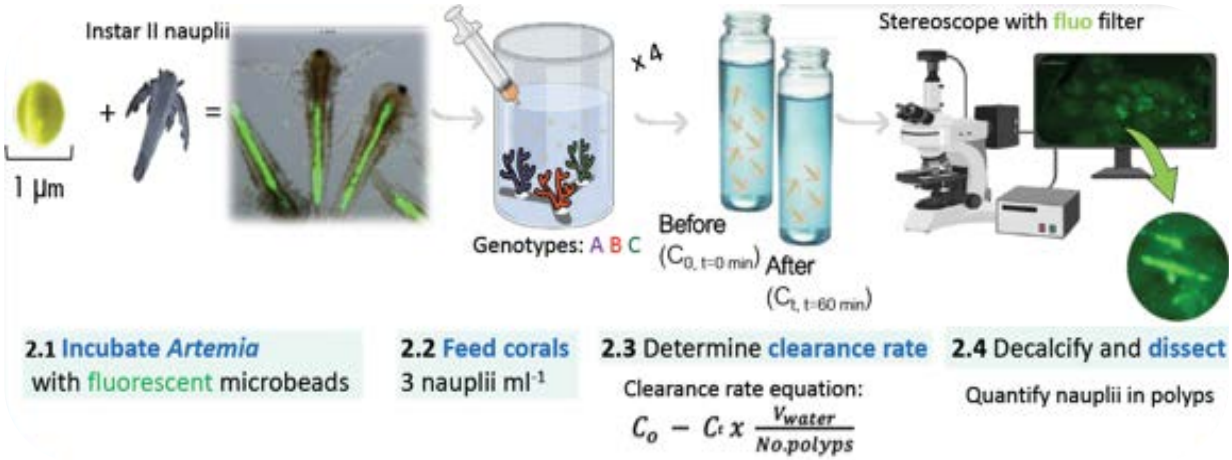


Figure S2-2 Experimental methods for Ingestion Rate experiment. *Artemia* instar II nauplii incubated in culture flasks with 1µm fluorescent microbeads, rinsed, counted and delivered to triplicate corals in 4 chambers per species. Sample aliquots (20 mL) were taken before and after 1 hour for capture rate calculations and compared results ingested prey detected in coral polyps via fluorescence microscopy.

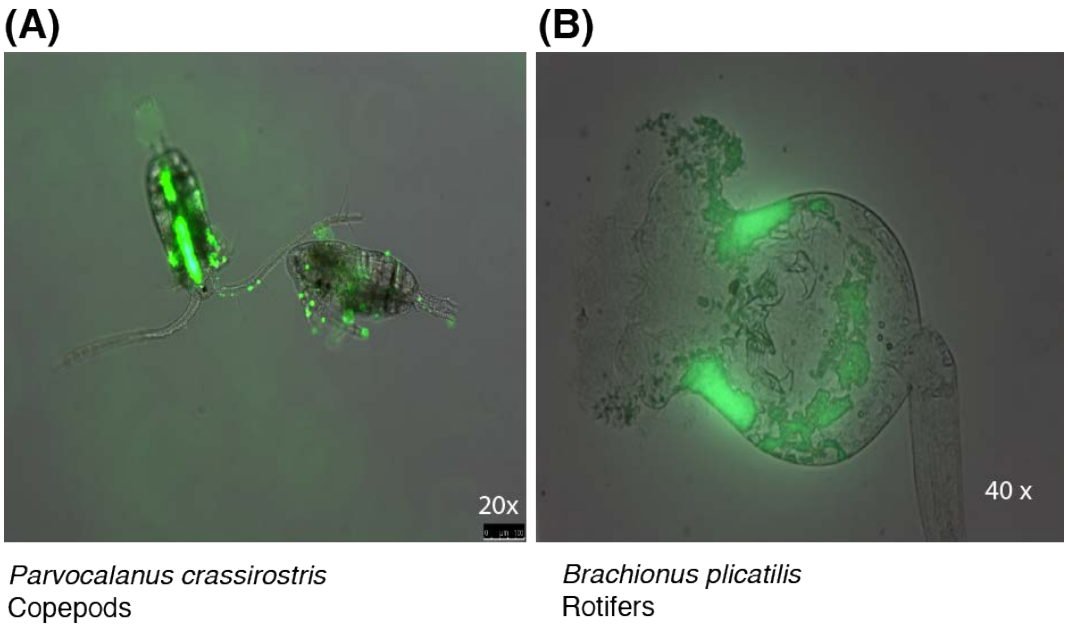


Figure S2-3 Copepods (A) and Rotifers (B) incubated with Yellow-Green fluorescent microbeads

Table S4-2 Two Sample T-test assuming unequal variances comparing mean feeding rates (ind./polyp/hour) per replicate chambers. *

