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The effects of artificial substrate and stocking density on Pacific white shrimp (*Litopenaeus vannamei*) performance and water quality dynamics in high tunnel-based biofloc systems

Leo J. Fleckenstein*, Nathan A. Kring, Thomas W. Tierney, Jill C. Fisk, Benjamin C. Lawson, Andrew J. Ray

School of Aquaculture, Kentucky State University Land Grant Program, 103 Athletic Drive, 40601Frankfort, KY, USA

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ABSTRACT

The use of artificial substrates in shrimp aquaculture may allow for production of shrimp at increased densities while providing a growth medium for microbes that assist with water quality processes and provide supplemental nutrition for shrimp. Greenhouse-based shrimp production systems can extend the shrimp production season in temperate climates while conserving water and energy. For this study, we evaluated the effects of providing extra substrate and shrimp density on water quality and shrimp production in greenhouse-based biofloc systems. Four 11-m^3, wood framed, and rubber-lined tanks were constructed in each of four high tunnel greenhouses (for a total of 16 tanks). Four treatments were evaluated: high-density stocking with substrate (HDS), high-density stocking with no substrate (HDNS), low-density stocking with substrate (LDS), and low-density stocking with no substrate (LDNS). Each treatment was randomly assigned to one tank in each tunnel to block for location. No artificial heat was used, and shrimp were grown for 120 days. High-density systems were stocked at 200 shrimp/m^3 while low-density tanks had 100 shrimp/m^3. Adding substrate increased total in-tank surface area by 13.4%. The addition of substrate had no significant effect on any shrimp production or standard water quality parameters. Shrimp had significantly greater final weight, faster growth rate, and lower feed conversion rate in low-density treatments ($P \leq 0.02$ for all). Total shrimp biomass production was significantly higher in high-density treatments (HD: 4.0 kg/m^3; LD: 2.3 kg/m^3; $P < 0.05$). There were no significant differences in survival between densities (HD: 91.3%; LD: 94.5%; $P = 0.43$). Peak and overall mean nitrite levels were significantly higher in high-density treatments compared to low-density treatments. Dissolved oxygen levels and pH over the course of the study were significantly lower in high-density treatments, likely due to increased respiration rates in the water column. This project shows the feasibility of shrimp production in temperate climates with no artificial heat using high tunnel greenhouses, few impacts of added substrate on shrimp production, and increased shrimp density can result in much larger harvests with few negative impacts on production metrics.

1. Introduction

As global demand for seafood increases, producers and researchers are looking to increase shrimp production, reduce costs, and limit waste outputs (Neori et al., 2007; Smith et al., 2010). One intensive production method that uses very little water exchange is the biofloc approach (Avnimelech, 2009). Biofloc particles are aggregations of bacteria, algae, fungi, and detritus that form naturally as a result of intensive nutrient inputs and minimal filtration (Crab et al., 2007; Xu and Pan, 2014). In contrast to clear-water (CW) recirculating aquaculture systems (RAS), which use external biofilters to house nitrifying bacteria, biofloc particles provide substrate for beneficial bacteria (Ebeling and Timmons, 2012). Biofloc particles can provide supplemental nutrition and may offer probiotic effects to animals like the Pacific white shrimp (*Litopenaeus vannamei*) (Coyle et al., 2011; Kent et al., 2011; Crab et al., 2012; Xu et al., 2012). The density of biofloc particles needs to be carefully managed. If levels are too high, issues with gill fouling, high oxygen consumption, and bacterial infections may occur (Hargreaves, 2013; Schveitzer et al., 2013). If biofloc levels are too low, nitrification or nitrogen assimilation rates may not be adequate.

The management of nitrogenous wastes is performed by different microbial communities in biofloc systems. Chemoautotrophic microbes

* Corresponding author.
E-mail address: leo.fleckenstein@kysu.edu (L.J. Fleckenstein).

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perform nitrification, which requires high levels of dissolved oxygen and pH. The nitrification process results in relatively low solids accumulation rates and low oxygen demand but consumes carbonate and produces nitrate (Ray and Lotz, 2014). Heterotrophic microbes assimilate dissolved inorganic nitrogen to build proteins. This process can be enhanced by the addition of organic carbon, such as sugars, to increase the dissolved C/N ratio and provide energy to the microbes (Pérez-Fuentes et al., 2016). Heterotrophic-dominated biofloc systems have low levels of nitrate accumulation compared to chemoautotrophic systems; however, the rates of solids accumulation and oxygen demand are greatly increased (Hargreaves, 2013). In situations where biofloc systems are exposed to light, photosynthetic organisms can reduce nitrogenous waste levels through assimilation (Ju et al., 2008). These organisms utilize light, carbon dioxide, and dissolved ammonia and nitrate to build organic material (Ju et al., 2009; Baloi et al., 2013). Biofloc systems contained in greenhouses tend to have a mixture of all of the above processes, making it important that managers understand the nuances of each (Ebeling et al., 2006; Wasielesky et al., 2006).

