Whole gut microbiome composition of damselfish and cardinalfish before and after reef settlement

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The Pomacentridae (damselfish) and Apogonidae (cardinalfish) are among the most common fish families on coral reefs and in the aquarium trade. Members of both families undergo a pelagic larvae phase prior to settlement on the reef, where adults play key roles in benthic habitat structuring and trophic interactions. Fish-associated microbial communities (microbiomes) significantly influence fish health and ecology, yet little is known of how microbiomes change with life stage. We quantified the taxonomic (16S rRNA gene) composition of whole gut microbiomes from ten species of damselfish and two species of cardinalfish from Lizard Island, Australia, focusing specifically on comparisons between pelagic larvae prior to settlement on the reef versus post-settlement juvenile and adult individuals. On average, microbiome phylogenetic diversity increased from pre- to post-settlement, and was unrelated to the microbial composition in the surrounding water column. However, this trend varied among species, suggesting stochasticity in fish microbiome assembly. Pre-settlement fish were enriched with bacteria of the Endozoicomonaceae, Shewanellaceae, and Fusobacteriaceae, whereas settled fish harbored higher abundances of Vibrionaceae and Pasteurellaceae. Several individual operational taxonomic units, including ones related to *Vibrio harveyi*, *Shewanella sp.*, and uncultured *Endozoicomonas* bacteria, were shared between both pre and post-settlement stages and may be of central importance in the intestinal niche across development. Richness of the core microbiome shared among pre-settlement fish was comparable to that of settled individuals, suggesting that changes in diversity with adulthood are due to the acquisition or loss of host-specific microbes. These results identify a key transition in microbiome structure across host life stage, suggesting changes in the functional contribution of microbiomes over development in two ecologically dominant reef fish families.

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14 **ABSTRACT**

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16 common fish families on coral reefs and in the aquarium trade. Members of both families
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26 column. However, this trend varied among species, suggesting stochasticity in fish
27 microbiome assembly.   Pre-settlement fish were enriched with bacteria of the
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30 taxonomic units, including ones related to *Vibrio harveyi*, *Shewanella sp.*, and uncultured
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34 individuals, suggesting that changes in diversity with adulthood are due to the acquisition
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39

40 **INTRODUCTION**

41   Animals harbor diverse microbial communities that influence key aspects of host

42 health, development, and behavior. In most vertebrates, the majority of microorganisms

43 inhabit the gastrointestinal (GI) tract at an abundance of potentially trillions of cells whose

44 collective genome may be orders of magnitude larger than that of the host (Whitman et al.

45 1998; Backhed et al. 2005; Gill et al. 2006).   A wealth of studies in both fish and

46 mammalian systems now confirm the importance of a gut microbiome to host health,

47 fitness, and development (Rawls et al. 2004, Bates et al. 2006, Chung et al. 2012; Lee &

48 Hase 2014), with alterations of microbiome composition affecting such properties as host

49 immunity, susceptibility to toxins, adiposity, efficiency of nutrient acquisition, behavior and

50 mood, and chemical signaling among conspecifics (Turnbaugh et al. 2006; Round &

51 Mazmanian 2009; Heijtz et al. 2011; Greenblum et al. 2012; Theis et al. 2013; Zheng et al.

52 2013, Wada-Katsumata et al. 2015).

53   Despite recognition of the important role of microbiomes in host ecology (Wong and

54 Rawls, 2012), microbiomes remain unexplored for the vast majority of ecologically relevant

55 taxa, including the most diverse of the vertebrate groups, the teleost fishes. Fish account

56 for almost half of all vertebrate species on earth and span a wide spectrum of habitats, host

57 ranges, physiologies, and ecological strategies. Studies of fish gut microbiomes have

58 focused primarily on commercial or model species, with most targeting only a small

59 number of host or microbial taxa (Roeselers et al. 2011; Clements et al. 2014; Llewellyn et

60 al. 2014). These studies raise the possibility of a core set of fish gut microbes shared across

61 diverse hosts, notably a dominance by bacteria of the Gammaproteobacteria and

62   Firmicutes, including unique fish-associated strains of the Vibrionales and Clostridia

63   (Spanggaard et al. 2000; Al-Harbi & Naim Uddin 2004; Martin-Antonio et al. 2007; Sullam

64   et al. 2012; Xing et al. 2013; Llewellyn et al. 2014). Significant variation in the fish gut

65   microbiome has also been reported, with changes in taxonomic composition shown to

66   affect host immunity, nutrient acquisition, and epithelial differentiation (Rawls et al. 2004;

67   Bates et al. 2006, 2007; Cheesman & Guillemin 2007; Cheesman et al. 2010; Kanther &

68   Rawls 2010, Ghanbari et al. 2015).   Fish microbiome composition has been linked to

69   diverse factors including host type (Ye et al. 2014; Givens et al. 2015, Hennersdorf et al.

70   2016), trophic ecology and diet (Bolnick et al. 2014a,b; Miyake et al. 2015; Sullam et al.

71   2015), and environmental conditions (e.g., salinity; Sullam et al. 2012; Schmidt et al. 2015).

72   The composition of the fish microbiome can also change significantly over an

73   individual's lifespan (Bakke et al. 2015; Burns et al. 2015, Llewellyn et al. 2015). However,

74   the timing, determinants, and magnitude of these shifts are unclear for most taxa, and likely

75   highly variable among species with differing life histories. On coral reefs, for example,

76   >90% of fish species undergo a dispersing or planktonic stage as either larvae or juveniles,

77   followed by an adult phase marked by settlement into a constrained territory on the reef

78   (Leis et al. 1991). For such species, microbiome structure is hypothesized to be shaped by

79   a combination of factors that co-associate with host life stage, including diet shifts, immune

80   system and GI tract development, and differences in the composition of the external

81   microbial community between pre- and post-settlement habitats.

82   Comparing reef fish microbiomes over developmental stages will help identify shifts

83   in the contribution of the microbiome to host health and behavior, thereby helping reach a

84   holistic understanding of reef ecology.   Collectively, coral reefs harbor over 2500 fish

85   species engaged in a complex web of interactions, including predation, herbivory,

86   corallivory, and symbiosis (Bellwood et al. 2004; Allen 2014). These interactions together

87   modulate material and energy transport and ecosystem structuring on coral reefs, with

88   important consequences for reef-building corals that rely on fish waste for nutrients, or

89   fish herbivory to limit competition with benthic macroalgae (Mumby et al. 2006; Burkepile

90   & Hay 2008; McCauley et al. 2010).   Such interactions may also be important vectors for

91   moving microbes between reef habitats. A handful of studies have characterized gut

92   microbiomes of coral reef fish, identifying a role for both diet type and host identity in

93   shaping microbiome composition (Smriga et al. 2010, Miyaki et al. 2015).   However, the

94   microbiomes of the vast majority of reef fishes, including some of the most abundant and

95   ecologically relevant taxa, remain uncharacterized.