	Capture I vs. Capture II		Capture I vs. Ingestion		Capture II vs. Ingestion	
A. millepora						
Mean	0.74	0.68	0.74	0.22	0.68	0.22
df	8		21		4	
t Stat	0.32		3.93		2.99	
P one-tail	0.38		0.0004***		0.020*	
P two-tail	0.754		0.001**		0.040*	
P. acuta						
Mean	0.233	1.198	0.233	3.478	1.198	3.48
df	4		3		5	
t Stat	-7.41		-14.84		-9.18	
P one-tail	0.001**		0.0003***		0.0001***	
P two-tail	0.002**		0.001**		0.0003***	
G. fascicularis						
Mean	29.27	59.25	29.27	75.45	59.25	75.45
df	3		3		6	
t Stat	-2.20		-3.15		-0.83	
P one-tail	0.058		0.026		0.219	
P two-tail	0.115		0.051		0.439	

* Capture rate I mean values are from the different trials and chambers as Capture rate II values. Ingestion rate values are derived from the same trials and chambers as Capture Rate II values, using different methods of assessment. Capture I results are derived from corals fed instar I *Artemia* nauplii in the first experiment whereas Capture II and Ingestion rate results are derived from corals fed instar II *Artemia* nauplii in the second experiment. Mean feeding rates included in this analysis were from initial prey densities of 3 ± 1.5 ind./mL.

Chapter 3 - Supplemental Methods and Results

Key Terms Chapter 3

Conjugation: A mode of gene transfer in bacteria involving direct cell-to-cell contact (Bott, 2014; Feiss, 1996) and is common in Gram-negative bacteria due to the ubiquitous presence of pili, long hair like surface appendages, which help form a mating pair.

Plasmid: A circular extrachromosomal DNA, that can replicate independently of a bacterial host

Probiotic: live microorganisms which when administered in adequate amounts confer a health benefit on the host

Fluorescent tagging: The transfer of a gene from an organism, whose genome contains a fluorescent protein () gene, into an unrelated organism.

Transconjugants: Recombinant bacterial cells containing DNA with certain plasmid encoded genes.

Transposon: a genetic element that moves from one DNA molecule to another

Transposases: Enzymes encoded by the transposon that activate and carry out a transposition pathway which results in the movement of genes from the transposon onto the host's DNA.

4.3.1 Supplementary Methods Chapter 3

Day 1: Prepare liquid cultures w/respective antibiotics and inoculate selective growth media (TCBS Cm15) with each individual strain to make sure growth is inhibited

Day 2: Wash 1 mL of each culture (4650 RPM, 5 min) and resuspend pellet in 500 µL LB broth. Mix 400 µl of each of the three washed cultures into an Eppendorf tube and incubate at 30°C for 30 min (no shaking).
Prepare conjugation plates using two methods:
Spot: Place 75 µL of the donor/helper mix on LB agar. Add 25 µL of the recipient directly on top
Filter: On a 0.2 µm polycarbonate filter, spot 75 µL of the donor/helper mix and add 25 µL of the recipient on top
Incubate the plates overnight at 30 °C without shaking.

Day 3: Inoculate selective growth media with growth from spot and filter conjugation plates. Incubate overnight at 30 °C.

