Effects of tank cleaning frequency and sea cucumber co-culture on larval sablefish growth and survival, water quality, and microbial communities

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Abstract

The larval period is the most labor-intensive and highest-mortality stage in marine fish aquaculture, due in part to the need to maintain good water quality. Organic matter from feed, fish waste, and algae (greenwater) can promote bacterial proliferation, including pathogens, and deteriorate water quality. Frequent cleaning methods are often employed to mitigate these effects, but cleaning is labor intensive and may have negative effects such as stress and physical damage to larvae, and disruption of potentially probiotic biofilm communities. In this study, we compared three tank cleaning methods: high cleaning frequency, low cleaning frequency, and low cleaning frequency with sea cucumbers (*Parastichopus californicus*). We assessed water quality, microbial community composition and abundance, and growth and survival of larval sablefish (*Anoplopoma fimbria*). Sea cucumbers have been shown to extract organic content in other aquaculture systems, but co-culture with sea cucumbers caused 98% larval mortality and is not recommended for sablefish larviculture. High and low cleaning frequencies did not differ in the types or numbers of bacteria present in tank seawater, or dissolved oxygen, ammonia, and nitrite levels. Further, high and low cleaning frequencies did not differ in larval sablefish growth or survival, indicating that low cleaning frequencies can be used to reduce labor costs without negative effects on larvae.

Key Words: Larviculture, husbandry, siphon, water quality, frequency, microbiome

1. Introduction

High organic inputs can compromise tank hygiene in marine fish larviculture. Since larvae are weak predators with poor visual acuity, feed and algae must be frequently added to tanks in order to prevent starvation and create suitable visual environments for detecting prey (i.e., greenwater, Faulk and Holt, 2005; Lazo et al., 2000; Naas et al., 1992; Stuart and Drawbridge, 2011). Decaying larvae and feces provide additional organic material. Excess organic matter can reduce water quality by promoting the growth of pathogenic bacteria (e.g., *Salmonella*, *Vibrio*, and *Listeria* spp., Rajkowski, 2009) and increasing biochemical oxygen demand.

Manual cleaning of larviculture tanks is labor intensive. In flow-through systems with juvenile or adult fish, high flow rates are often used to flush excess feed and feces out of rearing tanks (Davidson and Summerfelt, 2004). However, in larval rearing tanks, flow rates are limited by poor larval swimming abilities and by the need to minimize flushing of expensive live feeds and greenwater. Effective methods to reduce organic matter in larval rearing tanks include mechanical self-cleaning tanks (e.g., Oceans Design, Colorado Springs, CO), clay as a turbidity agent (Attramadal et al., 2012; Lee et al., 2017; Lee et al., 2021), and passive larval transfer (Stuart et al., 2016), but manual tank cleaning is the most common method. Tank cleaning usually includes siphoning of organic debris from tank bottoms, scrubbing biofilms with pole-mounted scrub pads, and center-standpipe rinsing.

Tank cleaning can remove organic matter, but excessive tank cleaning can waste labor and potentially cause direct physical damage and stress to larvae, especially early in larval life. For

example, larvae can be accidentally removed during debris siphoning or crushed during wall scrubbing. Additionally, cleaning could disrupt beneficial aspects of biofilm communities that might include probiotics and organisms that reduce nitrogenous wastes (Baskaran et al., 2020; Gichana et al., 2018). Previous experiments revealed that sablefish rearing tank biofilm communities are diverse, vary temporally, and contain bacteria related to both common pathogens (e.g., *Colwelliaceae*, *Altermonadaceae*) and probiotics (e.g., *Rhodobacteraceae*) (Dodd et al., 2020; Pierce et al., 2019). The early stages of fish development are particularly sensitive to water quality and disease outbreak, partly due to an early window of microbiome colonization and succession (Llewellyn et al., 2014; Pierce et al., in prep). Frequent cleaning at this stage may remove many of the commensal bacteria necessary to maintain fish health. Intentional biofilm use has resulted in enhanced survival of larval fish and shellfish in aquaculture (Avendaño-Herrera and Riquelme, 2007; Mata et al., 2017).

This study focused on sablefish (*Anoplopoma fimbria*), an emerging species for aquaculture. As for most marine fish species, sablefish larviculture is characterized by high organic inputs and high labor requirements. Sablefish require greenwater in the first week after first feeding, and live rotifers before transitioning to live *Artemia* and dry prepared feeds (Cook et al., 2015; Lee et al., 2017; Lee et al., 2021). Traditional sablefish larviculture methods involve frequent siphoning, scrubbing, and center standpipe rinsing, starting at first feeding. We investigated two possible techniques to reduce tank-cleaning frequencies.

First, traditional tank cleaning methods (hereafter, “high frequency”) may be more frequent than necessary to maintain water quality. Rearing tanks are typically disinfected before stocking, and

there may be a time lag before water quality begins to deteriorate. Thus, lower cleaning frequencies in the earlier stages of larval rearing may come with no or low costs to water quality. Low frequencies during early stages may also be beneficial since young larvae are weak swimmers and thus more likely than older larvae to be unintentionally siphoned out of tanks or crushed against tank side walls. Even if water quality is reduced slightly due to reduced frequency, there may be a net benefit to larval growth and survival if larval stress and mechanical damage are sufficiently reduced.

Second, if low frequencies leave too much organic matter in rearing tanks, marine invertebrates could potentially help remove some of the organic matter without causing larval stress and physical damage, and without requiring extra human labor. Sea cucumbers are slow-moving omnivorous deposit feeders that can consume detritus, algae, feces, and uneaten food from tank bottoms and tank walls. While sea cucumbers produce their own waste products, they can extract organic content with net-positive effects (Ahlgren, 1998; Nelson et al., 2012; Palzat et al., 2008; Zamora et al., 2018). They also produce secondary metabolites that have been described as anti-inflammatory, anti-microbial, anti-predatory, anti-fouling and anti-tumor (for review see Kamyab et al., 2020), which may help to improve rearing tank water quality and total bacterial loads. We tested whether California sea cucumbers (*Parastichopus californicus*) could help to maintain tank water quality and improve larval growth and survival, in tanks with low cleaning frequencies.

In this study, we compared larval growth, larval survival, and water quality parameters among three cleaning methods from larval first feeding to weaning. Tanks were either 1) cleaned on our

114 traditional larval sablefish schedule ("high frequency"), 2) cleaned on a low frequency schedule,
115 or 3) cleaned on a low frequency schedule and supplemented with sea cucumbers.
116
117 **2. Methods**
118
119 *2.1. Spawning and production*
120 Broodstock spawning and larval production are detailed in Cook et al. (2015). Briefly, wild
121 broodstock were collected from the Pacific Ocean off the Washington coast, transported to the
122 Manchester Research Station, and artificially spawned. Embryos and yolk sac larvae were held at
123 5-6°C in the dark for about 50 days. Embryos hatched after 14 or 15 days and larval yolk sacs
124 were depleted about 49-50 days after the start of embryo incubation. At depletion, silo
125 temperatures were raised to 8°C, and larvae were stocked into experimental tanks.
126
127 *2.2. Tanks and cleaning*
128 Experimental tanks were 91 cm tall with 100 cm diameters, with center standpipes. Cutouts in
129 the center standpipe allowed water to exit the tank but the mesh on the standpipes was small
130 enough to prevent larvae from being flushed out. The mesh was 300 microns at stocking, 500
131 microns starting day 4, and 1000 microns starting on day 18. External standpipes maintained the
132 water height at 78 cm, for a total water volume of 610 L. Seawater was pumped from Puget
133 Sound, UV sterilized, sand filtered, and bag-filtered to 1.0 µm before being heated and delivered
134 to tanks. The flow rate to each tank was 1.4 liters per minute (LPM) at stocking and increased to
135 1.6 LPM on day 8 and 2.0 LPM on day 17. Water temperatures were 9°C at stocking, 12°C by
136 day four, and maintained at 14 - 15°C starting on day six.

137

138 Water turbidity was maintained following methods detailed in Lee et al. (2021). Briefly, starting
139 at stocking, peristaltic pumps metered *Nannochloropsis oculata* paste (Reed Mariculture,
140 Campbell, CA, USA) and green dye ("green shade color," Esco Foods, San Francisco, CA, USA)
141 into rearing tanks. Clay is superior to algae for sablefish larvae, as long as algae is used in the
142 first week after stocking (Lee et al., 2017; Lee et al., 2021). We gradually transitioned from
143 *Nannochloropsis* algae to clay between days 16 and 19. Kentucky Ball clay OM4 (Kentucky-
144 Tennessee Clay Company, Roswell, GA, USA) was mixed with water using a blender, mixed
145 with the green dye, and then distributed into rearing tanks. Clay continued to provide turbidity
146 until the end of the experiment on day 32.

