Research Study

BiOWiSH® AquaFarm & BiOWiSH® MultiBio 3PS

Improved Hatchery Post Larvae Yield and Health in Vannamei Shrimp using BiOWiSH® AquaFarm and BiOWiSH® MultiBio 3PS

Background

Vietnam is among the world leaders in shrimp production. Producers face increasing pressure in controlling water quality and disease which impact production and profits. Optimal hatchery production is a critical step towards improving yield and shrimp quality. Ensuring the quality and health of hatchery post larvae (PL) is crucial to the overall production process. PL must be strong, and disease free. This requires control of nitrogen and organic waste buildup (ammonia, nitrite, and nitrate), and keeping *Vibrio* bacteria levels low to maintain optimum PL health and increase survival rate through the hatchery production.

The United Nations has issued a report on the challenges facing aquaculture. Availability of quality PL from hatcheries that are disease free was one of four critical global challenges facing the industry.

Objectives

To address this issue and provide products, and a hatchery process that ensure high quality, antibiotic free post larvae for the shrimp industry in Vietnam, BiOWiSH is conducting ongoing hatchery tests in cooperation with ShrimpVet (Dr. Loc Tran) in support of BiOWiSH Vietnam. The objective is to validate that the addition of BiOWiSH® AquaFarm and BiOWiSH® MultiBio 3PS maintain optimal water quality, inhibit *Vibrio* bacteria during production in tank water, and establish a healthy gut in PL that suppresses *Vibrio* growth or accumulation. The results have demonstrated that BiOWiSH® products not only produce stronger PL, but also increase PL survival rate and biomass.

Key Conclusions

- BiOWiSH® AquaFarm helps maintain optimum tank water quality and reduce *Vibrio* levels. It is important to first establish and maintain a high concentration of beneficial heterotrophic bacteria in hatchery tank water (~105 - 106 CFU/mL or higher) prior to stocking using BiOWiSH® AquaFarm. This level of heterotrophs suppresses fast growing *Vibrio* bacteria by inhibition and competitive exclusion, and ensures *Vibrio* levels remain low, and do not accumulate in, or on, growing PL keeping them healthy. Results show that maintaining a level of total beneficial bacteria, and suppressing yellow *Vibrio* and green *Vibrio* with a ratio of around 100:10:1 produces good results. (For example, with a heterotroph concentration of (~105 - 106 CFU/mL), it is important to maintain yellow *Vibrio* species (which largely accumulate when excess feed builds up) to <104 – 105 CFU/mL and green *Vibrio* species to less than about 102 CFU/mL with very low, or nondetectable levels of *V. parahaemolyticus*, and any luminous *Vibrio* species. (See Chart 1)
- Ammonia, nitrite, and nitrate buildup from undigested feed, feces, and detritus are toxic to shrimp. It is important that these are especially monitored regularly. Daily use of BiOWiSH® AquaFarm maintains total ammonia nitrogen (TAN) to <3 mg/L, and nitrite below 0.3 mg/L with no detectable nitrate. Based on tank pH, salinity, and temperature it is important to keep TAN levels below 3 ppm, as at these concentrations the level of free un-ionized ammonia can be maintained below a critical level of 0.5 mg/L. This work will lead to the development of a calculator-based approach to optimizing dosages of BiOWiSH® AquaFarm levels to best control ammonia and nitrite in hatcheries as well as nursery and grow-out ponds.

BiOWiSH® AquaFarm

- Increases growth rate and weight of fish and shrimp
- Improves water quality
- Inhibits the growth of pathogenic Vibrio bacteria in the pond
- Accelerates the decomposition of pond bottom sludge and minimizes suspended organic solids in the pond water
- Reduces odor
- Reduces labor and associated costs for pond treatment
- Increases quality of shrimp and fish, increasing economic value

Available Sizes

- 100g/3.5oz
- 1kg/2.2lbs

- In vitro and in vivo testing shows BiOWiSH® MultiBio 3PS protects gut health, and reduces *Vibrio* colonization in PL. Daily inclusion of BiOWiSH® MultiBio 3PS with hatchery feed (algae, artemia, and artificial feed) helps maintain PL gut health against problematic *Vibrio* through cytoprotection, and increases their resistance to stress events. Stress events can be due to unexpected surges in nitrogen or organic waste from feed, temperature, or salinity changes. BiOWiSH® MultiBio 3PS increases survival rates, produces stronger/healthier PLs, and provides an important element of improved food quality with no antibiotic usage. BiOWiSH® MultiBio 3PS was shown to suppress *Vibrio* colonization in PL, even if tank water *Vibrio* levels increased temporarily due to surges in organic waste, ammonia, or nitrite. This is due to BiOWiSH® MultiBio 3PS providing a high level of cytoprotection in the PL during its microbiome development.
- While it is important to specify general dosage rates for both BiOWiSH® AquaFarm and BiOWiSH® MultiBio 3PS, changes in feed regimens, and growth of PL may generate conditions where dosages may need to be increased to control surges in *Vibrio* growth as well as maintain water quality parameters. Two key stages in PL development where these can occur are at the zoea 2 stage and the mysis to PL stage. Frequent monitoring of water quality can detect these changes early and allow for adjustments to dosage levels to control these events.

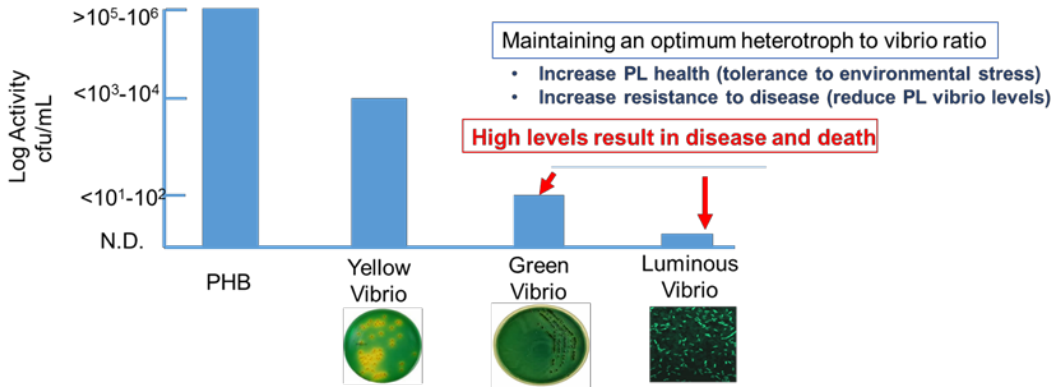


Chart 1: Maintaining an optimum ratio of beneficial heterotrophic bacteria to yellow and green species of *Vibrio* ensures competitive exclusion and inhibition. *V. parahaemolyticus* and luminous *Vibrio* should be as close to nondetectable as possible.