96   Damselfish (Pomacentridae) and cardinalfish (Apogonidae) are among the most

97   diverse and abundant families of reef fishes and exhibit similar ecological strategies.

98   Species within these families occupy critical positions in reef food webs as generalist

99   planktivorous consumers, subsisting on a variety of algae, plankton, and benthic

100 invertebrates (Emery et al. 1973, Emery et al. 1980, Marnane and Bellwood, 2002). These

101 fish also constitute major fractions of the diet of larger piscivorous reef fish (Beukers-

102 Stewart and Jones, 2004). Most species of damselfish and cardinalfish spend their first 12

103 to 35 days of life as members of the off-reef plankton community before settling back onto

104 the reef as juveniles (Wellington and Victor, 1989, Leis et al. 1991, Leis et al. 1996). Post-

105 settlement, damselfishes (including members of the *Chromis*, *Pomacentrus*, and *Dascyllus*

106 genera) occupy distinct microhabitats using various types of coral for habitat and cover

107   (Wilson et al. 2008). Many Pomacentrids are highly territorial and influence turf algae

108   composition through grazing (Hinds, 1987, Klumpp and Polunin, 1989). Apogonids also

109   occupy coral microhabitats during the day when they are mostly inactive but leave coral

110   cover at night to feed throughout the water column (Gardiner and Jones, 2005). Fish

111   species of the Apogonidae often dominate nocturnal planktivore assemblages (Manane and

112   Bellwood, 2002). In this study, we quantified microbiome taxonomic composition (16S

113   rRNA gene diversity) in whole guts from replicate individuals from ten species of

114   damselfish and two species of cardinalfish (Table S1). We focused on comparisons

115   between pre-settlement planktonic larvae and post-settlement individuals (juveniles

116   through to adults) to determine whether microbiome composition varied between

117   ontogenetic stages and, if so, whether this variation was related to differences in the

118   microbial composition of the surrounding water column.

119   **MATERIALS AND METHODS**

120   **Sample collection**

121   Fishes were collected over a 3-4 day period from the fringing reefs surrounding

122   Lizard Island, Australia (14°40.08'S 145°27.34'E) in February 2014. Water depths at

123   collection sites were approximately 5-10 meters. Larval, pre-settlement individuals (8-16

124   mm total length, Table S1) were defined as samples collected from floating light traps

125   deployed overnight at a single site. These traps sampled the upper meter of the water

126   column above the reef. Post-settlement stage juveniles/adults (14-36 mm total length,

127   Table S1) were individuals hand-collected on SCUBA at depth from nearby reefs using dip

128   nets. Post-settlement fish in this study were likely sexually immature (juveniles) and

129   recently settled, given their small size (sexual maturity in most Pomacentrids typically

130   occurs at lengths >60 mm, Schmale et al. 1986, Kavanaugh, 2000). Net capture of settled

131   fish followed mild sedation with a clove oil/ethanol mixture dispensed via spray bottle. All

132   fish were euthanized by immersion in a seawater-ice slurry combined with an overdose of

133   clove oil and stored in ethanol for further analysis. Water column samples (n=11) were

134   collected on SCUBA at the same depth and location of post-settlement fish collection sites

135   and the light trap site using sterile plastic 1L bottles. Bottles were first opened within the

136   water column to ensure collection at the desired location and prevent contamination.

137   Microbial biomass in the sampled water was concentrated onto 0.2 μm Polyvinylidene

138   Flouride (PVDF) filters through filtration. Filters were immediately placed in ethanol in

139   cryovials and stored at room temperature until processing. Fish were identified to the

140   lowest possible taxonomic unit, typically either genus or species, using Indo-Pacific reef

141   fish field guides based on morphology. In total, 49 pre-settlement individuals were

142   sampled (Table S1), including individuals of the damselfish *Pomacentrus moluccensis* (n=4),

143   *P. chrysurus* (n=4), *P. nagasakiensis* (n=4), *P. amboinensis* (n=4), *P. wardii* (n=4), *P.*

144   *bankanensis* (n=4), *P. coelestis* (n=4), *Dascyllus aruanus* (n=3) and an unidentified Chromis

145   species (n=7). Two species of pre-settlement stage cardinalfish, *Ostorhinchus doederleini*

146   (n=4) and an unidentified Apogon species (n=7), were also collected.   A total of 24 post-

147   settlement individuals were sampled (Table S1), including individuals of 5 species of

148   damselfish (*P. moluccensis* (n=6), *P. chrysurus* (n=3), *A. polyacanthus* (n=3), *Dascyllus*

149   *aruanus* (n=5) and an unidentified Chromis sp. (n=3)) and 1 cardinalfish (*O. doederleini*

150   (n=4)). Note that the individuals of the unidentified *Chromis* species (collected both pre

151   and post-settlement) were almost certainly either *Chromis viridis* or *Chromis atripectoralis*,

152   both of which are similar ecologically and closely related. All research was reviewed and

153   conducted under the guidelines of Georgia Tech IACUC #A14063 and Great Barrier Reef

154   Marine Park Authority collection permits G13.36166.1 and animal ethics permits A1920.

155   These methods were approved by Georgia Tech IACUC and Great Barrier Reef Marine Park

156   Authority for use in this study.

157   **DNA extractions**

158   Whole fish samples were removed from storage containers and rinsed thoroughly

159   with fresh, filter-sterilized ethanol to remove (potentially) surface-attached microbes. As

160   dissection of only the intestinal contents was not possible due to the small size of most pre-

161   settlement individuals, whole gut contents (stomach + intestines) were excised by

162   dissection with a sterile razor from each individual. Excised contents were placed in a

163   sterile 1.5 ml centrifuge tube and frozen until extraction. Bulk DNA was extracted from gut

164   contents using the Qiagen DNeasy blood and tissue kit according to manufacturer

165   instructions. To process water samples, each collection filter was transferred from ethanol

166   into a clean, 1.5 ml centrifuge tube. The ethanol remaining in the original storage tube was

167   vacuum-filtered onto a new 0.2 μm PVDF filter, which was then dried at room temperature