147

148 Larvae were stocked into rearing tanks at a density of 7.2 larvae per L of tank water. From
149 stocking until day 23, larvae were fed rotifers (*Brachionis plicatilis*) three times per day at a
150 density of 10-15 rotifers per mL of tank water. *Artemia* (1-1.5 nauplii per mL) were fed to the
151 sablefish larvae three times per day from days 17 to 31. Dry prepared feed (Otohime^{TM}; Reed
152 Mariculture, Campbell, CA, USA) was offered *ad libitum* from days 26 to 32 (Table 1).

153

154 Tank cleaning consisted of four activities:

155

156 1) Siphoning involved using a vacuum siphon fitted with a 25-cm wide vacuum head to remove
157 debris from the tank bottom. Siphoning continued until the effluent ran clear (after
158 approximately five to ten liters of water was removed from each tank bottom). A bristle brush

159 insert in the vacuum head lightly scrubbed the tank bottom as the vacuum head was slowly
160 moved around.
161
162 2) Partial draining and scrubbing consisted of draining the tanks by 50% and using a pole-
163 mounted abrasive pad to scrub the exposed tank sides.
164
165 3) Lower scrubbing consisted of using the same pole-mounted abrasive pad to slowly scrub the
166 lower 50% of tank sides, below the water line.
167
168 4) Screen cleaning involved removing the external standpipe and draining until the water level
169 was lowered by four cm, then reinserting the external standpipe. Draining ensured that there
170 would be no water flow out of the tank during screen cleaning. After the reinsertion of the
171 external standpipe, the center internal standpipe was removed and quickly sprayed down with
172 pressurized water, before being immediately reinserted into the tank.
173
174 *2.3. Experimental design*
175 Twelve tanks were divided into three treatments (n=4 per treatment). The "high frequency"
176 treatment followed methods traditionally used by our laboratory (Table 1). In the high frequency
177 treatment, siphoning and partial draining and scrubbing of the upper exposed tank walls were
178 performed every third day for the first week, every other day for the second week, and then daily
179 until the end of the experiment. Lower scrubbing below the water line and screen cleaning were
180 done daily starting on days 16 and 3, respectively. For the "low frequency" treatment (Table 1),
181 siphoning was done every three days starting day 18, then daily starting day 27 (65% reduction

182 in siphoning relative to high). Partial draining and scrubbing were performed every other day
183 starting day 6, then daily starting day 18 (13% reduction relative to high). Scrubbing below the
184 water line never occurred (100% reduction relative to high), and screens were cleaned every
185 fourth day (76% reduction relative to high). The overall reduction across all activities was 62%.
186 Procedures for the "low frequency plus sea cucumber" treatment were exactly the same as the
187 low frequency treatment, except ten sea cucumbers were added to each tank on day 2. The
188 average weight of each sea cucumber was 339 grams. The experiment ended 32 days after larval
189 sablefish stocking.
190
191 *2.4. Weight and survival*
192 At the end of the experiment, all surviving fish in each tank were counted and group-weighed
193 while wet ("biomass," one group weight per tank). The biomass was divided by the number of
194 surviving larvae in the tank to calculate average wet weight per larva.
195
196 *2.5. Water quality sampling*
197 Dissolved oxygen (Hach HQ40D), ammonia (Hanna Instruments HI700, Nessler Method), and
198 nitrite levels (Hanna Instruments HI764) were measured immediately before stocking on day 1,
199 and every eight days starting on day 8, with the final sampling date on the final day of the
200 experiment (day 32).
201
202 *2.6. Microbial community sampling*
203 Prior to stocking and every seven days thereafter, 300 mL of tank seawater was sampled from
204 each tank using a vacuum filter with 0.22 µm polycarbonate filters (Millipore). On day 16, all

sampling was done prior to the start of clay addition. Tanks were then partially drained and tank surfaces were swabbed using a sterile polyester tipped applicator stored in a dry transport tube (Puritan Medical Products, LLC). Filters and swabs were stored at -20°C until processing for 16S rRNA gene amplicon sequencing. Tank seawater was also collected and preserved in 10% formalin (for a final concentration of 4% formalin) to assess total bacterial cell counts. Samples were held at 4°C until further processing.

2.7. DNA extractions & PCR

DNA from tank seawater samples was extracted using the DNeasy PowerWater Kit (Qiagen), and swab samples were extracted using the DNeasy PowerSoil Kit (Qiagen). Swab extractions were modified to enhance yield by reducing the volume of reagents used for the IRT steps. A targeted amplicon sequencing (TAS) protocol was employed as previously described by Green et al. (2015) with modifications to the 16S rRNA gene hypervariable region and polymerase used (Pierce et al., 2019). Briefly, the V3-V4 region of the 16S rRNA gene was amplified using the primers 341F (5'-ACGGGAGGCAGCAG-3') and 806R (5'-GGACTACH VGGGTWTCTAAT-3') plus CS1/CS2 linkers, followed by a second PCR to attach the Fluidigm sequencing primers. PCR conditions can be found in Green et al. (2015).

2.8. 16S rDNA amplicon sequencing

Libraries were prepared and sequenced on the Illumina MiSeq platform at the DNA Services Facility at the University of Illinois at Chicago (V3 chemistry, 2 x 300 paired-end sequencing). Raw sequencing reads are available for download from the NCBI Sequence Read Archive, BioProject accession number PRJNA594400.

228

229 Read pairs were trimmed, quality and length filtered, merged, chimera checked, denoised, and
230 assigned taxonomy using the DADA2 (v.1.12.1) package in R (v. 3.6.1) (Callahan *et al.* 2016).
231 Amplicon Sequence Variants (ASVs) were assigned taxonomy using the SILVA database (v.
232 132) (Quast et al., 2013).

233 Sequences were filtered to remove singletons and ASVs accounting for less than 0.005% of the
234 total abundance. Sequences identified as chloroplasts and mitochondria were removed prior to
235 analyses as well. Further analysis was done using the phyloseq (v. 1.28.0) and vegan (v. 2.5.5)
236 packages in R (McMurdie and Holmes, 2013).

237 *2.9. Cell counts*

238 For details on slide preparation, imaging, and processing, see Pierce et al. (2019). Briefly,
239 preserved water samples were diluted 1:100 with 1X phosphate buffered saline (PBS), filtered on
240 black polycarbonate track etched membrane filters (0.2 μm, 25 mm; GVS North America,
241 Sanford, ME, USA), which were stained with 5 μg/mL 4',6-diamidino-2-phenylindole (DAPI)
242 and mounted on microscope slides. Imaging was done using fluorescence microscopy (Leica
243 DM5500 B, Leica Microsystems, Wetzlar, Germany; 100x/1.4 Oil Plan Apochromat objective
244 lens; QImaging EXiAqua Bio-Imaging Microscopy camera) with ImagePro software (v. 7.0),
245 and processed using ImageJ software (v. 1.51j8, Schneider et al. 2012). Counting was done
246 according to the guidelines outlined in Muthukrishnan et al. (2017) with 30 random fields of
247 view per sample.

248

249 *2.10. Statistics*

250

251 *2.10.1. Weight and survival*

252 One-way Analysis of Variance (ANOVA) tests followed by Tukey HSD were used to examine
253 the effect of treatment on biomass, number of survivors, and wet weight per larva. Rearing tanks
254 were the units of replication. All statistical analyses in this study assessed significance using an
255 alpha level of 0.05.

256

257 *2.10.2. Water quality*

258 One-way Analysis of Variance (ANOVA) tests were run to test the effect of treatment on water
259 quality levels (nitrite, ammonia, dissolved oxygen) using the aov function in R.

260

261 *2.10.3. 16S rDNA amplicon sequencing*

262 Permutational multivariate analysis of variance (PERMANOVAs) were carried out in R (adonis
263 function, vegan package) using Bray-Curtis OTU-based distance matrices to test the effect of the
264 factors treatment and time on the microbial community structure of each sample type.
265 Homogeneity of variance was evaluated with the betadisper function which utilizes a
266 multivariate analogue of Levene's Test. Results that did not violate the homogeneity assumption
267 of the PERMANOVA test were interpreted as the most reliable. Those comparisons with a
268 balanced design that were statistically heterogenous were also reported as they are still
269 considered admissible as outlined in Anderson and Walsh (2013).