Materials & Methods (Trial 1)

1. Nauplii source

This trial used Nauplii from Konabay's broodstock, which were checked for important infectious diseases including WSSV, TSV, IMNV, EMS/AHPND, and EHP using both histopathology and PCR techniques. Nauplii obtained from newly hatched eggs were washed with disinfectants to eradicate any potential pathogen contamination. Then the washed Nauplii were checked for all diseases listed above to confirm again the disease-free status of the stock.

2. Experimental design

The experiment was set up with 2 treatments x 8 replicates. The trial was set up as a completely randomized design (CRD) in which every treatment was randomly designed with the different tanks. Total period of the trial was 18 days.

This study was conducted at ShrimpVet's hatchery in Vietnam using 16 5000 L tanks. Seawater was passed through a gravity sand filter, then treated with Chlorine (30 ppm), chelated with EDTA (10 ppm), and filtered through bag filters with pore sizes of 5 microns, 2 microns, 1 microns and 0.5 microns. Then the water was treated with UV light before being pumped into larval tanks.

Shrimp stocking density was at 200 Nauplii per liter from stage Z1 to PL10. Feeding amount was calculated based on the estimated biomass of the whole shrimp tank and consumption rate of shrimp larvae. Feed was dispersed evenly into the tanks. Feeding frequency was eight times per day. Uneaten feed was siphoned out of the tanks.

All products were applied directly to tanks at the fixed dosage levels below without activation for comparison to the competitive product.

BiOWiSH® MultiBio 3PS

- Improves fish and shrimp growth rate and biomass
- Increases feed efficiency and digestibility
- Improves animal gut health by preventing vibrio colonization for pathogenic bacteria such as EMS and White Feces
- Reduces mortality
- Replaces selected antibiotics
- Improves feed consumption, reducing feed waste

Available Sizes

- 1kg/2.2lbs
- 5kg/11lbs
- 10kg/22lbs
- 25kg/55lbs

Treatment Definition

Treatment	Probiotic	Water Treatment	Live Algae
Treatment T1 (Commercial probiotics)	Product A (2 ppm/day)	Product B (4 ppm/day)	ShrimpVet's algae
Treatment T2 (BiOWiSH® probiotics)	BiOWiSH® MultiBio 3PS (2 ppm/day)	BiOWiSH® Aquafarm (4 ppm/day)	ShrimpVet's algae

3. Water quality parameters

Temperature, salinity, dissolved oxygen (DO), and pH was recorded daily with a DO meter and a pH meter. Water samples were collected daily from each culture tank. Samples were analyzed for TAN (total ammonia nitrogen) by the Phenate procedure, NO_2-N (nitrite-nitrogen) by the diazotization method, and total alkalinity by titration.

4. Bacteriological analysis

Bacterial counts were performed everyday with water sampled from larval tanks. The samples were serially diluted by log scale and 100 µL plated on both TCBS and TSA+ (TSA + 2% salt) media. The plates were incubated at 28°C for 18 hours before being counted for colonies. The TCBS plates were also be differentially counted for yellow and green colonies. Total and differential plate counts were be recorded for each treatment and replicate sample.

5. Survival rates and total production per tank

During each stage of the culture (Zoae1-3, Mysis 1-3, and PL10), estimated survival rates were checked in all tanks.

The total number of shrimp per tank was calculated by division of the weight of the whole shrimp population per tank to the average weight of individual shrimp PL.

6. Larvae resistance test

Salinity stress test and formalin stress test were done when the PL reach PL4 and PL10 stage.

Results

In side by side hatchery testing versus a leading competitive probiotic control, BiOWiSH® two product approach (BiOWiSH® AquaFarm for water treatment and BiOWiSH® MultiBio 3PS for feed) BiOWiSH® outperformed the competitor and increased survival rate (by as much as 3x) and improved strength and vigor (by salinity and formalin tests) (Figures 1 and 2; and Tables 1 and 2).

Table 1. **Survival of PL till the Mysis Stage and at the End of the Trial**

	Survival Rates (%)	
Stage	**Control (T1)**	**BiOWiSH® (T2)**
Z1	82.08 ± 2.64	100.00 ± 0.00
Z2	65.83 ± 12.47	99.58 ± 1.18
Z3	31.46 ± 14.04	94.79 ± 5.73
Z3-M1	29.79 ± 12.29	81.88 ± 3.83
M1	23.96 ± 8.59	69.17 ± 5.27
M2	23.13 ± 8.93	65.00 ± 7.92
M3	20.83 ± 8.64	55.63 ± 4.36
PL10	19.36 ± 10.447	56.46 ± 13.026

Figure 1: Survival Rates of the Larva Stages in the Treatments

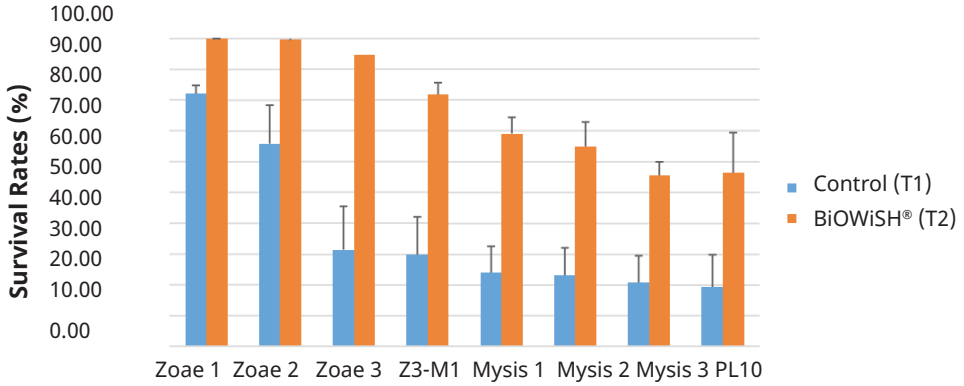


Table 2. **Survival Rates of the Larva Post-stress Tests in the Treatments**

Survival Rate (%)	Control (T1)	BiOWiSH® (T2)
Salinity Test (PL4)	13.88 ± 19.27	51.88 ± 23.08
Formalin Test (PL10)	89.06 ± 12.59	99.33 ± 0.82