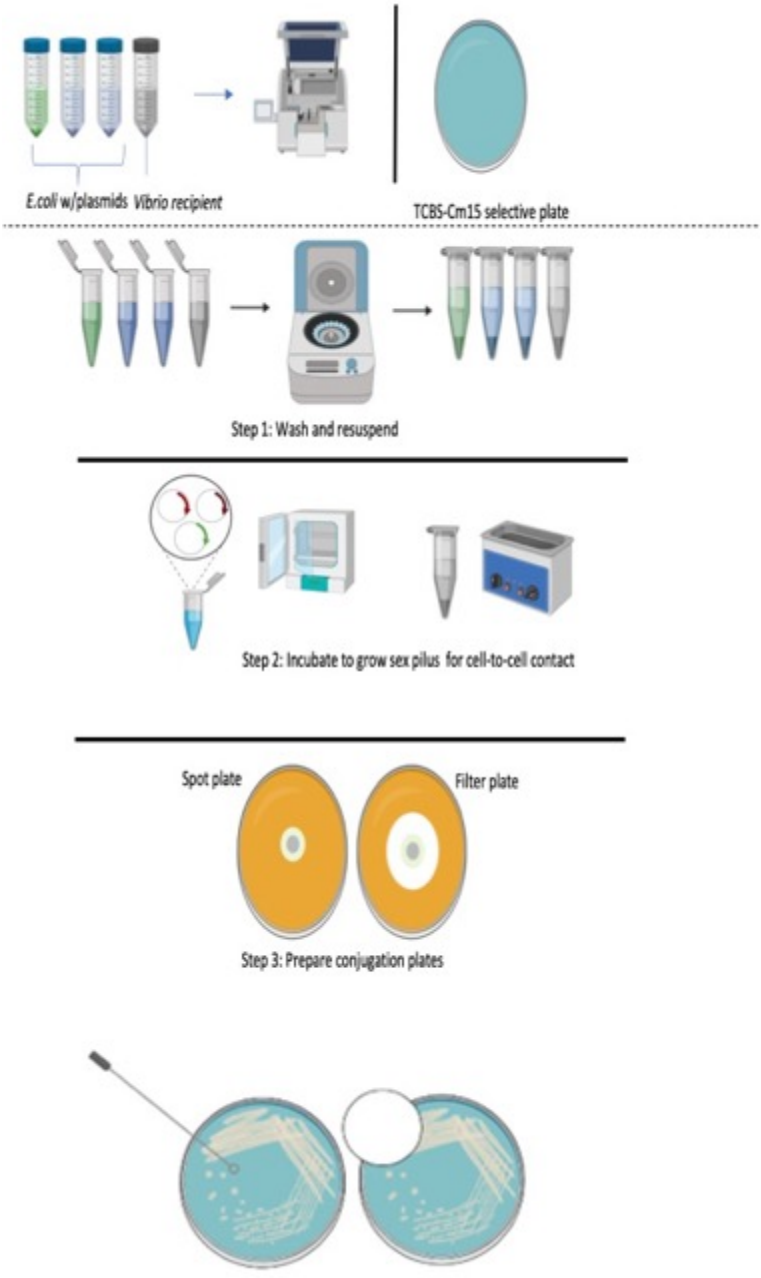


Figure S3-1 Mobilization of fluorescent genes via four-parental conjugation laboratory procedure.

4.3.2 Supplementary Results

Table S3-1 Transconjugant *V. alginolyticus* tnsD binding site sequences assembled to the type strain

Consensus attTn7-glmS *V. alginolyticus*	**CCACGTAACCTAGCGAAAGCGGTAACGGTTGAGTAA**
V. alginolyticus ATCC 17749	CCACGTAACCTAGCGAAAGCGGTAACGGTTGAGTAA
G1_*V. alginolyticus* AMM-P-12:	CCACGTAACCTAGCGAAAGCGGTAACGGTTGAGTAA
Y3_*V. alginolyticus* 090405_12:	CCACGTAACCTAGCGAAAGCGGTAACGGTTGAGTAA

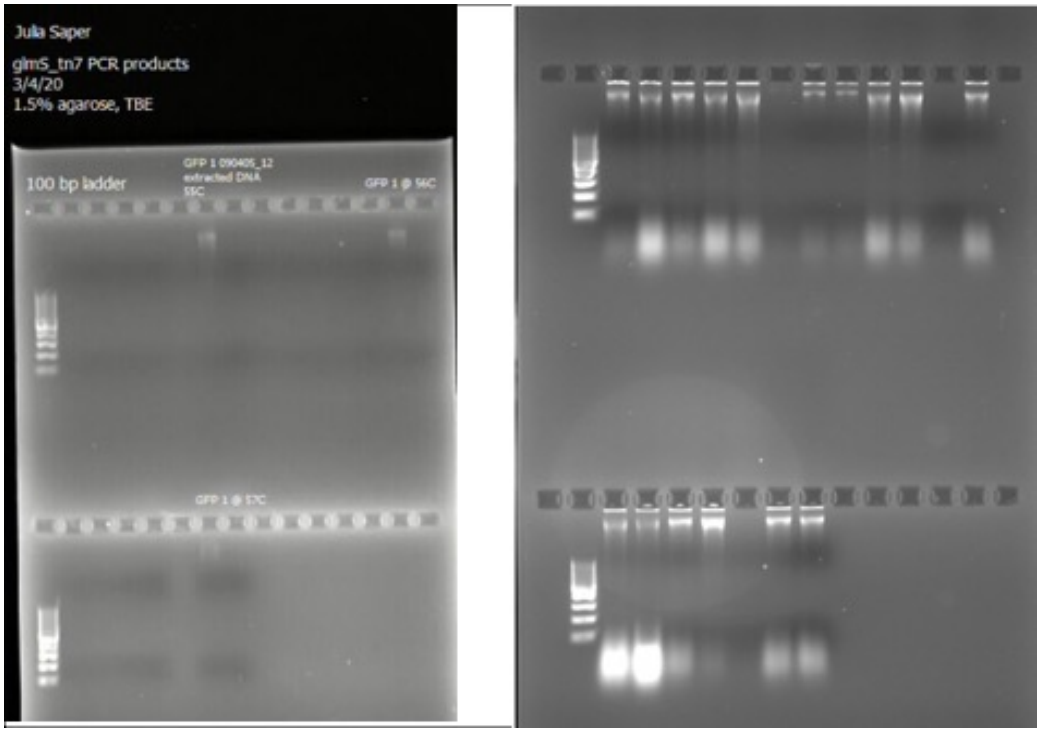


Figure S3-2 Gel Electrophoresis of Transconjugant DNA PCR products (*glmS*-tn7R).

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