168   and pooled with the initial collection filter. DNA was extracted from filters using a

169   phenol:chloroform method. Briefly, cells were lysed by adding lysozyme (2 mg in 40 μl of

170   lysis buffer per sample) directly to the pooled filters, capping, and incubating for 45 min at

171   37°C. Proteinase K (1 mg in 100 μl lysis buffer, with 100 μl 20% SDS) was added and

172   samples incubated for an additional 2 hours at 55°C. The lysate was removed, and DNA

173   was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with

174   chloroform:isoamyl alcohol (24:1). Extracted DNA was concentrated by spin dialysis using

175   Ultra-4 (100 kDa, Amicon) centrifugal filters and stored frozen.

PCR amplification and sequencing

177   High-throughput sequencing of dual-indexed PCR amplicons spanning the V3-V4

178   hypervariable regions of the 16S rRNA gene was used to assess gut microbiome taxonomic

179   composition. Amplicons were synthesized using Platinum® PCR SuperMix (Life

180   Technologies) with primers F515 (5'-GTGCCAGCMGCCGCGGTAA-3') and R806 (5'-

181   GGACTACHVGGGTWTCTAAT-3', Caporaso et al., 2011), with both primers modified to

182   include sample-specific barcodes and Illumina sequencing adapters according to Kozich et

183   al. (2013). Ten nanograms of starting DNA was used as template for each PCR reaction.

184   Amplification was performed using denaturation at 94°C (3 min), followed by 30 cycles of

185   denaturation at 94°C (45 sec), primer annealing at 55°C (45 sec), primer extension at 72°C

186   (90 sec), and a final extension at 72°C for 10 min. Amplicon products were verified using

187   gel electrophoresis, purified using Diffinity RapidTip2 PCR purification tips (Diffinity

188   Genomics, NY), and quantitated fluorometrically using the Qubit (Life Technologies).

189   Amplicons from different samples were pooled at equimolar concentrations and sequenced

190   on an Illumina MiSeq using a 500 cycle kit with 30% PhiX added to increase sequence

191   diversity. All raw sequences are available in the NCBI Sequence Read Archive under

192   BioProject ID PRJNA290348.

Sequence Analysis

194   Barcoded sequences were de-multiplexed and trimmed (length cutoff: 100 bp) and

195   filtered to remove low quality reads (cutoff: Phred score of 25, averaged over all bases)

196   using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/).

197   Paired-end reads were then merged using FLASH (Magoč & Salzberg 2011), with an

198   average read length of 250, and a fragment length of 300. Merged reads were analyzed

199   using the QIIME pipeline (Caporaso et al. 2010). In QIIME, chimeric sequences were

200   identified using USEARCH (Edgar, 2010) and removed from the input dataset. Merged,

201   non-chimeric sequences were clustered into Operational Taxonomic Units (OTUs) at 97%

202   sequence similarity using open-reference picking with the UCLUST algorithm (Edgar

203   2010). Singleton OTUs (those represented by only one sequence read in the combined

204   dataset) were also removed at this step. Taxonomy was assigned to each OTU by

205   comparison with the Greengenes database 13_5 release (DeSantis et al. 2006).

206   Cyanobacterial and chloroplast sequences were manually removed from the OTU tables as

207   these sequences can be assumed to reflect non-host-associated taxa, potentially those

208   brought into the gut through food passage. Selected OTUs were queried via BLASTN

209   against the NCBI-nr database to identify closest relatives.

210   OTU counts were rarefied (10 iterations) to 4262 sequences per sample and the

211   rarified OTU table was used in downstream analysis. A Bonferroni-corrected ANOVA was

212   used to identify microbial families significantly enriched in pre vs post-settlement

213   individuals, with family composition data expressed as percentage of total reads. Alpha

214   and beta diversity statistics for pre- and post-settlement individuals were calculated in

215   QIIME using the core_diversity_analyes.py command. The unweighted Unifrac metric was

216   used to conduct principal coordinates analysis to visualize beta diversity. Significant

217   differences in beta diversity were identified using a two-sided t-test (Bonferroni-

218   corrected). Core microbiome analysis was performed in QIIME using the

219   compute_core_microbiome.py script to identify individual OTUs shared by at least 70% of

220   all individuals per sample grouping (all pre-settlement individuals vs. all post-settlement

221   individuals). This script was also used to estimate the average number of OTUs shared

222   between random pairs of individuals from each life stage, based on 100 random iterations

223   per life stage. Indicator analysis was performed with the indicspecies package in R to

224   identify genera (indicator taxa) significantly enriched according to life stage, taking into

225   consideration both the extent to which an OTU is exclusive to a life stage (A component), as

226   well as the relative frequency at which the OTU occurs in all individuals within a stage (B

227   component)(Dufresne and Legendre 1997; Fortunato et al. 2013). Only those taxa

228   occurring in more than half of all samples (B component >0.5) and for which permutation

229   testing yielded p-values <0.05 are included in the output.   Indicator values range from 0 to

230   1 with higher values reflecting stronger relationships between indicator taxa and the tested

231   sample groupings (pre vs. post-settlement).

232   **RESULTS AND DISCUSSION**

233   **General microbiome characteristics**

234   A total of 49 pre-settlement and 24 post-settlement individuals representing ten

235   Pomacentridae species and two Apogonidae species were collected from Lizard Island,

236   Australia. Of these, five species - the pomacentrids *Pomacentrus moluccensis*, *P. chrysurus*,

237   *Dascillus aruanus*, and an unidentified *Chromis* sp. (either *C. viridis* or *C. atripectoralis*) and

238   the apogonid *Ostorhinchus doederleini* - were represented by both pre- and post-settlement

239   individuals, with the remaining species represented only in the pre-settlement sample set

240   (n = 6 species) or only in the post-settlement set (n = 1).

241   A total of 3,503,605 16S rRNA gene sequences were obtained after quality control

242   filtering (average: 41,709 per sample; range: 4262-238,858). After rarefaction, OTU counts

243   per sample were highly variable, from 44 to 557 OTUs, with averages of 163 (±59 standard

244   deviation (SD)) and 245 (±102 SD) for pre- and post-settlement stages, respectively. As

245   observed in other fish species (Spanggaard et al. 2000; Al-Harbi & Naim Uddin 2004;

246   Martin-Antonio et al. 2007; Sullam et al. 2012; Xing et al. 2013), Pomacentrid and Apogonid

247   gut microbiomes were dominated by Gammaproteobacteria of the

248   Pseudoaltermonadaceae, Endozoicimonaceae, Vibrionaceae, and Shewanellaceae, with

249   these Families constituting 80% (±10.3 SD) and 67% (±6.7 SD) of all sequence reads in pre-

250   and post-settlement fishes, respectively (Figure S1), although key differences in the relative

251   abundance of microbial families were also evident (discussed below, Table 1).