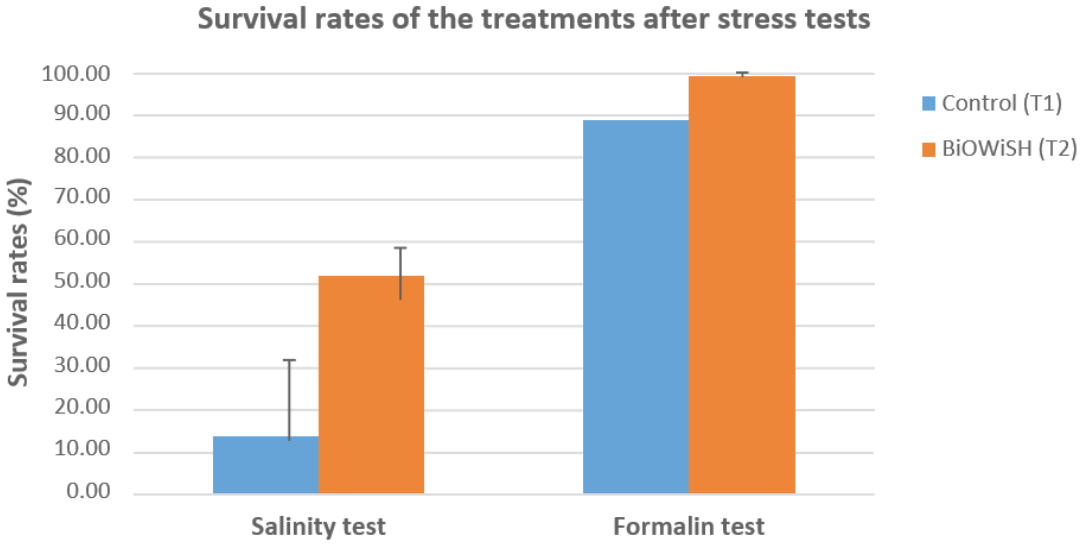


Figure 2: Survival Rates of the Larva Post-stress tests in the treatments

Ensuring good post larvae health is a combination of maintaining good water quality, that is control of nitrogen and *Vibrio* in water, combined with the reduction of *Vibrio* colonization in PL. We've determined that flexible dosing regimens are important as hatchery tank conditions change.

Water control is a continual challenge as tank conditions can change suddenly and require dosage adjustment. This was observed in this early trial where (as noted in the above experimental details) dosages were fixed with no pre-activation throughout the test for comparison purposes to the control. This resulted in higher levels of ammonia and nitrite – even though BiOWiSH® AquaFarm maintained levels below critical thresholds (shown in Figures 3 - 6).

Subsequent to this initial trial, BiOWiSH® has learned that adjusting BiOWiSH® AquaFarm levels based on a feed and biomass basis offers the best approach to controlling ammonia and nitrite levels.

Ongoing measurements in actual hatchery production are in progress using this approach across 64 hatchery tanks for ammonia and nitrite levels as well as *Vibrio* over multiple hatchery production lots. A portion of this data over a two-month period is shown in figures (10, 11, and 12).

An addendum to this data covering the current 8 months of hatchery production (representing over 1000 data measurements) is now being updated and will be issued to BiOWiSH® Vietnam shortly. As represented in figures 10-12 below, these additional results demonstrate excellent control of ammonia, nitrite, and *Vibrio* when a flexible dosing strategy is used along with pre-activation of the BiOWiSH® AquaFarm.

Water quality parameters of the treatments over 18 days

Total ammonia nitrogen (TAN) of the treatments over 18 days

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Control (T1) (ppm)	0.1573	0.1486	0.0951	0.1131	0.0951	0.1580	0.1374	0.2996	0.3293
BiOWiSH® (T2) (ppm)	0.1010	0.1284	0.0796	0.2298	0.3298	0.4921	0.3848	0.3865	0.5793

Treatment	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18
Control (T1) (ppm)	0.2353	0.3652	0.2918	0.4376	0.4178	0.4432	0.2739	0.1830	0.2023
BiOWiSH® (T2) (ppm)	0.8190	1.0627	1.3929	1.7268	2.2962	1.8748	1.9040	2.0258	2.0103

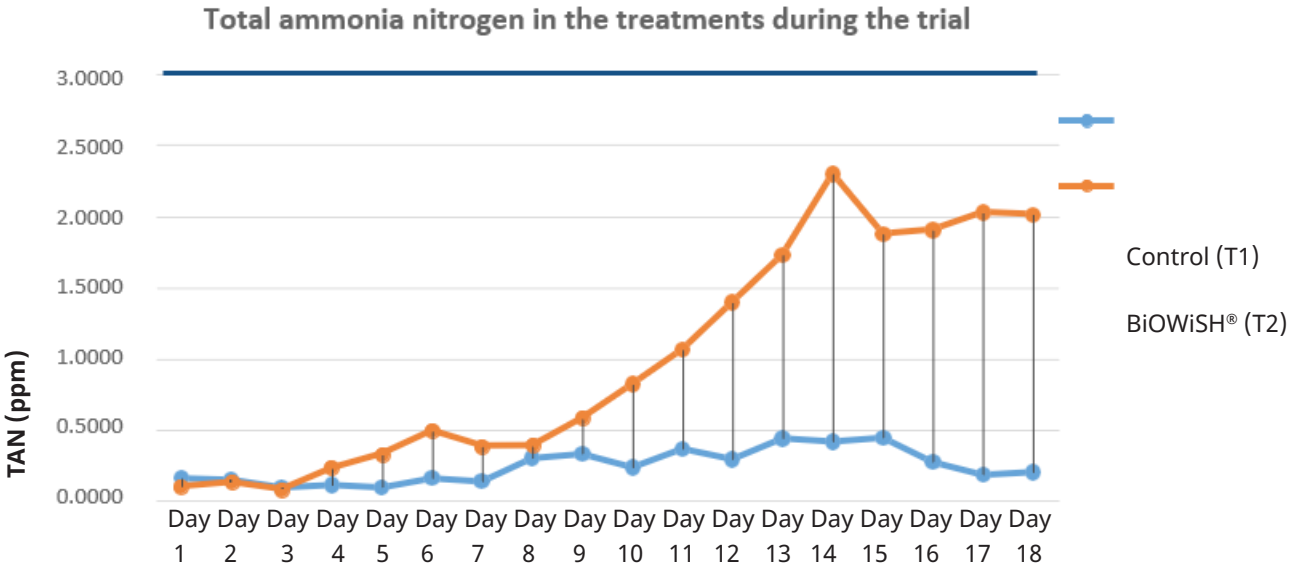


Figure 3: Total ammonia nitrogen (TAN) during 18 days of trial (Mean values).

Target level for Ammonia is < 3.0 mg/L

Nitrite-nitrogen of the treatments over 18 days.