270

271 *2.10.4. Cell counts*

272 Treatment effects on cell counts were modeled in R statistical software using a negative binomial
273 generalized linear model (GLM). Due to significant interaction effects of treatment and date,

these two factors were modeled separately in order to evaluate treatment effects on each day and the effect of time on a single treatment.

3. Results

3.1. Larval weight and survival

There were no significant differences between the high frequency treatment and the low frequency treatment in biomass, survival, or wet weight per larva ($p > 0.05$ for all measures). Casual visual observations revealed an obvious reduction in larval numbers in tanks with sea cucumbers within the first week of the experiment. At the end of the experiment, the "low frequency plus sea cucumber" treatment had lower biomass (Figure 1; $F = 51.816$; $p < 0.0001$) and fewer survivors (Figure 1; $F = 123.346$; $p < 0.0001$) than the high and low frequency treatments, but no significant differences in wet weight per larva (Figure 1; $F = 0.057$; $p > 0.05$).

3.2. Water quality

For the duration of the experiment, all water quality metrics remained within acceptable ranges (Brownell, 1980). There was no significant effect of treatment on dissolved oxygen ($F = 1.032$, $p = 0.312$; Figure 2), ammonia ($F = 0.232$, $p = 0.631$; Figure 2), or nitrite ($F = 0.046$, $p = 0.831$; Figure 2). Dissolved oxygen means for the duration of the experiment were 7.6 ± 0.6 mg/L (high frequency treatment), 7.7 ± 0.7 mg/L (low frequency treatment), and 7.5 ± 0.6 mg/L ("low frequency plus sea cucumber" treatment). Ammonia means for each treatment were all < 0.01 ppm. Average nitrite levels in rearing water were 10.5 ± 2.5 ppb (high frequency treatment), 9.3

± 3.1 ppb (low frequency treatment), and 10.3 ± 2.9 ppb ("low frequency plus sea cucumber" treatment).

3.3. Microbial communities

We obtained between 13 - 78k raw sequences per sample, with an average of 41k reads. After denoising, merging, chimera removal, and filtering, reads per sample were between 5 – 18k, with a mean of 9,918 reads per sample. A total of 1,866 bacterial taxa were identified from 139 samples in post-processing.

Cleaning frequency did not impact the types or number of bacteria found in sablefish rearing tanks. No significant effect of treatment was observed on the structure of tank seawater microbial communities ($p = 1.000$, Pseudo-F = 0.24; PERMANOVA, Table 2) or on tank surface microbial communities ($p = 0.878$, Pseudo-F = 0.635; Table 2). Date significantly affected both tank seawater and tank surface communities (Table 2; $p < 0.001$, Pseudo-F = 33.4 and 15.2, respectively). Date accounted for 71.6% of the variability observed in tank seawater communities, and 50.9% in tank surface communities (Table 2).

Tank seawater community composition shifted over time, corresponding with larval stocking, the transition from greenwater to claywater, and changes in feed. Prior to the addition of sablefish larvae, the tanks were dominated by the bacterial families *Microcystaceae* and *Saccharospirillaceae* (Figure 3). In the first week of larval rearing, tank seawater communities were dominated (>50%) by *Rhodobacteraceae* (Figure 3). Many taxa were present throughout the experiment, including *Nitrincolaceae*, *Vibrionaceae*, *Rhodobacteraceae*, *Moritellaceae*,

319 *Marinomonadaceae*, *Pseudomonadaceae* and *Colwelliaceae*. Tank seawater communities
320 sampled on days when treatments were receiving greenwater (days 8 & 16) clustered separately
321 from those tank seawater communities sampled on days when treatments were receiving
322 claywater (days 24 & 32) (Figure 4).
323
324 *Colwelliaceae* dominated tank surface communities (ca. 50%), regardless of treatment, and
325 persisted up to one week following the transition to claywater (Figure 3). By week 5 of the
326 experiment, tank surface communities were largely composed of *Rhodobacteraceae* and
327 *Flavobacteriaceae*. Over time, relative abundances of *Rhodobacters* decreased in tank seawater,
328 but increased in the tank surface communities.
329
330 Low cleaning frequencies did not significantly impact the total number of bacterial cells in tank
331 seawater. Cell counts in tank seawater were significantly affected by date ($p < 0.0001$), but not
332 treatment ($p = 0.829$). Due to significant interaction effects, negative binomial GLMs were run
333 on individual days to determine treatment effects, and on individual treatments to determine the
334 effect of date. On days 1 and 8 there was no significant effect of treatment on cell counts ($p >$
335 0.05, Figure 5). On day 16, the "low frequency plus sea cucumber" treatment had significantly
336 fewer total bacterial cells than the low frequency treatment ($\beta = -0.554$, SE = 0.271 , $z = -2.04$, p
337 = 0.0415), but no differences were seen between the high and either of the two low frequency
338 treatments ($p > 0.05$). Significant differences were largely seen after tanks were transitioned to
339 claywater, during the final two weeks of the experiment. On day 24, the "low frequency plus sea
340 cucumber" treatment had significantly fewer cells compared to the control ($\beta = -0.894$, SE =
341 0.269, $z = -3.33$, $p < 0.001$), but no differences were seen between the high frequency treatment

and the low frequency treatment, or between the low frequency treatment and the "low frequency plus sea cucumber" treatment ($p > 0.05$). On day 32, both the high and low frequency treatments had significantly fewer cells compared to the "low frequency plus sea cucumber" treatment ($p < 0.0001$), and no differences were detected between the high and the low frequency treatment ($p = 0.897$). The high and low frequency treatments had significant increases in total cells only on day 24. The "low frequency plus sea cucumber" treatment, however, saw a significant increase in cells on days 8, 24, and 32 compared to the first day of the experiment.

4. Discussion

Compared to the high frequency treatment, the low frequency treatment had no negative impacts on water quality, tank-associated microbial communities, or larval growth or survival. Since the high frequency treatment approximated traditional larval sablefish cleaning protocols, these data suggest that those traditional cleaning protocols are more frequent than necessary, and that cleaning labor can be reduced by more than half without negative impacts on larvae. We had hypothesized that sea cucumbers might be necessary to remove excess organic matter in tanks with low frequencies, but any excess organic matter in those tanks did not appear to have negative effects.

4.1. Sea cucumbers

Sea cucumbers themselves were harmful to larvae, causing mortality rates greater than 98%. Sea cucumbers are soft-bodied, slow-moving invertebrates with few physical defenses against predators. Secondary metabolites, such as saponins, are a main part of their chemical defense,

365 used to deter predators and keep their bodies free from fouling organisms (Kamyab et al., 2020;
366 Zhao et al., 2018). Such chemical defenses may negatively impact fish during co-culturing, but
367 only a few studies have been conducted on toxicity, and to our knowledge, none with larval fish
368 stages. Bakus (1974) fed pieces of different sea cucumber species to fish and found that the body
369 wall of many species of sea cucumbers were toxic to fish, but results were dependent on
370 geography and species. *P. californicus* was not tested. Another study found that adding tissue
371 from the sea cucumber *Holothuria vagabunda* (now classified as *H. luecospilota*) to aquaria
372 caused mortality in marine fishes (Yamanouchi, 1955). Hannah et al. (2013) co-cultured
373 sablefish with California sea cucumbers and saw an increase in sea cucumber growth in
374 cages/net pens in the Pacific Ocean. The ability of sea cucumbers to utilize fish waste and reduce
375 total organic carbon and nitrogen has been observed in co-culture studies (Hannah et al., 2013;
376 Yu et al., 2012). Most studies that have found positive effects of sea cucumber co-culture have
377 been conducted in high flow or open environments. In contrast, the detrimental effects of sea
378 cucumbers observed in our study could relate to our low-flow, enclosed tank environment, which
379 may have allowed sea cucumber metabolites to concentrate to harmful levels. In addition to low
380 flow rates, our study used larvae, which may be more sensitive than adult fish to sea cucumber
381 metabolites. The majority of larval mortality was observed within the first week, and survival
382 appeared relatively steady after that. Perhaps the weaker larvae or the larvae that were most
383 sensitive to sea cucumber metabolites died in the first week. Alternatively, younger larvae may
384 be sensitive to sea cucumber metabolites, while older larvae may be less vulnerable.
385
386 *4.2. Cleaning frequency*