252   It is important to note that our data do not differentiate communities based on

253   location in the GI tract, as the small size of individuals prohibited separation of intestine

254   from stomach contents. Thus, we cannot distinguish between resident gut microbiome

255   members, which might be relatively more common in the intestine, versus members

256   derived from food contents or passively from seawater intake, which might be relatively

257   enriched in the stomach. Both host-adapted as well as transient microbiome members may

258   nonetheless exert effects on host health, as either symbionts or potentially as inocula for

259   resident populations.   However, gut microbiome composition (weighted Unifrac;

260   Pomacentridae + Apogonidae combined) of both pre- and post-settlement stages differed

261   significantly from that of the surrounding seawater (p=0.028; Figure 1), which was instead

262   enriched in Gammaproteobacteria of the Halomonadaceae family, Alphaproteobacteria,

263   and Bacteroidetes (Figure S2). Moreover, microbiome composition did not differ

264 significantly between water collection sites (Figure S3), suggesting fine-scale variation in

265 local environments alone should not influence microbiome composition. The microbiome

266 composition data also suggests minimal effect of source water on gut microbiome

267 structure.

Microbiome diversity and composition

269 Microbiomes showed a general trend toward greater complexity following

270 settlement, although this trend was variable among species. The phylogenetic composition

271 of the combined (Pomacentrid + Apogonid) microbiomes changed significantly from pre- to

272 post-settlement groups (weighted Unifrac metric; p=0.028), with pre-settlement

273 microbiomes showing less intra-individual variation compared to those of post-settlement

274 fish (Figure 1) and overall lower phylogenetic diversity (PD) (average: 20.3 ± 7 SD, versus

275 28.7 ± 12 SD post-settlement; p=0.001, t-stat = 3.5; Figure 2). Both pre- and post-

276 settlement microbiomes exhibited substantially lower PD than the surrounding water

277 column community (PD: 79.4 ± 23.5 SD; p=0.001). Of the five species for which both pre-

278 and post-settlement samples were available, three - *Chromis sp., O. doederleini,* and *D.*

279 *aruanus* - exhibited significantly higher PD post-settlement (Figure 3, p=0.001). Greater

280 microbiome diversity with age has been observed in other fish species, with suggested

281 linkages to increases in diet complexity (Bolnick et al. 2014a,b; Miyake et al. 2015; Sullam

282 et al. 2015), or potentially to differentiation of the GI tract into distinct niches with

283 development. In contrast, elevated microbiome diversity in early life was observed in *P.*

284 *chrysurus* and *P. moluccensis* (Figure 3, p=0.001), consistent with data from lab-reared

285 zebrafish (Stephens et al 2015). This pattern suggests that pre-settlement microbiomes in

286   these species are influenced primarily by transient associations with bacteria from the

287   exterior environment or by higher diet complexity at the planktonic stage. As an individual

288   matures, its microbiome may become more specialized in response to diverse factors,

289   including diet shifts, immune system development, physiological and chemical changes

290   along the GI tract, or decreased connectivity with the external environment. Indeed,

291   species-specific microhabitat usage (i.e., selection of distinct coral types by different

292   species) has been observed in some of the hosts examined here, including *P. moluccensis*

293   and *P. nagasakiensis*. However, all settled hosts in our dataset are assumed to be generalist

294   planktivores with fairly similar post-settlement diets based on prior work (Emery 1973,

295   Emery and Thresher, 1980, Marnane and Bellwood, 2002). Although this study does not

296   allow us to quantify the influence of each of the factors discussed above on developmental

297   shifts in microbiome composition, our data, combined with results from prior studies,

298   suggest that microbiome diversity is dynamic over life stages and assembly processes vary

299   on a per-species basis.

300   Key microbial taxa differed in abundance between pre- and post-settlement

301   microbiomes (Table 1), although microbiome composition was relatively consistent within

302   hosts per age group (Figure S3). At the microbial family level, pre-settlement microbiomes

303   were significantly enriched (>70-fold) in bacteria of the Endozoicimonaceae, while settled

304   fish harbored over 2-fold higher median abundances of the abundant Vibrionaceae and an

305   80-fold enrichment of planctomycetes of the Pirellulaceae (Table 1). Differences in the

306   relative abundance of microbes at the family level did not appear to be driven by a single

307   host. For example, all pre-settlement individuals were enriched in bacteria of the

308   Endozoicomonaceae compared to post-settlement hosts (Figure S4). At the microbial

309 genus level, both life stages were marked by sets of indicator taxa (Table 2). Indicator taxa

310 represent individual OTUs showing significant enrichment (as a percent of total sequence

311 reads) in one life stage vs. the other according to permutation testing (p<0.05, Dufresne

312 and Legendre 1997; Fortunato et al. 2013). The number of indicators was considerably

313 higher for settled fish (38) compared to larvae (5; Table 2), consistent with the increase in

314 diversity associated with post-settlement, perhaps reflective of an overall expansion in

315 microbial niche breadth as discussed above.   The marine bacterial genera *Kordia*

316 (Flavobacteriia) and *Halomonas* (Gammaproteobacteria, Oceanospirillales) were the

317 strongest indicators of pre-settlement stage (Table 2), followed by members of the

318 *Arcobacter* (Epsilonproteobacteria, Campylobacterales), *Oceanospirillum*

319 (Gammaproteobacteria, Oceanospirillales), and *Idiomarina* (Gammaproteobacteria,

320 Alteromonadales), all taxa common to the marine environment but not often observed in

321 fish microbiomes. Pre-settlement enrichment implicates these taxa as important during

322 early development, more successful at colonizing and growing in young fishes, or

323 commonly associated with pre-settlement food sources. Indicators of post-settlement

324 spanned a wide phylogenetic breadth, with a number classifiable only to the order or

325 family level, and the strongest indicators including diverse members of the

326 Gammaproteobacteria (Vibrionaceae, Portiera), Firmicutes (Epulopiscium),

327 Alphaproteobacteria (Kiloniellales, Hyphomicrobiaceae), Verrucomicrobia

328 (Coraliomargarita), and Planctomycetes (Pirellulaceae) (Table 2). Enrichment of these taxa

329 in fully developed, settled fish suggests the host GI tract as a specific niche for members of

330 these groups.