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Control (T1) (ppm)	0.0012	0.0000	0.0013	0.0003	0.0007	0.0007	0.0000	0.0010	0.0009
BiOWiSH® (T2) (ppm)	0.0008	0.0010	0.0008	0.0030	0.0008	0.0004	0.0027	0.0070	0.0059

Treatment	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18
Control (T1) (ppm)	0.0019	0.0026	0.0022	0.0026	0.0019	0.0041	0.0139	0.0001	0.0004
BiOWiSH® (T2) (ppm)	0.0181	0.0063	0.0059	0.0102	0.0037	0.0097	0.0140	0.0062	0.0097

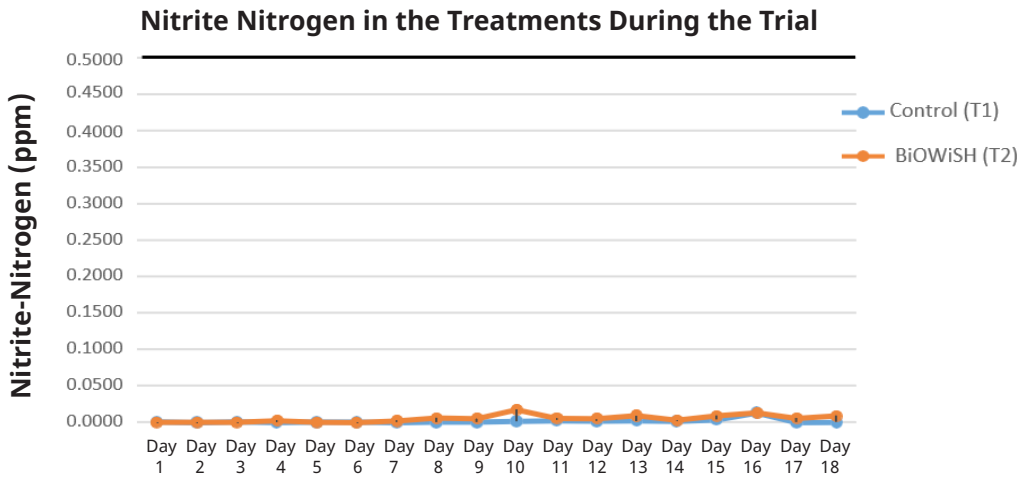


Figure 4: Nitrite nitrogen (NO_2-N) during 18 days of trial (Mean values).

Target level for Nitrite is < 0.5 mg/L. Values shown above are 10x lower than the threshold level.

The increases noted above in figures 3 and 4 for ammonia and nitrite versus the control for BiOWiSH® treatments were due to significant increases in feed used for the BiOWiSH® post larvae treatments due to their rapid and higher growth rate versus the control probiotic.

Because dosage levels were fixed by the protocol, BiOWiSH® AquaFarm was under-dosed, and with excess feed being used in the BiOWiSH® treatments, there was also an increase in yellow *Vibrio*.

However, because of BiOWiSH® strong inhibitory effects, pathogenic species of *Vibrio* green and luminous *Vibrio* were still significantly reduced and no pathogenic *Vibrio* were detected in PL.

Total Alkalinity of the treatments over 18 days

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Control (T1) (ppm)	143	140	132	132	134	135	138	135	135
BiOWiSH® (T2) (ppm)	120	118	125	119	124	122	124	125	124

Treatment	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18
Control (T1) (ppm)	134	131	131	131	135	130	126	125	128
BiOWiSH® (T2) (ppm)	128	132	130	129	128	133	129	128	128

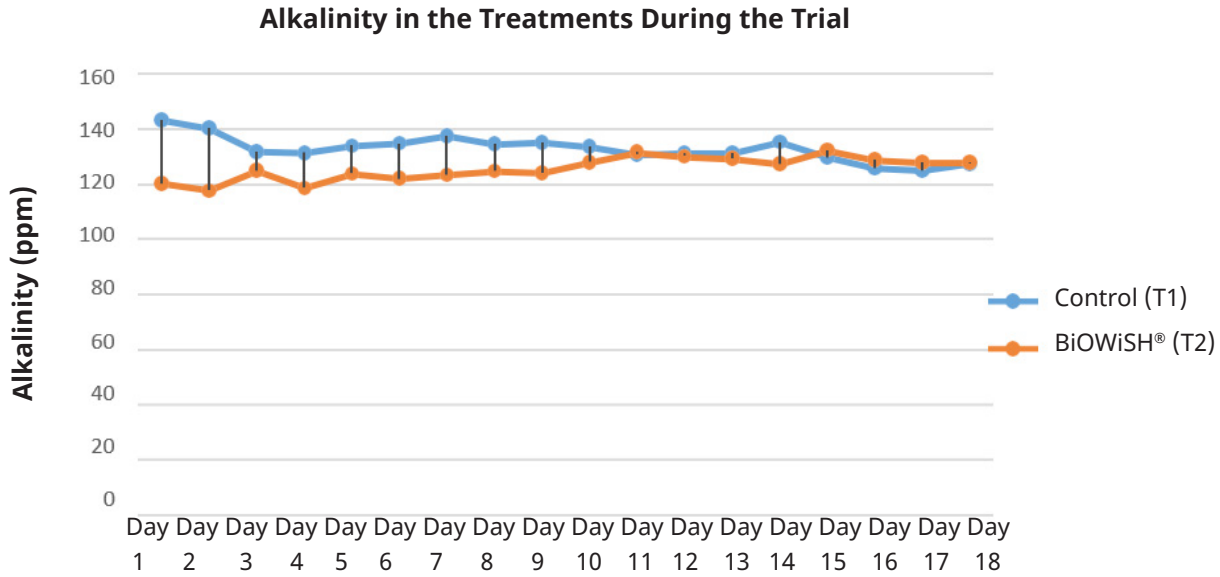


Figure 5: Alkalinity measurement during 18 days of trial (Mean values)

Bacteria measurement over the 18-day trial: Yellow and Green *Vibrio*

Yellow Colonies (CFU/mL)									
Treatment	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**
Control (T1) (ppm)	1.00E+01	9.55E+04	1.14E+05	6.16E+04	8.05E+03	5.91E+03	6.24E+03	6.23E+03	1.35E+04
BiOWiSH® (T2) (ppm)	2.07E+04	7.95E+03	2.16E+05	8.19E+04	9.44E+03	1.61E+04	6.06E+04	7.98E+04	7.86E+04

Yellow Colonies (CFU/mL)									
Treatment	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**	**Day 16**	**Day 17**	**Day 18**
Control (T1) (ppm)	6.93E+03	5.09E+03	5.46E+03	1.51E+04	1.03E+04	5.90E+03	5.04E+04	2.80E+04	3.00E+04
BiOWiSH® (T2) (ppm)	7.46E+04	1.02E+05	1.12E+05	1.02E+05	3.06E+04	4.05E+05	5.59E+04	7.60E+04	1.07E+05