387 Optimal tank cleaning frequencies may be a balance between removal of excess organic matter
388 and potential larval damage, and this balance may change through ontogeny. In the low
389 frequency treatment, there was no siphoning in the first half of the larval period, but siphoning
390 occurred every day beginning on day 27. This strategy was chosen because damage to larvae
391 from siphoning may decrease, and benefits of siphoning may increase, with larval age. Siphoning
392 may reduce larval survival by unintentionally removing larvae from tanks, and may be more
393 harmful to younger larvae, which are weaker swimmers than older larvae (Fisher et al., 2000).
394 Siphoning may be more important for maintaining water quality near the end of the larval period,
395 when dry prepared feeds are used (starting on day 26), and are more likely to accumulate and
396 decay than live feeds (van der Meeren et al., 1998). Live feeds generally remain in the water
397 column and are continually flushed out of tanks, whereas dry prepared feeds are more likely to
398 sink to tank bottoms and decay (Charlon and Bergot, 1986; People Le Ruyet et al., 1993). Early
399 in the sablefish weaning process, most of the dry prepared feed that is added to tanks is not eaten,
400 as larvae only gradually learn to accept dry feeds (personal observations). Future studies may
401 explore the possibility of lower frequencies near the end of the larval period, but this may have
402 negative effects on water quality and larval growth and survival. Sea cucumbers that are added to
403 rearing tanks near the end of the larval period may be beneficial if they consume excess dry
404 prepared feeds from tank bottoms, and if older larvae are not harmed by sea cucumbers.
405
406 Cleaning frequency may have had no effect on larval growth or survival because a) low
407 frequencies did not affect water quality or microbial communities, or b) low frequencies harmed
408 water quality and microbial communities, but was offset by reduced larval damage. Our data do
409 not support the hypothesis that low frequencies harmed water quality and microbial

communities. If low frequencies reduced larval damage, we should have detected increased larval growth and survival, relative to the control treatment. While the growth and survival data trended in that direction, it was not statistically significant.

Cleaning frequency had no significant effect on the number of bacterial cells on tank surfaces or in tank seawater, suggesting that low frequencies did not increase microbial proliferation. The increase in total bacterial cells in all tanks on day 24 was likely due to the shift from greenwater to claywater on day 19, as well as changes in feed. Our previous work has shown similar increases in cell counts with a shift from greenwater to claywater (Pierce et al., 2019). This increase could be accounted for by dissolved organic carbon adsorbing to clay and providing a favorable substrate for bacterial adhesion, as clay-organic aggregates have been linked to bacterial production (Lind et al., 1997; Tietjen et al., 2005). However, bacteria may be bound to charged clay particles and thus no longer biologically available to larvae. Further, clay-organic aggregates can further reduce biological availability by sinking out of the water column, where they can be easily removed by siphoning (Attramadal et al., 2012). If clay indeed binds and precipitates bacteria, the use of claywater in the second half of this study may have contributed to our ability to use low frequencies without negative effects on water quality, larval growth, or larval survival.

Cleaning frequency had no significant effect on tank seawater microbial community composition. Consistent with previous work, tank seawater microbial community composition was significantly influenced by the transition from greenwater to claywater, and also shifted outside of these transitions (Dodd et al., 2020; Pierce et al., 2019; Pierce et al., in prep). These

433 shifts may reflect temporal variation in incoming seawater from Puget Sound. Many of the
434 bacterial families abundant in the current study, *Acrobactereace*, *Vibrionaceae*,
435 *Rhodobacteraceae*, *Pseudomonadaceae* and *Colwelliaceae,* are all common marine bacteria, and
436 are regular components of seawater-associated microbial communities in marine fish and
437 shellfish aquaculture (Carda-Diéguez et al., 2017; Liu et al., 2018; Michaud et al., 2009; Powell
438 et al., 2013; Roalkvam et al., 2019).
439
440 Cleaning frequency showed no significant effect on tank surface microbial community
441 composition, and thus no evidence for effects on pathogens or probiotics. Tank surface microbes
442 showed delayed shifts in community composition after the transition from greenwater to
443 claywater. Similarly in previous work, greenwater-associated operational taxonomic units
444 (OTUs) persisted on larval skin after the transition to claywater (Dodd et al., 2020). Compared to
445 tank seawater, tank surface biofilms may increase stability and slow the rate of community shifts
446 after transitions from greenwater to claywater. Additionally, tank surface microbial community
447 compositions were consistent with previous biofilm research. Rhodobacteraceae are common
448 and dominant members of marine biofilms, including Loktanella sp. detected in our current
449 study (Dang and Lovell, 2002; Elifantz et al., 2013). The abundance of Rhodobacters in both
450 tank seawater and tank surface communities may be related to their varied metabolisms,
451 especially with respect to carbon, nitrogen, and sulfur (Luo and Moran, 2014). Rhodobacters
452 may have played a role in maintaining water quality despite low frequencies.
453
454 *4.3. Conclusions*

455 In summary, while sea cucumbers are not recommended for co-culture with sablefish larvae, low
456 cleaning frequencies were used without adverse effects on water quality, microbial communities,
457 or larval sablefish growth or survival. Further work that tests for effects of low frequency
458 schedules under varying conditions (e.g. flow rates, feed types, and use of greenwater versus
459 claywater) will help further optimize methods and determine how they may apply to other
460 marine finfish species.
461
462 **Acknowledgements**
463
464 Ryan Crim of the Puget Sound Restoration Fund provided sea cucumbers and advice on sea
465 cucumber biology and husbandry. Charlotte Regula-Whitefield provided advice on sea cucumber
466 stocking/experimental design. Emily Dodd conducted cell counts. Chris Tatara commented on
467 the manuscript. Funding was provided by The Saltonstall-Kennedy Grant Program (National
468 Oceanic and Atmospheric Administration), Award 18WCR007-050 to RP. The views expressed
469 herein are those of the authors and do not necessarily reflect the views of the National Oceanic
470 and Atmospheric Administration or any of its sub-agencies.

Literature Cited

Ahlgren, M.O., 1998. Consumption and assimilation of salmon net pen fouling debris by the red sea cucumber *Parastichopus californicus*: implications for polyculture. J. World Aquacult. Soc. 29, 133-139, https://doi.org/10.1111/j.1749-7345.1998.tb00972.x.

Anderson, M. J., Walsh, D. C. I., 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? Ecol. Monogr. 83, 557–574, https://doi.org/10.1890/12-2010.1.

Attramadal, K.J.K., Tøndel, B., Salvesen, I., Øie, G., Vladstein, O., Olsen, Y., 2012. Ceramic clay reduces the load of organic matter and bacteria in marine fish larval culture tanks. Aquacult. Eng. 49, 23-34, https://doi.org/10.1016/j.aquaeng.2012.02.003.

Avendaño-Herrera, R.E., Riquelme, C.E., 2007. Production of a diatom-bacteria biofilm in a photobioreactor for aquaculture applications. Aquacult. Eng. 36, 97-104, https://doi.org/10.1016/j.aquaeng.2006.08.001.

Bakus, G.J., 1974. Toxicity in Holothurians: a geographical pattern. Biotropica. 6, 229-236, https://doi.org/10.2307/2989667.

Baskaran, V., Patil, P., Antony, M.L., Avunje, S., Nagaraju, V.T., Ghate, S.D., Nathamuni, S., Dineshkumar, N., Alavandi, S.V., Vijayan, K.K., 2020. Microbial community profiling of

494 ammonia and nitrite oxidizing bacterial enrichments from brackish water ecosystems for
495 mitigating nitrogen species. Sci. Rep. 10, 5201, https://doi.org/10.1038/s41598-020-62183-9.
496
497 Bjornsdottir, R., Smaradottir, H., Sigurgisladottir, S., Gudmundsdottir, B.K., 2011. Effects of
498 different environmental shading on the cultivable bacterial community and survival of first
499 feeding Atlantic halibut larvae. Iceland. Agr. Sci. 24, 33–41.
500
501 Brownell, C.L., 1980. Water quality requirements for first-feeding in marine fish larvae. I.
502 Ammonia, nitrite, and nitrate. J. Exp. Mar. Biol. Ecol. 44, 269-283, https://doi.org/10.1016/0022-
503 0981(80)90158-6.
504
505 Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016.
506 DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581-
507 583, https://doi.org/10.1038/nmeth.3869.
508
509 Carda-Diéguez, M., Ghai, R., Rodríguez-Valera, F., Amaro, C., 2017. Wild eel microbiome
510 reveals that skin mucus of fish could be a natural niche for aquatic mucosal pathogen evolution.
511 Microbiome 5, 162, https://doi.org/10.1186/s40168-017-0376-1.
512
513 Charlon, N., Bergot, P., 1986. An improved automatic dry food dispenser for fish larvae. The
514 Progressive Fish-Culturist. 48, 156-158,
515 https://doi.org/10.1577/1548-8640(1986)48<156:AIADFD>2.0.CO;2.
516