331 **Comparison of the core microbiome between pre and post-settlement individuals**

332   Although egg-tending behavior is common in many pomacentrids, fish are

333   presumed sterile at birth and fish gut microbiome is assumed to be inoculated primarily

334   from the environment and via food items early in life (Nayak 2010). If the pre-settlement,

335   pelagic diet is similar across species, it is reasonable to predict that larval individuals of

336   different fish species will share a greater number of microbial taxa (i.e., larger core

337   microbiome). The core microbiome might then be expected to shrink post-settlement as

338   species adapt to specific diets and microhabitats. We explored this prediction in two ways.

339   First, we determined the average number of OTUs shared between randomly sampled pairs

340   of pre-settlement individuals compared to post-settlement individuals. Contrary to our

341   expectation, the average count of shared OTUs did not vary with life stage (22.8 vs 23.2 for

342   pre- and post-settlement, respectively). Second, we compared the richness and

343   composition of the core microbiome between life stages, following normalization to a

344   uniform sample size (# of host individuals) per stage. Consistent with a recent analysis of

345   microbiomes from 15 coastal fish species (Givens et al. 2015), no single microbial OTU was

346   present in all individuals of either group (pre or post) in our study. Contrary to our

347   prediction, the richness of the core microbiome was similar for both pre and post-

348   settlement stages; for example, 17 OTUs were shared across 70% of pre-settlement

349   individuals, compared to 15 for post-settlement (Figure 4). Furthermore, many of the same

350   OTUs were present in the core microbiomes identified at each stage. For example, of the

351   OTUs occurring in 70% of total microbiome samples (pre- and post combined), 12 (of 16

352   total) were present in both the pre- and post-settlement core sets (70% threshold) when

353   evaluated separately (Figure 4), indicating conservation of a subset of key microbial

354   members across settlement. Together, these results suggest processes affecting assembly

355   and diversity of the core microbiome, when defined at the OTU level, are relatively constant

356   from pre- to post-settlement.

357   Many of these core OTUs were absent from or at much lower abundances in

358   seawater samples (with the exception of 3 OTUs), indicating selective accumulation in the

359   fish gut (Figure 4). Notably a single OTU with high sequence similarity to *Vibrio harveyi*

360   (Vibrionaceae, Genbank accession KX380754.1) comprised an average of 18-20% of the

361   total sequence reads in both pre- and post-settlement fish, but only 2% of the seawater

362   community (Figure 4). *Vibrios* are often described as opportunistic pathogens

363   (Karunasagar et al. 1994; Austin & Zhang 2006). For example, metagenome sequences

364   related to *Vibrio* species, including *V. harveyi*, from the guts of farmed adult turbot were

365   enriched in genes encoding potential virulence functions (Xing et al. 2013).   However, the

366   wide distribution of *V. harveyi* in healthy marine hosts, observed here and in other studies

367   of diverse invertebrates and fish (Onarheim et al. 1994; Guerrero-Ferreira et al. 2013;),

368   suggests that the primary niche of this bacterium may be commensal or even mutualistic

369   with the host, potentially with a role in protein degradation and digestion (Ray et al. 2012).

370   Interestingly, the Vibrionaceae family as a whole (multiple OTUs) was over twice as

371   abundant in post-settlement fish (Table 1) and therefore may be more typical of mature gut

372   microbiome communities.

373   The proportional abundance of other core microbiome taxa varied significantly

374   between life stages. The 16 taxa detected in 70% of all microbiome samples, a core set

375   dominated by OTUs belonging to the Gammaproteobacteria (Figure 4), constituted ~60%

376   of the total gut microbiome sequence reads in pre-settlement fish but only ~25% in settled

377 fish, indicating that these OTUs may be important early in life. Notably, OTUs closely

378 related to *Shewanella putrafaciens* (Genbank accession KP967510.1) and to an uncultured

379 bacterium of the family Endozoicomonaceae (Genbank accession LN626318.1) constituted

380 8 and 18% of the pre-settlement microbiome, respectively, but less than 1% of the post-

381 settlement community (Figure 4). To our knowledge, this is the first report of

382 Endozoicomonaceae occupying a large proportion of the fish gut microbiome. Bacteria of

383 this family, within the widely distributed Oceanospiralles group, exhibit diverse

384 heterotrophic metabolisms (Neave et al. 2014) and are commonly identified as symbionts

385 in marine invertebrates (Bayer et al. 2013a; Bayer et al. 2013b; Forget & Juniper 2013;

386 Nishijima et al. 2013; Beinart et al. 2014; Hyun et al. 2014), suggesting invertebrate prey as

387 a potential vector of transport into the fish microbiome. The functional properties and

388 potential for long-term residency of these bacteria in reef fishes remain to be verified. In

389 contrast, Gammaproteobacteria of the genus *Shewanella*,have been detected widely in fish.

390 Indeed, *Shewanella putrefaciens*, the species most closely related (97%) to the core OTU in

391 this study, has been used as a probiotic to increase growth and pathogen resistance in

392 juvenile sole (Lobo et al. 2014).

393   Other members of the core OTU set spanned diverse bacterial divisions, but

394 occurred at lower abundances (Figure 4). These included members of genera containing

395 known fish pathogens, such as *Photobacterium* (Chabrillon et al. 2005), *Pseudoalteromonas*

396 (Pujalte et al. 2004), *Pseudomonas* (Wakabayashi et al. 1996), and *Halomonas* (Austin

397 2005). However, these groups also contain potentially beneficial members, as certain

398 *Pseudoalteromonas* and *Alteromonas* strains can inhibit bacterial pathogens in fish

399 (Gatesoupe 1999) and *Photobacterium* species are known bioluminescent symbionts in

400   diverse fish hosts (Ruby & Nealson 1976; Ast et al. 2007). Core OTUs also included a

401   Chromatiales-affiliated taxon with 100% similarity to the uncultured gill symbiont of

402   *Ifremeria nautilei* (Beinart et al. 2013, Genbank accession KF780855.1), a member of the

403   *Kordia* genus resembling a heterotrophic isolate from the gut of marine polychaetes (Choi

404   et al. 2011, Genbank accession NR_117471.1), and *Salinivibrio costicola*, a halotolerant,

405   facultatively anaerobic bacterium first isolated from a hypersaline pond (Huang et al.