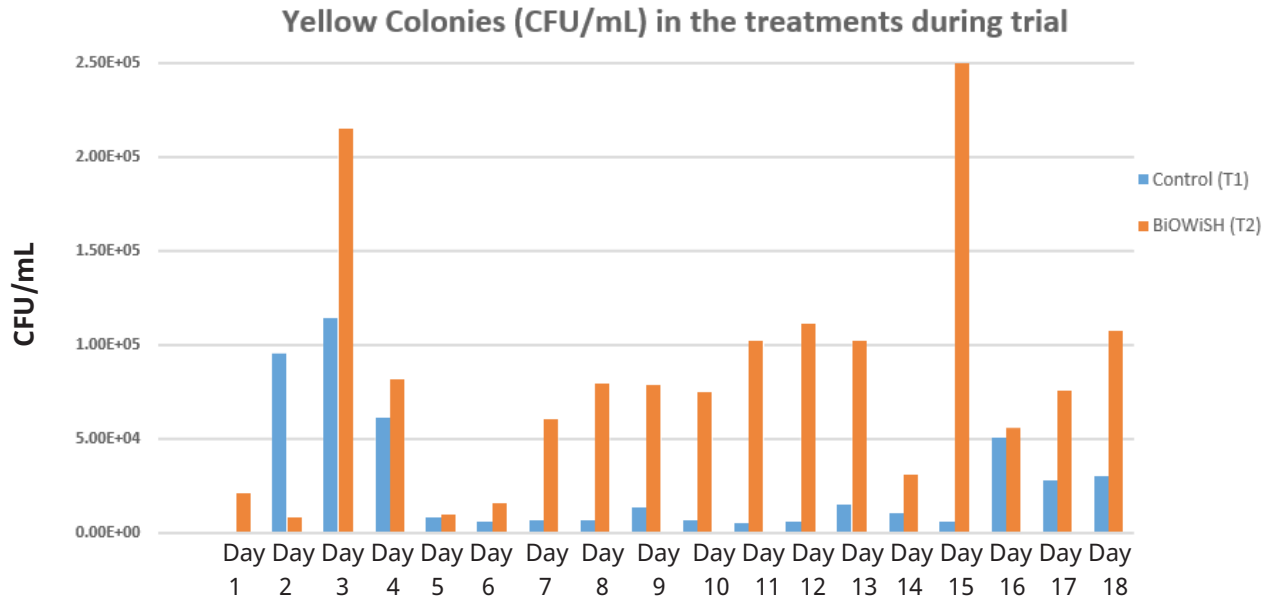


Figure 6: Yellow colonies count during 18 days of trial (Mean value)

Green Colonies (CFU/mL)									
Treatment	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**
Control (T1) (ppm)	1.00E+01	2.76E+03	4.16E+03	1.38E+03	3.44E+02	4.63E+01	1.00E+01	2.13E+01	1.00E+01
BiOWiSH® (T2) (ppm)	1.00E+01	1.00E+01	1.00E+01	3.19E+02	2.28E+02	3.12E+03	2.61E+03	7.06E+02	2.03E+03

Green Colonies (CFU/mL)									
Treatment	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**	**Day 15**	**Day 16**	**Day 17**	**Day 18**
Control (T1) (ppm)	1.00E+01	8.56E+02	1.70E+02	3.38E+03	6.43E+02	1.93E+03	7.50E+03	1.38E+04	1.00E+01
BiOWiSH® (T2) (ppm)	4.05E+02	3.43E+01	2.59E+02	1.00E+01	1.00E+01	3.84E+02	1.45E+02	3.83E+02	2.29E+03

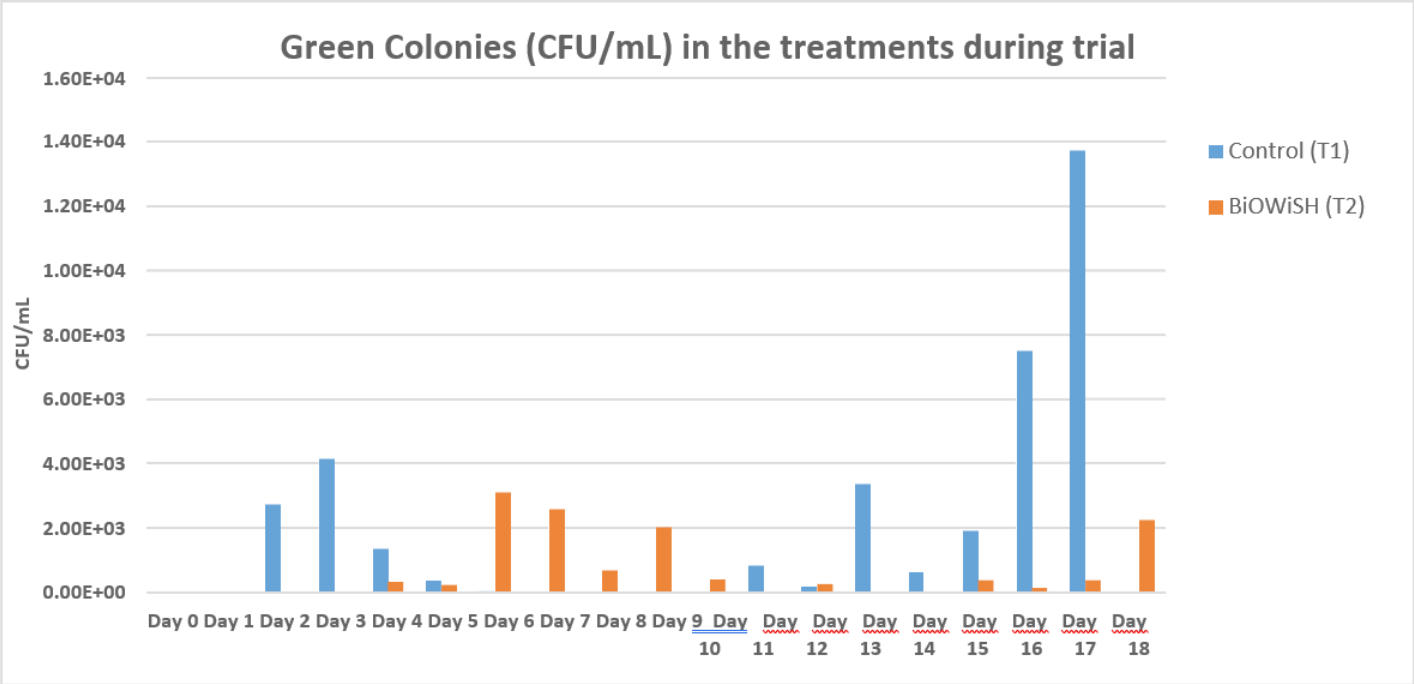


Figure 7: Green colonies count during 18 days of trial (Mean value)

The key factor in the high survival rate and health of the post larvae (figures 1 and 2) under the stressful conditions in this initial trial were due in large part to not only maintaining water nitrogen and *Vibrio* levels below critical levels (even at underdosage levels of BiOWiSH® AquaFarm), but also to the gut protection and inhibition of *Vibrio* colonization that BiOWiSH® MultiBio 3PS provided post larvae in the BiOWiSH treatments.

BiOWiSH® MultiBio 3PS acts in two important ways to improve post larvae resistance to stress and disease. First, as figure 8 shows, in vitro studies show that BiOWiSH® MultiBio 3PS provides a high level of cytoprotection to epithelial cells against *Vibrio,* and better cytoprotection that leading probiotics.