517 Cook, M.A., Massee, K.C., Wade, T.H., Oden, S.M., Jensen, C., Jasonowicz, A., Immerman,
518 D.A., Goetz, F.W., 2015. Culture of sablefish (*Anoplopoma fimbria*) larvae in four experimental
519 tank designs. Aquacult. Eng. 69, 43-49, https://doi.org/10.1016/j.aquaeng.2015.09.003.
520
521 Dang, H., Lovell, C.R., 2002. Numerical dominance and phylotype diversity of marine
522 *Rhodobacter* species during early colonization of submerged surfaces in coastal marine waters as
523 determined by 16S ribosomal DNA sequence analysis and fluorescence in situ hybridization.
524 Appl. Environ. Microbiol. 68, 496-504, https://doi.org/10.1128/aem.68.2.496-504.2002.
525
526 Daugherty, Z. N., 2013. Effects of algae paste substitutes on the larval rearing performance and
527 microbial communities in the culture of cobia *(Rachycentron canadum)* and yellowtail kingfish
528 *(Seriola lalandi)*. Ph. D. thesis, University of Miami Miami, FL.
529
530 Davidson, J., Summerfelt, S., 2004. Solids flushing, mixing, and water velocity profiles within
531 large (10 and 150 m^3) circular Cornell-type dual-drain tanks. Aquac. Eng. 32, 245-271,
532 https://doi.org/10.1016/j.aquaeng.2004.03.009.
533
534 Dodd, E., Pierce, M.L., Lee, J.S.F., Poretsky, R.S., 2020. Influences of claywater and greenwater
535 on the skin microbiome of cultured larval sablefish (*Anoplopoma fimbria*). Animal Microbiome.
536 2 (27), 1-13, https://doi.org/10.1186/s42523-020-00045-5.
537

538 Elifantz, H., Horn, G., Ayon, M., Cohen, Y., Minz, D., 2013. *Rhodobacteraceae* are the key
539 members of the microbial community of the initial biofilm formed in Eastern Mediterranean
540 coastal seawater. FEMS Microbiol. Ecol. 85, 348-357, https://doi.org/10.1111/1574-6941.12122.
541
542 Faulk, C.K., Holt, G.J., 2005. Advances in rearing cobia *Rachycentron canadum* larvae in
543 recirculating aquaculture systems: live prey enrichment and greenwater culture. Aquaculture
544 249, 231–243, https://doi.org/10.1016/j.aquaculture.2005.03.033.
545
546 Fisher, R., Bellwood, D.R., Job, S.D., 2000. Development of swimming abilities in reef fish
547 larvae.
548 Marine Ecology Progress Series. 202, 163-*173,* https*://*doi.org/10.3354/meps202163.
549
550 Gichana, Z.M., Liti, D., Waidbacher, H., Zollitsch, W., Drexler, S., Waikibia, J., 2018. Waste
551 management in recirculating aquaculture system through bacterial dissimilation and plant
552 assimilation. Aquac. Int. 26, 1541-172, https://doi.org/10.1007/s10499-018-0303-x.
553
554 Green, S. J., Venkatramanan, R., Naqib, A., 2015. Deconstructing the polymerase chain reaction:
555 understanding and correcting bias associated with primer degeneracies and primer-template
556 mismatches. PLoS One 10, e0128122, https://doi.org/10.1371/journal.pone.0128122.
557
558 Hannah, L., Pearce, C.M., Cross, S.F., 2013. Growth and survival of California sea cucumbers
559 (*Parastichopus californicus*) cultivated with sablefish (*Anoplopoma fimbria*) at an integrated

560 multi-trophic aquaculture site. Aquaculture 406-407, 34-42,
561 https://doi.org/10.1016/j.aquaculture.2013.04.022.
562
563 Ji, T., Dong, Y., Dong, S., 2008. Growth and physiological responses in the sea cucumber,
564 *Apostichopus japonicus* Selenka: aestivation and temperature. Aquaculture 283, 180-187,
565 https://doi.org/10.1016/j.aquaculture.2008.07.006.
566
567 Kamyab, E., Kellermann, M.Y., Kunzmann, A., Schupp, P.J., 2020. Chemical biodiversity and
568 bioactivities of saponins in Echinodermata with an emphasis on sea cucumbers (Holothuroidea),
569 in: Jungblut S., Liebich V., Bode-Dalby M. (Eds.), YOUMARES 9 - The Oceans: Our Research,
570 Our Future. Springer, Cham, pp. 121-157, https://doi.org/10.1007/978-3-030-20389-4_7.
571
572 Lazo, J.P., Dinis, M.T., Holt, G.J., Faulk, C., Arnold, C.R., 2000. Co-feeding microparticulate
573 diets with algae: toward eliminating the need of zooplankton at firstfeeding in larval red drum
574 (*Sciaenops ocellatus*). Aquaculture 188, 339–351, https://doi.org/10.1016/S0044-
575 8486(00)00339-2.
576
577 Lee, J.S.F., Cook, M.A., Berejikian, B.A., Goetz, F.W., 2017. Temporal changes in the
578 suitability of claywater as a greenwater substitute for rearing larval sablefish (*Anoplopoma*
579 *fimbria*). Aquaculture 470, 11-16, https://doi.org/10.1016/j.aquaculture.2016.12.011.
580
581 Lee, J.S.F., Pierce, M.L., Poretsky, R.S., Cook, M.A., Berejikian, B.A., Goetz, F.W., 2021.

582 Transitioning from algae to clay as turbidity agents: Timing, duration, and transition rates for
583 larval
584 sablefish (*Anoplopoma fimbria*). Aquaculture. 541, 736825,
585 https://doi.org/10.1016/j.aquaculture.2021.736825.
586
587 Lind, O., Chrzanowski, T., Dávalos-Lind, L., 1997. Clay turbidity and the relative production of
588 bacterioplankton and phytoplankton. Hydrobiologia 353, 1–18,
589 https://doi.org/10.1023/A:1003039932699.
590
591 Liu, Z., Qiuqian, L., Yao, Z., Wang, X., Huang, L., Zheng, J., Wang, K., Li, L., Zhang, D., 2018.
592 Effects of a commercial microbial agent on the bacterial communities in shrimp culture system.
593 Front. Microbiol. 9, 2430, https://doi.org/10.3389/fmicb.2018.02430.
594
595 Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N., 2014. Teleost microbiomes: the state
596 of the art in their characterization, manipulation and importance in aquaculture and fisheries.
597 Front. Microbiol. 5, 207, https://doi.org/10.3389/fmicb.2014.00207.
598
599 Luo, H., Moran, M.A., 2014. Evolutionary ecology of the marine *Roseobacter* clade. Microbiol.
600 Mol. Biol. Rev. 78, 573-587, https://doi.org/10.1128/MMBR.00020-14.
601
602 Mata, M.T., Luza, M.F., Riquelme, C.E., 2017. Production of diatom-bacteria biofilm isolated
603 from *Seriola lalandi* cultures for aquaculture application. Aquac. Res. 48, 4308-4320,
604 https://doi.org/10.1111/are.13253.

McMurdie, P. J., Holmes, S., 2013. Phyloseq: an r package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217, https://doi.org/10.1371/journal.pone.0061217.

Michaud, L., Lo Giudice, A., Troussellier, M., Smedile, F., Bruni, V., Blancheton, J. P., 2009. Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system. J. Appl. Microbiol. 107, 1935-1946, https://doi.org/10.1111/j.1365-2672.2009.04378.x.

Muthukrishnan, T., Govender, A., Dobretsov, S., Abed, R. M. M., 2017. Evaluating the reliability of counting bacteria using epifluorescence microscopy. J. Mar. Sci. Eng. 5, 4, https://doi.org/10.3390/jmse5010004.

Naas K.E., Naess T., Harboe T., 1992. Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L.) in green water. Aquaculture 105, 143–156, https://doi.org/10.1016/0044-8486(92)90126-6.

Nelson, E.J., MacDonald, B.A., Robinson, S.M.C., 2012. The absorption efficiency of the suspension-feeding sea cucumber, *Cucumaria frondosa*, and its potential as an extractive integrated multi-trophic aquaculture (IMTA) species. Aquaculture 370-371, 19-25, https://doi.org/10.1016/j.aquaculture.2012.09.029.