406   2000). Three OTUS matching marine Rhodobacteraceae, a broadly distributed aquatic

407   group that has also been detected in reef surgeonfish (Miyake et al. 2015), were also

408   prevalent across samples. However, two of these OTUs were more abundant in water

409   samples, suggesting these taxa may be transient members of the gut community. Together,

410   these data identify a relatively species-poor (e.g., 16 OTUs at 70%) but abundant (35-65%

411   of total 16S sequences) core microbiome across the damselfish and cardinalfish sample set,

412   including potentially pathogenic or beneficial bacteria, and a subset of core OTUs (12 of 16)

413   that persist across pre- and post-settlement life stages. Persistence over development may

414   be an important indicator of commensal or mutualistic taxa ubiquitous to reef fish.

415   **Conclusion**

416   These results describe a diverse gut microbiome in two abundant reef fish families

417   and highlight the importance of life stage in structuring microbiome composition. A trend

418   toward greater microbiome diversity in settled (older) individuals was observed in the

419   pooled dataset, potentially explained by the hypothesis that pre-settlement fish of diverse

420   species are more similar to each other physiologically compared to adults, occupy the same

421   niche (pelagic zone), and acquire gut microbes from a common environmental pool,

422   perhaps due to similarities in diet. These factors could homogenize the pre-settlement

423   microbiome across diverse host species, with microbiomes diversifying after fish settle on

424   the reef and transition to adult feeding roles. However, this pattern is not uniform across

425   host species, suggesting a need for discretion in concluding general trends in microbiome

426   succession over development. Our data also identify core microbes common to pre- and

427   post-settlement fish of several host species.   Persistence of these microbes over major life

428   transitions, regardless of variable trends in bulk microbiome complexity, may implicate

429   these microbes as particularly important to host health and physiology.   Differences in

430   microbiome assembly between pre and post-settlement fish are likely driven by a

431   combination of factors, including physiological changes associated with development,

432   changes in diet and potentially feeding frequency, and varying connectivity with microbes

433   from the external environment. More research is needed to disentangle the relative

434   contributions of these determinants over the settlement transition.

435   **Acknowledgements**

436   We thank the staff of Lizard Island Research Station for logistical support.

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889   **Table and Figures**

890   **Table 1.** Median % abundance of bacterial Families* in pre- versus post-settlement

891   damselfish and cardinalfish (pooled).

Family	Pre		Post		
	med	MAD	med	MAD	Fold
Vibrionaceae	18.12	14.20	41.91	28.69	2.31
Endozoicimonaceae**	11.02	9.50	0.14	0.14	76.40
Pseudoalteromonadaceae	1.28	1.15	0.45	0.42	2.83
Rhodobacteraceae	1.24	1.01	1.35	1.22	1.09
Flavobacteriaceae	1.07	0.89	0.61	0.60	1.75
Alteromonadaceae	0.71	0.63	0.85	0.75	1.20
Shewanellaceae	0.68	0.65	0.11	0.11	5.99
Oceanospirillaceae	0.56	0.51	0.15	0.15	3.74
Pseudomonadaceae	0.29	0.24	0.49	0.45	1.70
Moraxellaceae	0.26	0.23	0.02	0.02	10.31
Pirellulaceae**	0.01	0.01	0.83	0.81	81.97
Chromatiales, Unknown	0.10	0.08	0.21	0.20	2.03

*only includes Families with median abundance > 0.2% in either pre or post datasets
**significant change pre vs. post (P < 0.05, ANOVA, Bonferroni corrected)
MAD, median absolute deviation
Fold, fold increase in median (pre to post); underlined indicates post to pre

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894   **Table 2.** Indicator genera associated with pre- and post-settlement damselfish and

895   cardinalfish (pooled).

Indicator genera	Indicator for pre or post settlement fish	Indicator value	p-value
Kordia	Pre	0.943	0.001
Halomonas	Pre	0.896	0.001
Arcobacter	Pre	0.869	0.001
Oceanospirillum	Pre	0.823	0.001
Idiomarina	Pre	0.751	0.002
unclassified Vibrionaceae	Post	0.997	0.01
unclassified Pirellulaceae	Post	0.922	0.001
unclassified Kiloniellales	Post	0.904	0.001
unclassified Gammaproteobacteria	Post	0.9	0.001
Coraliomargarita	Post	0.863	0.001
Portiera	Post	0.857	0.001
Epulopiscium	Post	0.857	0.001
unclassified Hyphomicrobiaceae	Post	0.851	0.001
Verrucomicrobium	Post	0.816	0.001
unclassified Rhizobiales	Post	0.786	0.001
Rhodospirillaceae	Post	0.779	0.001
unclassified Acidimicrobiales OCS155	Post	0.778	0.001
unclassified Oceanospirillales	Post	0.771	0.001
unclassified Peptostreptococcaceae	Post	0.771	0.001
unclassified Myxococcales	Post	0.768	0.001
unclassified Altermonadales OM60	Post	0.763	0.013
unclassified Phyllobacteriaceae	Post	0.761	0.001
Ferrimonas	Post	0.75	0.001
unclassified Thiohalorhabdales	Post	0.746	0.001
unclassified Flavobacteriales	Post	0.743	0.001
unclassified Cryomorphaceae	Post	0.733	0.038
unclassified Altermonadales	Post	0.733	0.038
unclassified Myxococcales OM27	Post	0.728	0.001
Balneola	Post	0.724	0.001
Crocinitomix	Post	0.723	0.001
unclassified Flammeovirgaceae	Post	0.722	0.002
unclassified Altermonadaceae	Post	0.722	0.035
unclassified Francisellaceae	Post	0.712	0.001
Saprospria	Post	0.692	0.003
Clostridium	Post	0.692	0.016
unclassified Rickettsiales	Post	0.688	0.001
Turicibacter	Post	0.682	0.001
unclassified Deltaproteobacteria GMD14H09	Post	0.678	0.006
unclassified Pasteurellaceae	Post	0.676	0.002
unclassified Actinomycetales	Post	0.675	0.001
Altermonadaceae HTCC2207	Post	0.67	0.001
unclassified Comamonadaceae	Post	0.664	0.004
Flavobacterium	Post	0.656	0.003