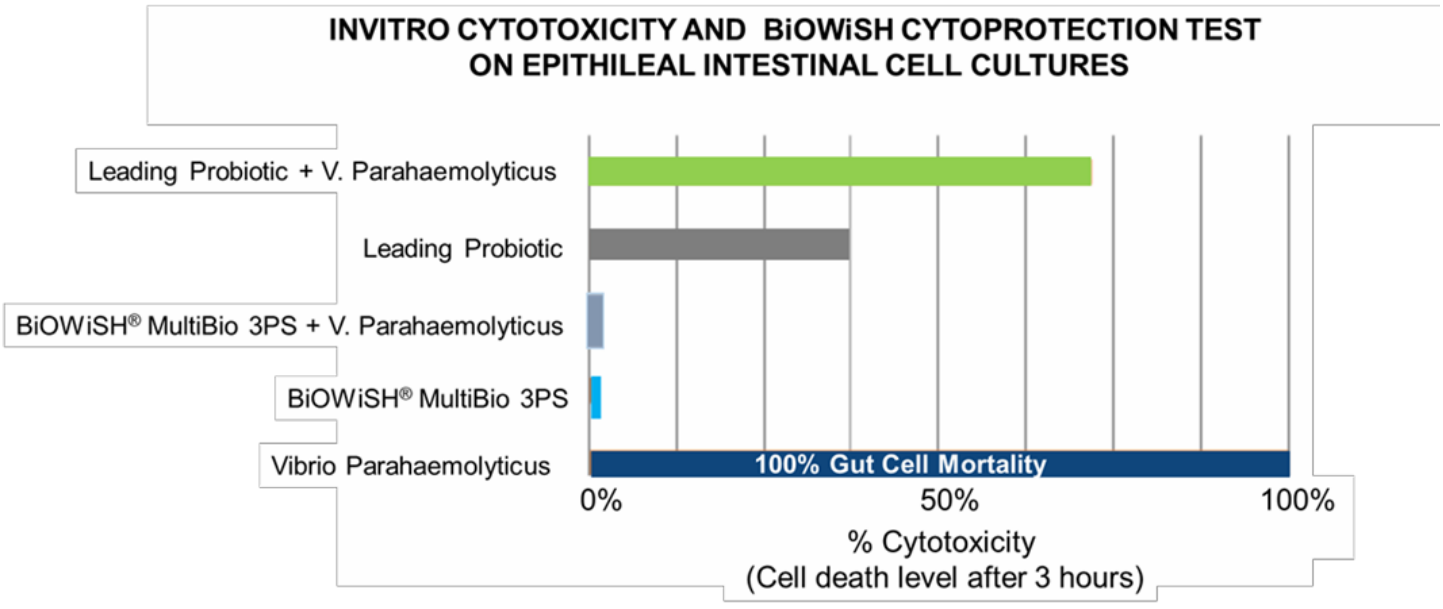


Figure 8: BiOWiSH® MultiBio 3PS cytoprotection versus *V. Parahaemolyticus* and a leading probiotic.

Figure 9 shows the very recent results of DNA analyses conducted at the Cal Poly University, San Louis Obispo from samples obtained in Vietnam hatchery production. The data are preliminary and further tests are being conducted at Cal Poly, but the results show as PL develop (PL 8-10) and their microbiome matures, the use of BiOWiSH® Aquafarm and BiOWiSH® MultiBio 3PS is associated with an increase in heterotroph population in the PL developing with beneficial heterotrophic bacteria. Analysis shows in particular an increase in *Firmicutes phyla* which contain beneficial *Lactobacillus*/Bacillus and no detectable *Vibrio* pathogens.

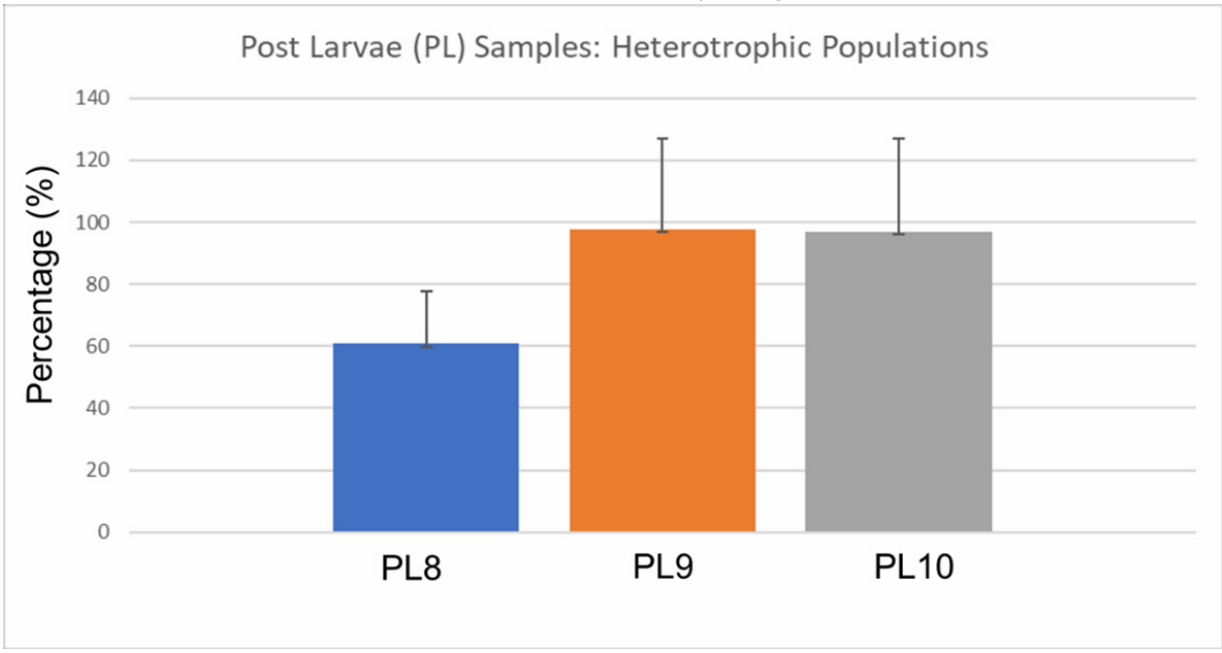


Figure 9: 16s Ribosomal RNA Microbiome Phyla Analysis on post larvae microbiome maturation from PL 8-10 with BiOWiSH® Aquafarm, and BiOWiSH® MultiBio 3PS.

More detailed genomic analysis at the species level of our hatchery results using BiOWiSH® AquaFarm and BiOWiSH® MultiBio 3PS is shown in Figure 10. These results, just recently obtained from Cal Poly, identified 200-300 bacteria species associated with PL as they develop from PL8-10. One species of bacteria dominates in particular: *Phaeobacter*.