628 Palzat, D.L., Pearce, C.M., Barnes, P.A., McKinley, R.S., 2008. Growth and production of
629 California sea cucumbers (*Parastichopus californicus* Stimpson) co-cultured with suspended
630 Pacific oysters (*Crassostrea gigas* Thunberg). Aquaculture 275, 124-137,
631 https://doi.org/10.1016/j.aquaculture.2007.12.014.
632
633 People Le Ruyet, J., Alexandre, J.C., Thébaud, L., Mugnier, C., 1993. Marine fish larvae
634 feeding:
635 formulated diets or live prey? Journal of the World Aquaculture Society. 24, 211-224,
636 https://doi.org/10.1111/j.1749-7345.1993.tb00010.x.
637
638 Pierce, M.L., Lee, J.S.F., Dodd, E., Poretsky, R.S., 2019. Algae and clay water additives
639 differentially impact survival and microbial community structure in sablefish (*Anoplopoma*
640 *fibria*) rearing tanks. Front. Mar. Sci. 6, 203, https://doi.org/10.3389/fmars.2019.00203.
641
642 Powell, S.M., Chapman, C.C., Bermudes, M., Tamplin, M.L., 2013. Dynamics of seawater
643 bacterial communities in a shellfish hatchery. Microb. Ecol. 66, 245-256,
644 https://doi.org/10.1007/s00248-013-0183-6.
645
646 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O.,
647 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-
648 based tools. Nucl. Acids Res. 41, D590-D596, https://doi.org/10.1093/nar/gks1219.
649

650 Rajkowski, K.T., 2009. Biofilms in fish processing. Fratamico, P.M., Annous, B.A., Gunther,
651 N.W. (Eds.), Biofilms in the Food and Beverage Industries. Woodhead Publishing, Cambridge,
652 pp. 499-516.
653
654 Roalkvam, I., Drønen, K., Dahle, H., Wergeland, H.I., 2019. Microbial communities in a flow-
655 through fish farm for lumpfish (*Cyclopterus lumpus* L.) during healthy rearing conditions. Front.
656 Microbiol. 10, 1594, https://doi.org/10.3389/fmicb.2019.01594.
657
658 Schneider, C. A., Rasband, W. S., Eliceiri, K. W., 2012. NIH Image to ImageJ: 25 years of
659 image analysis. Nat. Methods 9, 671–675, https://doi.org/10.1038/nmeth.2089.
660
661 Stuart, K., Drawbridge, M., 2011. The effect of light intensity and turbidity on survival and
662 growth of cultured larval yellowtail amberjack (*Seriola lalandi*). Aquaculture 321, 152–156,
663 https://doi.org/10.1016/j.aquaculture.2011.08.023.
664
665 Stuart, K., Rotman, F., Drawbridge, M., 2016. Methods of microbial control in marine fish larval
666 rearing: clay based turbidity and passive larval transfer. Aquac. Res. 47, 2470-2480,
667 https://doi.org/10.1111/are.12696.
668
669 Tietjen, T., Vähätlo, A.V., Wetzel, R.G., 2005. Effects of clay mineral turbidity on dissolved
670 organic carbon and bacterial production. Aquatic Sci. 67, 51-60, https://doi.org/10.1007/s00027-
671 004-0753-2.
672

van der Meeren, T., Harboe, T., Holm, J.C., Solbakken, R., 1998. A new cleaning system for rearing tanks in larval fish culture. ICES CM. L13.

Yamanouchi, T., 1955. On the poisonous substances contained in holothurians. Pub. Seto Mar. Bio. Lab. 4, 183–213.

Yu, Z., Hu, C., Zhou, Y., Li, H., Peng, P., 2012. Survival and growth of the sea cucumber *Holothuria leucospilota* Brandt: a comparison between suspended and bottom cultures in a subtropical fish farm during summer. Aquac. Res. 44, 114-124, https://doi.org/10.1111/j.1365-2109.2011.03016.x.

Zamora, L.N., Yuan, X., Carton, A.G., Slater, M.J., 2018. Role of deposit-feeding sea cucumbers in integrated multitrophic aquaculture: progress, problems, potential and future challenges. Rev. Aquacult. 10, 57-74, https://doi.org/10.1111/raq.12147.

Zhao, Y.C., Xue, C.H., Zhang, T.T., Wang, Y.M., 2018. Saponins from sea cucumber and their biological activities. J. Agr. Food Chem. 66, 7222-7237, https://doi.org/10.1021/acs.jafc.8b01770.

692 **Figure Captions**

693

694 Figure 1. Sablefish biomass, number of survivors, and weight per larva by treatment, taken at the
695 conclusion of the experiment (n = 4 tanks per treatment). Treatment H = high cleaning
696 frequency, L = low cleaning frequency, S = low cleaning frequency plus sea cucumbers.
697 Different letters denote significant differences between treatments ($p < 0.05$).

698

699 Figure 2. Water quality metrics. Data are presented as medians with upper and lower quartiles.
700 Outliers are represented by points. Treatment H = high cleaning frequency, L = low cleaning
701 frequency, S = low cleaning frequency plus sea cucumbers. No significant effect of treatment on
702 ammonia (n = 64), nitrite (n = 64), or dissolved oxygen (n = 108) was observed.

703

704 Figure 3. Relative abundance of bacterial families found in tank seawater and tank surfaces
705 during the experiment. Treatment H = high cleaning frequency, L = low cleaning frequency, S =
706 low cleaning frequency plus sea cucumbers. n = 4 tanks per treatment, per day.

707

708 Figure 4. Bray-Curtis Principal Coordinates Analysis (PCoA) of tank seawater and tank surface
709 microbial communities. Percent variation explained by each axis is in parentheses. Treatments
710 are denoted by shapes while experimental day is denoted by color. Treatment H = high cleaning
711 frequency, L = low cleaning frequency, S = low cleaning frequency plus sea cucumbers.

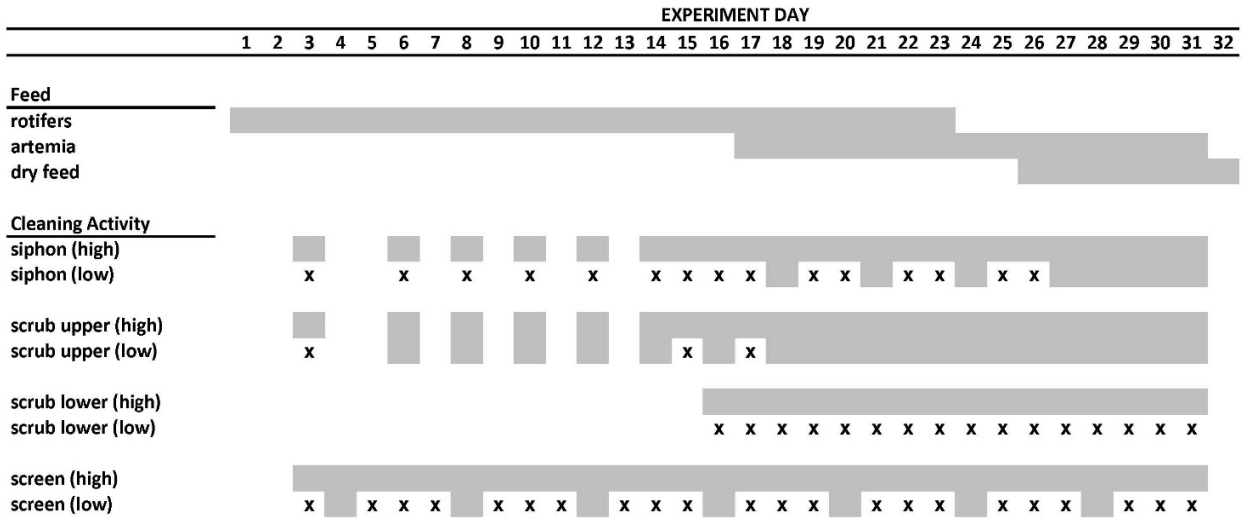
712

713 Figure 5. Total bacterial cell per mL of tank seawater in sablefish rearing tanks across the
714 duration of the experiment. Data are presented as means and standard deviations. Treatment H =

715 high cleaning frequency, L = low cleaning frequency, S = low cleaning frequency plus sea
716 cucumbers. Different letters indicate statistically significant differences between treatments on a
717 single day. N/S = no significance.

Tables & Figures

Table 1. Cleaning schedules for high and two low cleaning frequency treatments (low frequency treatment and "low frequency plus sea cucumber" treatment). Grey squares mark the days on which each cleaning activity was performed. X's mark deleted cleaning days in the two low frequency treatments.