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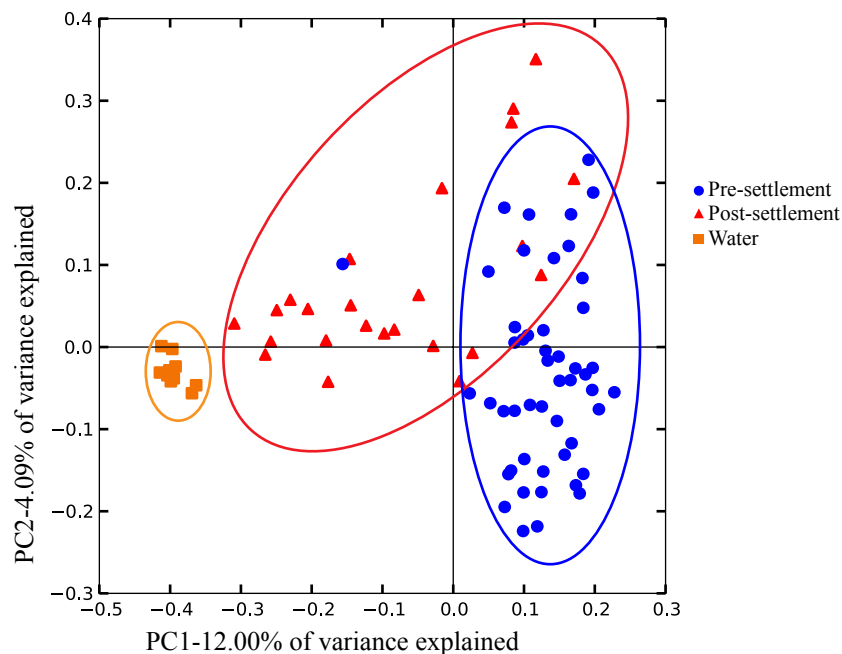
Figure 1. Compositional relatedness of microbiome samples. Principal coordinate analysis based on unweighted Unifrac distance with sequence data rarified to 4262 sequences per sample (49 pre-settlement, 24 post-settlement, 11 seawater samples). According to a two-sided t-test (Bonferroni-corrected), pre-settlement and post-settlement individuals are significantly different from one another ($p<0.05$) and both are significantly different from the water column ($p<0.05$) based on unweighted Unifrac distance.

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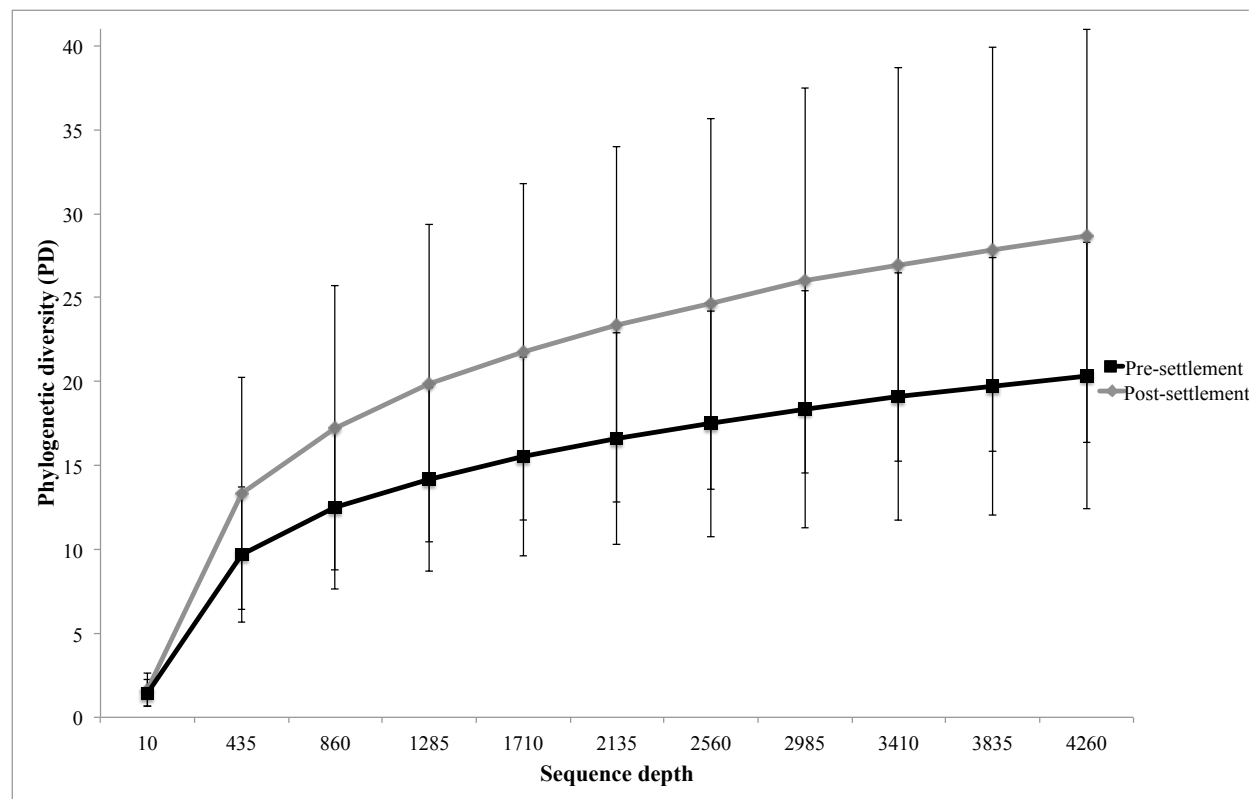
Figure 2. Microbiome phylogenetic diversity (PD) as a function of sequence depth in pre-

and post-settlement fish (all species combined, based on data rarified to 4262 sequences

per sample). Error bars are ± 1 standard error.