Phaeobacter has been shown to provide beneficial protection to aquatic animals in preventing Vibriosis. Consistent with BiOWiSH® probiotic learnings, research at the University of Denmark and the Norwegian Institute of Marine Sciences show that increasing *Phaeobacter* spp. levels in the guts of aquatic animals as early as possible, and in advance of the *Vibrio* pathogen, provides the best protection against disease. BiOWiSH® AquaFarm and BiOWiSH® MultiBio 3PS may provide an environment that selects for the increase of *Phaeobacter* as a key mode of action. This is now an active part of the ongoing BiOWiSH® aquaculture research program.

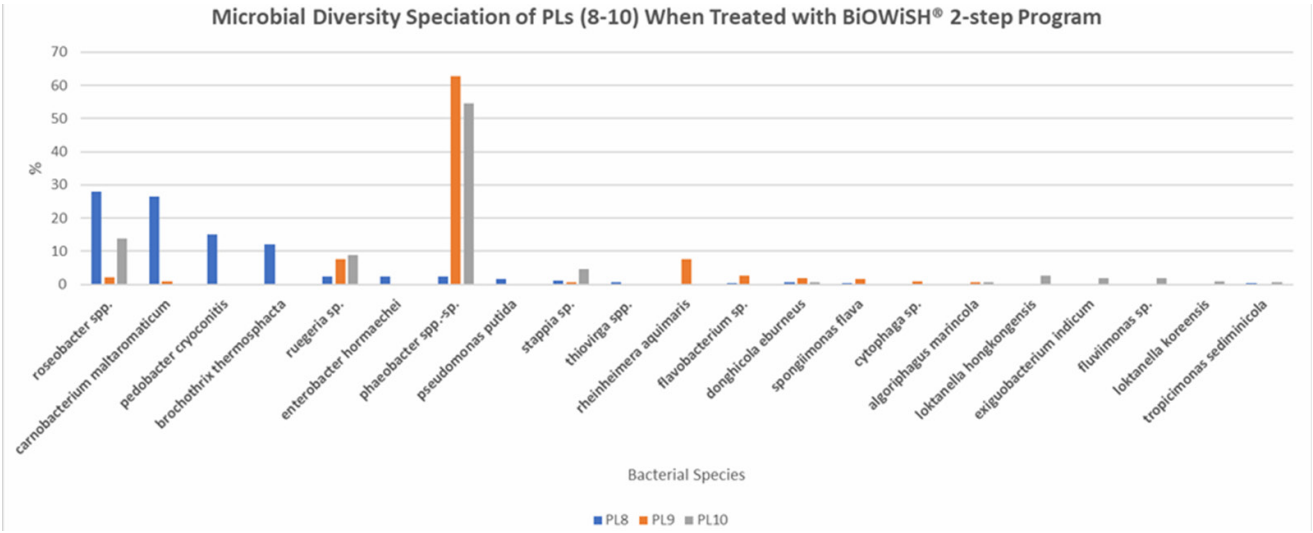


Figure 10: Genomic microbial diversity speciation of bacteria associated with post larvae as they develop from PL8-10 showing the predominant levels of increases in Phaeobacter spp. as post larvae mature from PL8-10.

Flexible Dosing Regimens

As noted earlier, fixed dosage requirements represent good practices during nominal hatchery conditions, but flexibility in dosing is required to maintain control of water quality and *Vibrio* levels when sudden changes occur during production. Daily monitoring of ammonia, nitrite, total bacteria, and *Vibrio* levels is critically important. Anticipating a change in water quality or *Vibrio* levels and then adjusting dosage up ahead of dangerous levels is more effective than trying to treat conditions after they reach critical levels with higher dosages.

Two particular points in hatchery post larvae production are important to focus on: the Zoae 2 stage and the transition to artificial feed. The Zoea 2 stage creates excess nutrients in the tank, providing a source for fast growing *Vibrio*. In the later stages of post larvae development artificial feed can lead to an excess of organic and nitrogen waste build up again, providing an opportunistic breeding ground for fast growing *Vibrio*. In both these situations, we have determined that increased dosage of BiOWiSH® Aquafarm from 4-8 mg/L, or higher when necessary, is required to maintain water quality and good post larvae health.

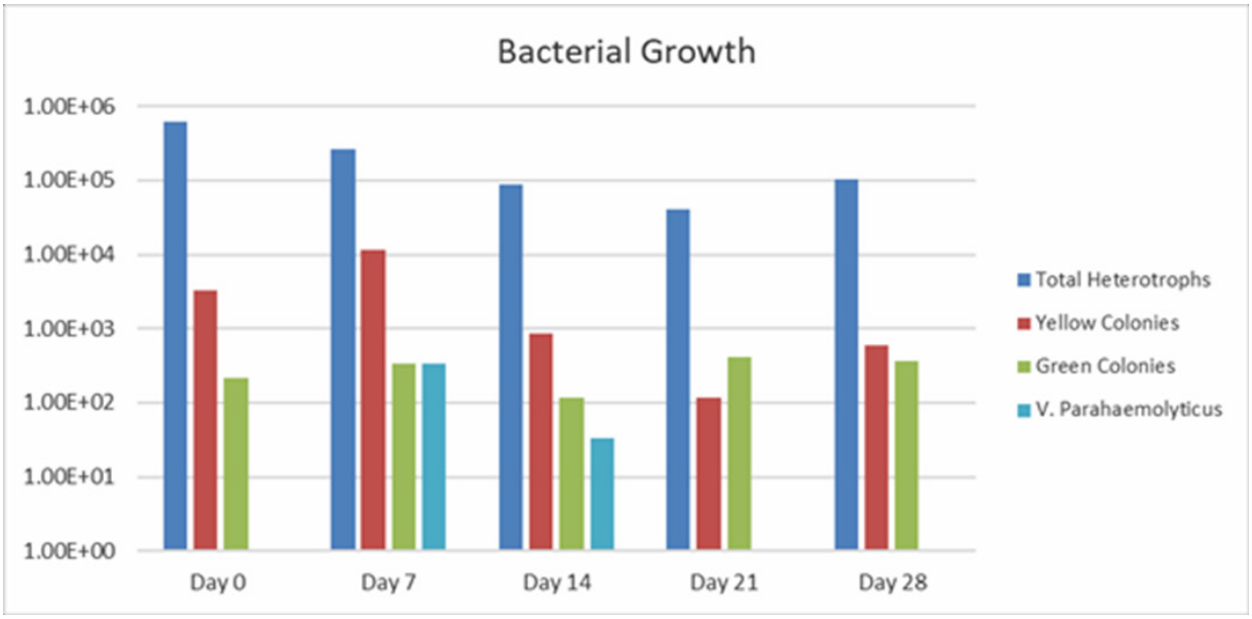
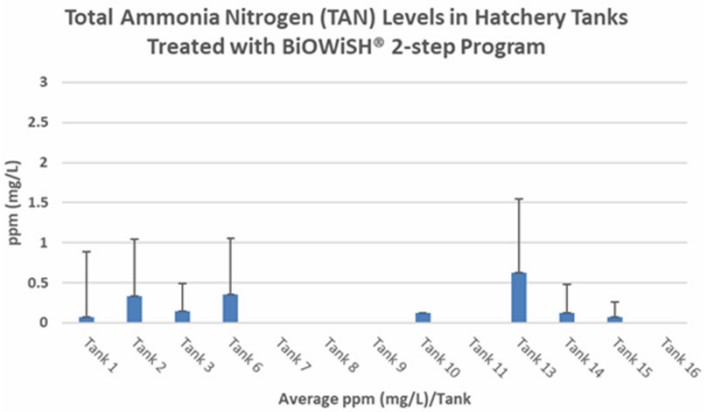
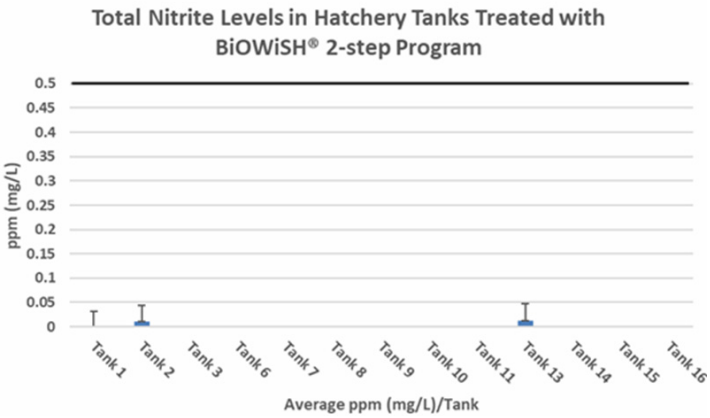