737 Table 2. PERMANOVA and Betadisper results using the Bray-Curtis dissimilarity metric for
738 tank seawater and tank surface microbial communities. P-values based on 999 permutations.
739 Homogeneity of variance p-values are in parentheses.

740

741

Sample Type	Factor	Df	SS	Pseudo-F	R^2	p-value	Homogeneity? (p-value)
Tank Seawater	Treatment	2	0.1794	0.23943	0.00689	1.000	Yes (0.958)
	Residuals	69	25.8516		0.99311		
	Total	71	26.0311		1		
	Date	5	18.1649	30.482	0.69782	0.001	No (0.001)
	Residuals	66	0.1192		0.30218		
	Total	71	26.0311		1		
Tank Surface	Treatment	2	0.4488	0.63471	0.02744	0.878	Yes (0.958)
	Residuals	45	15.91		0.97256		
	Total	47	16.3588		1		
	Date	3	8.3193	15.177	0.50855	0.001	No (0.001)
	Residuals	44	8.0395		0.49145		
	Total	47	16.3588		1		

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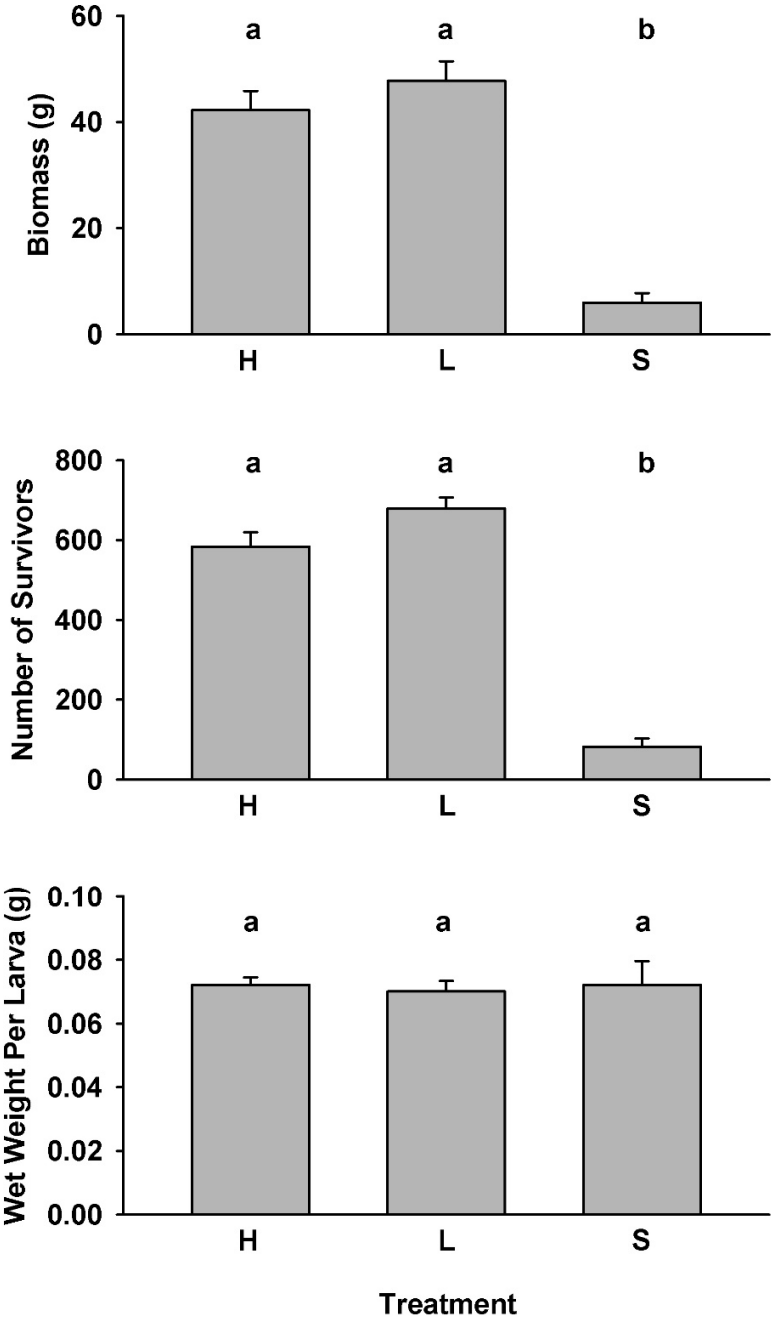


749
750 Figure 1

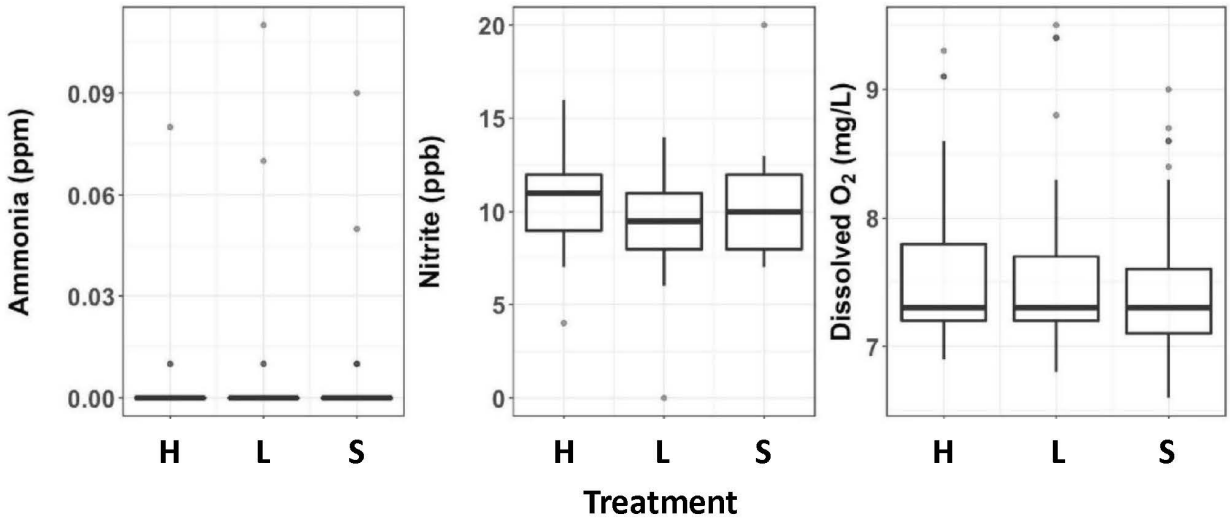
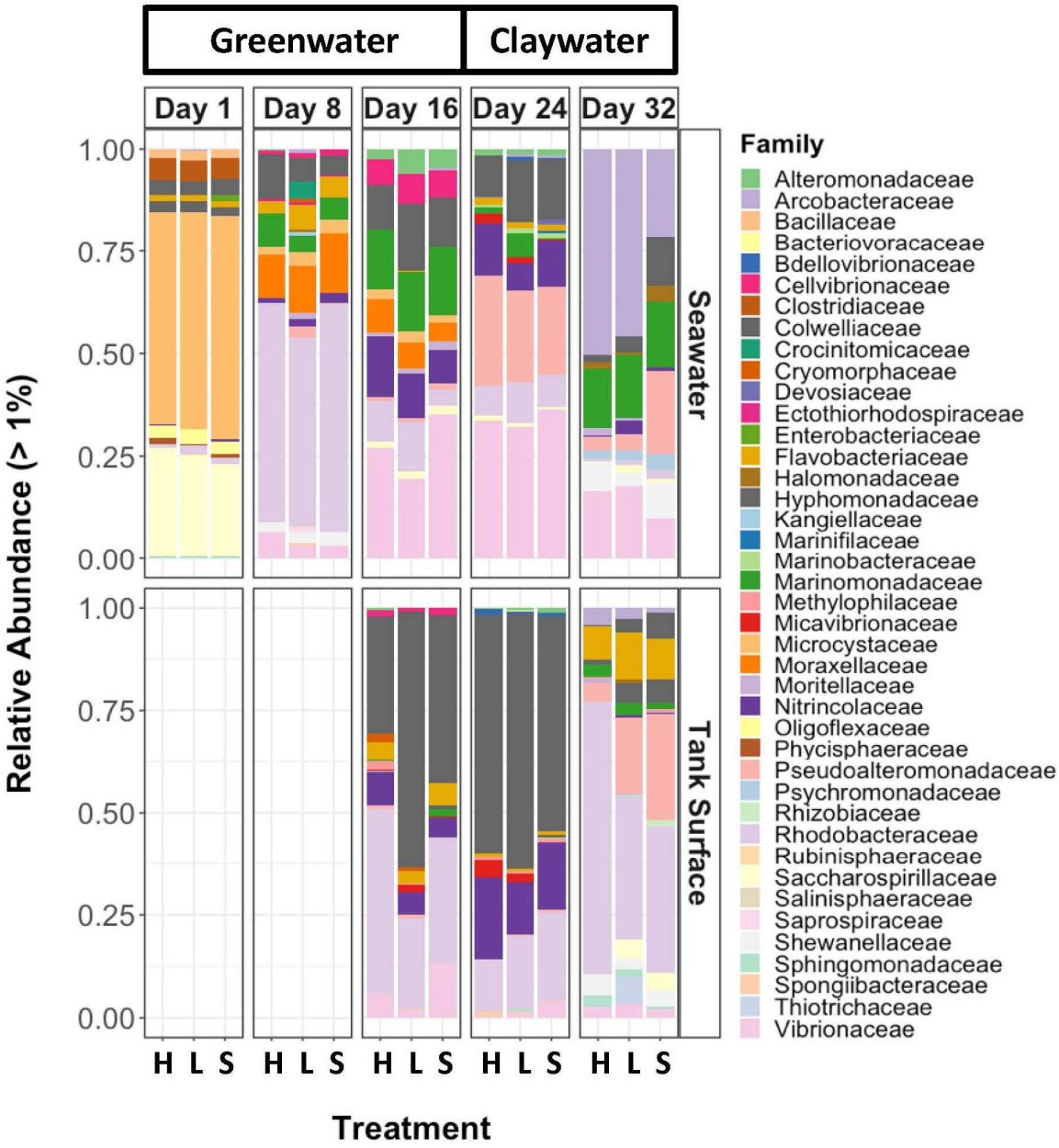