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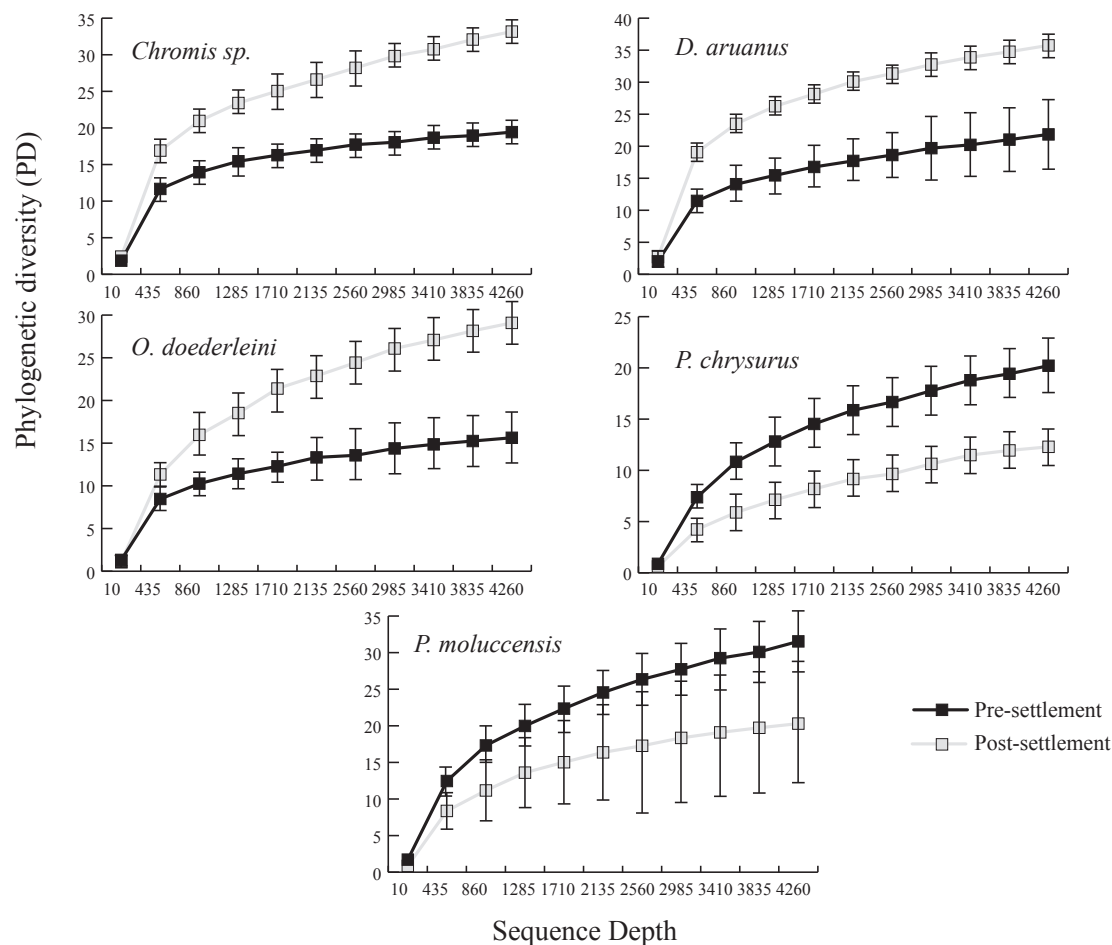
Figure 3. Microbiome phylogenetic diversity (PD) as a function of sequence depth in five species represented in both pre- and post-settlement datasets. Error bars are ± 1 standard error.

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Taxonomic classification

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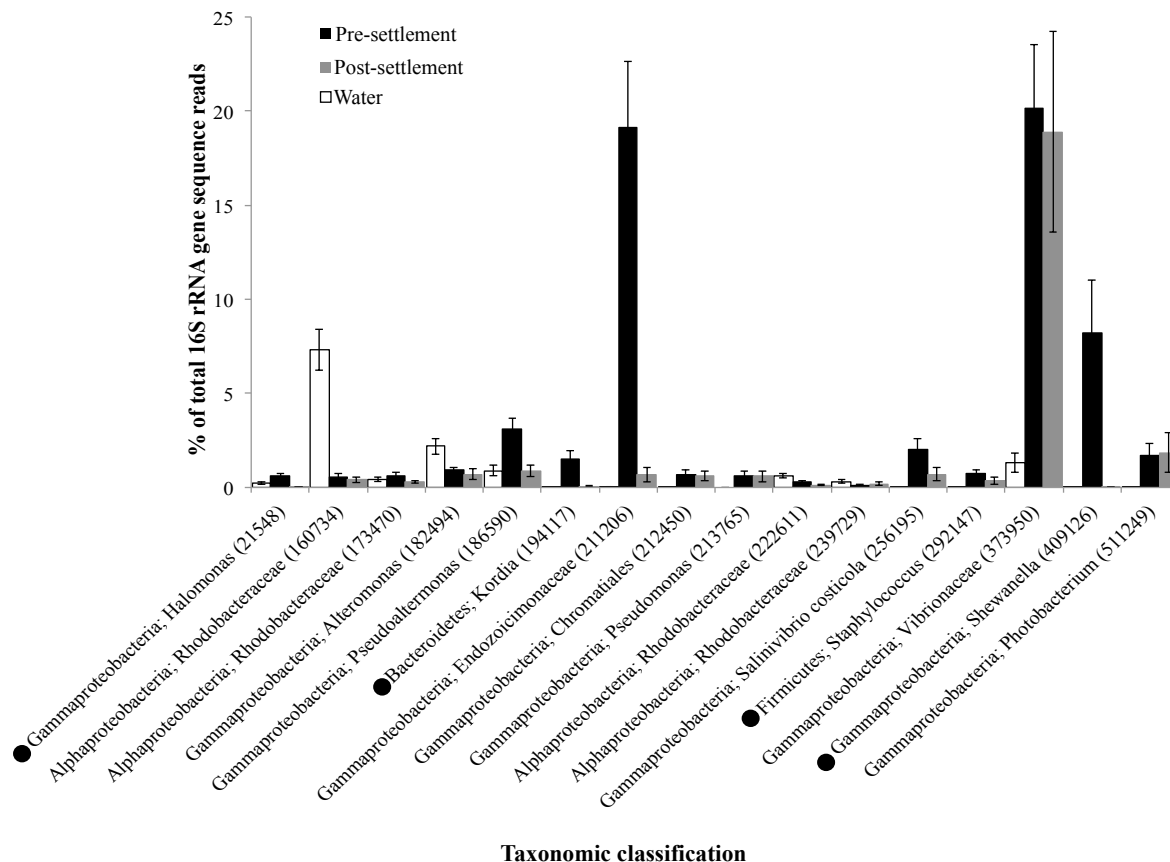
Figure 4. Taxonomic identity and relative abundance of OTUs detected in greater than 70%

of all fish microbiome samples (pre-settlement (n=49) and post-settlement (n=24)

samples) combined for analysis. ● indicates that an OTU was shared among 70% of the

pre-settlement samples when this sample set was evaluated independently, but was not

detected in >70% of samples in the post-settlement set when evaluated independently.

The relative abundances of these OTUs in the water column samples (n=11) are included

for comparison. Taxonomic classifications are at the level of genus whenever possible.

Error bars are ± 1 standard error.

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