Figure 11: results over a 28-day hatchery production showing how a flexible dosing strategy when necessary can best maintain a strong heterotrophic population of beneficial bacteria while suppressing vibrio and inhibiting *V. parahaemolyticus* and luminous *Vibrio* species.

This is further demonstrated in figures 12 and 13, where BiOWiSH® has been obtaining data from ongoing hatchery productions using a flexible dosing regimen over time to demonstrate BiOWiSH® stability in controlling ammonia and nitrite levels, maintaining a high level of beneficial heterotrophic bacteria population in tanks, and control of *Vibrio* species with very low or no V. parahaemolyticus - and no detectable luminous *Vibrio*. The data in figure 11 represent hatchery data collected daily for a two-month period (October to November of 2017) in 16 different tanks. As the data show, BiOWiSH® AquaFarm maintains very good control over ammonia and nitrite levels.





Figures 12 and 13: Ammonia and nitrite measurements in hatchery tanks over a 2-month period showing control of nitrogen waste buildup. Results show excellent control of ammonia and nitrite levels. Critical concentration levels for ammonia are to be <3mg/L and for nitrite <0.5 mg/L. Each data point represents a mean value covering the variability observed over two hatchery cycles and approximately 60 days of production data collected daily.

Additionally, figure 14 shows data for total beneficial heterotrophic bacteria development and controlling *Vibrio* levels over the same period of time.

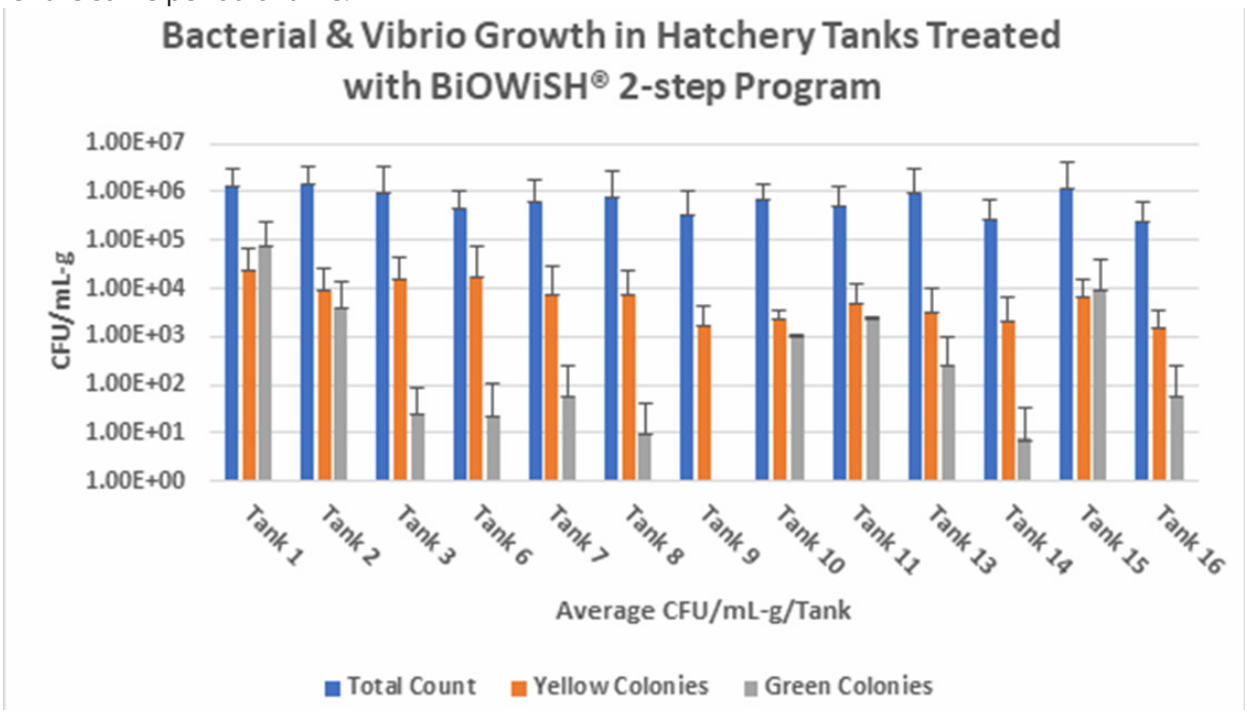


Figure 14: Total bacteria and vibrio measurements in hatchery tanks over a 2-month period (mean values for two months of daily data collection).

Summary

BiOWiSH® is continuing to collect and analyze data from ongoing hatchery production programs in cooperation with ShrimpVet Vietnam. An updated addendum to this data on hatchery water quality and bacteria monitoring will be reported shortly on further production monitoring.

Further research will be conducted using newly developed DNA profiling tools to better understand the role of heterotrophic bacteria development in improving shrimp health and resistance to disease.

User guides were developed as a result of this research and are available on the BiOWiSH® website.

Further studies through cooperation with ShrimpVet will report on the value of incorporating nursery technology into shrimp production and grow-out as soon as the data are available.

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