Ammonia (ppm)
0.09
0.06
0.03
0.00
H
L
S
Nitrite (ppb)
20
15
10
5
0
H
L
S
Dissolved O_2 (mg/L)
9
8
7
H
L
S
Treatment

Figure 2



774
775 Figure 3
776

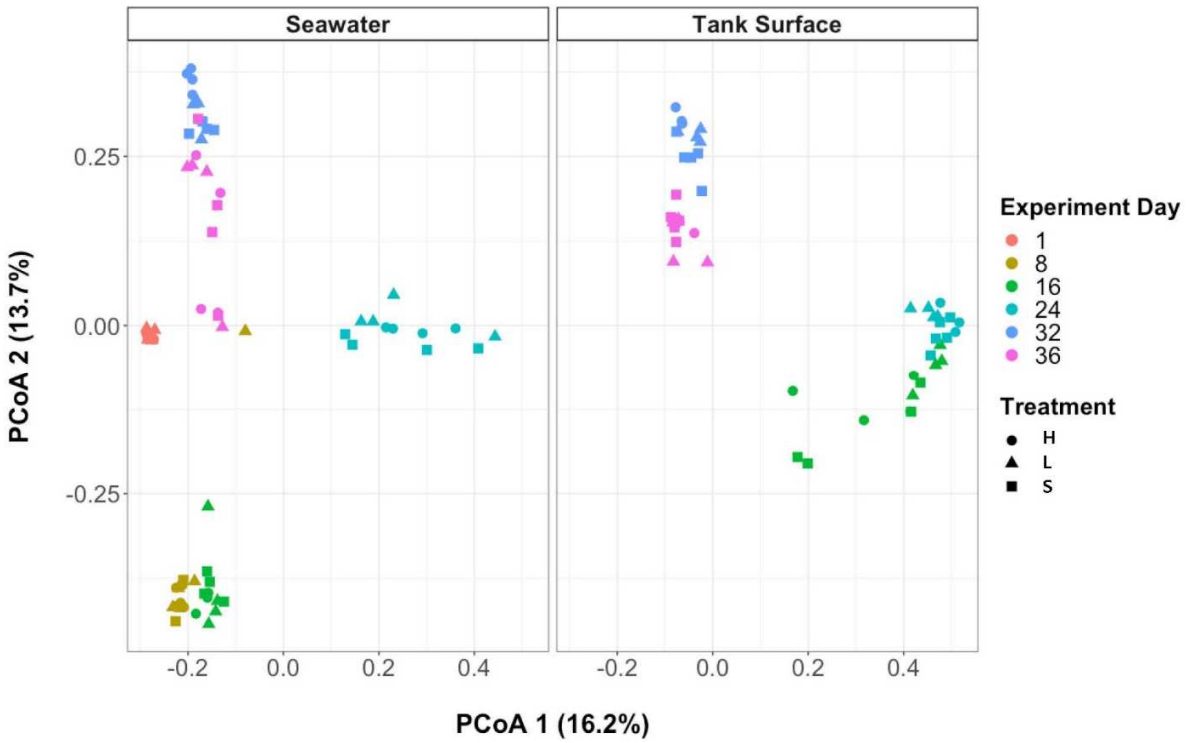

Seawater
Tank Surface
0.25
0.00
-0.25
PCoA 2 (13.7%)
-0.2
0.0
0.2
0.4
PCoA 1 (16.2%)
Experiment Day
1
8
16
24
32
36
Treatment
H
L
S

Figure 4

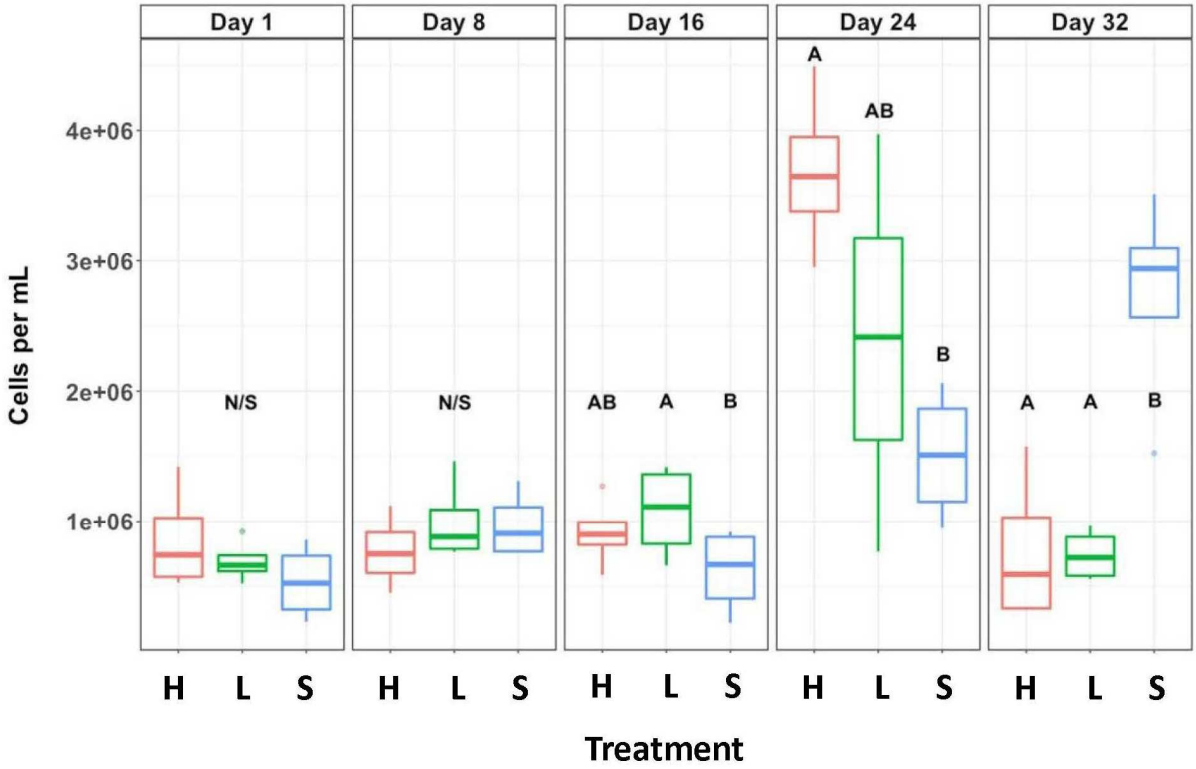

Day 1
Day 8
Day 16
Day 24
Day 32
Cells per mL
4e+06
3e+06
2e+06
1e+06
N/S
N/S
AB
A
B
A
AB
B
A
A
B
H
L
S
Treatment

Figure 5