High tunnels are a simple, low-cost greenhouse, typically consisting of a metal frame with wood-framed end walls covered in clear, thin, polycarbonate sheeting. High tunnels help retain energy from solar radiation, which can warm the water in aquaculture tanks (Krummenauer et al., 2011). This decreases the need for external energy sources and can extend the growing season for tropical animals like shrimp, especially in temperate climates (McAbee et al., 2003). High tunnels are common in temperate areas like Kentucky; however, no studies using them for marine shrimp production in the region have been published to date.

The stocking density at which shrimp are grown may affect nitrogen processing rates, dissolved nutrient concentrations, suspended and dissolved solids concentrations, dissolved oxygen consumption, pH balance, and intraspecies competition (Wyban et al., 1987; Martin et al., 1998; Prangnell et al., 2016). Adding substrates to pond production systems facilitates stocking shrimp at higher densities by reducing competition for space and decreasing potential cannibalism (Moss and Moss, 2004; Anand et al., 2019; Olier et al., 2020). Such substrates can be made of durable plastic or fabric and are suspended vertically or horizontally in the system (Bratvold and Browdy, 2001). The added surface area from substrates may be colonized by beneficial microbes, potentially helping improve water quality by reducing ammonia concentrations (Azim et al., 2002). Mixtures of bacteria, algae, and other organisms naturally colonize submerged surfaces, creating a biofilm often referred to as periphyton. Periphyton on substrate and tank walls may serve as a source of supplemental nutrition, similar to biofloc particles (Anand et al., 2013).

Carbon and nitrogen stable isotope analyses can be used to estimate contributions of these elements from potential food sources, such as prepared feeds and biofloc (Burford et al., 2004; Ray et al., 2017). The isotopic signature of these components can help map the flow of nutrients through a food chain (Schroeder, 1983) because the isotope levels of animal tissues are generally similar to the animal's diet. Fractionation is a phenomenon in which organisms retain a greater proportion of heavier isotopes (C^{13} and N^{15}) than their food source so this should be considered when interpreting isotope data. Due to the long water retention times in biofloc systems, complex biological activity of shrimp and microbes, and the consistent input of feed, it is possible to have fluctuations in isotope concentrations and the concentrations of important dissolved elements provided in the salt and the feed (Wu and Yang, 2011; Hargreaves, 2013). Measuring changes in mineral and metal concentrations over the course of a shrimp production cycle may prove useful in estimating how long water can be reused (McNevin et al., 2004).

The limits of shrimp stocking density in simple, high tunnel-based biofloc systems are unclear as well as whether the addition of artificial substrate has any impact or interactive effects (Kumlu et al., 2001; Samocha et al., 1993). The purpose of this study was to examine the effects of added substrate and two stocking densities on shrimp production in high tunnel-based biofloc systems, without the use of artificial heat sources. By increasing available surface area and optimizing shrimp density in simple, seasonally operated high tunnels, the project may help farmers optimize shrimp production and improve their return on investment.

2. Materials and methods

2.1. System design

This experiment took place at the Kentucky State University High Tunnel Complex (HTC) located in Frankfort, KY. The HTC is a 6070-m^2 fenced area with four high tunnel greenhouses; the entire HTC is certified by the United States Department of Agriculture (USDA) for organic production. All four high tunnels are identical in size and construction. Each high tunnel is 30.5 m long × 9.1 m wide and has a total covered area of 278.6 m^2; the longest dimension is in an east-west orientation. The high tunnels have a gothic-style arched design and the side walls are 1.5 m tall. The tunnels are covered in two layers of 1.5-mm polycarbonate plastic sheeting that allow light through and helps retain heat. No artificial heat sources were used in the high tunnels to affect air or water temperatures. High tunnel temperature was regulated by raising or lowering the side curtains and opening or closing the windows and doors at either end of the tunnel. The North half of each high tunnel was dedicated to aquaculture research while the South half was used for organic vegetable production; a walkway separates the two halves.

Each high tunnel contained four identical wooden tanks. The chemicals used in treated lumber are prohibited by USDA Organic regulations; therefore, the tanks used in the study were constructed from untreated lumber coated with two layers of latex paint. The frames of the tanks were constructed of 38 × 140 cm lumber and the inner wall was a layer of 12-mm plywood lined with a layer of 6-mm polystyrene insulation. The internal dimensions of the tanks were 5.5 m long and 2.6 m wide and oriented longitudinally down the length of the high tunnels. The water level in each tank was 76 cm deep, creating a total volume of 10.9 m^3. The liner of the tank was a 4.5-mm ethylene propylene diene monomer (EPDM) pond liner. The liner was supported by layer of sand (an approximately 15-cm deep) to prevent damage. To prevent the walls of the tanks from collapsing outwards, four plastic-coated steel cables ran across the width of the tank and one cable ran the length of the tank on the top and bottom.

The filtration system for each tank consisted of a foam fractionator (Fig. 1) and a settling chamber. The fractionators were built using 15.2-cm diameter polyvinyl chloride (PVC) pipe for the body. Water was pumped into the fractionator by an in-tank pump with the flow rate set to 150 L per hour (LPH). A 5-cm air diffuser generated bubbles in the fractionator. The settling chambers were 567-L cone-bottomed tanks with a 10.2-cm diameter baffle suspended in the middle of the chambers based on the design of Ray et al. (2011). The settling chambers contained plastic mesh material (1.4-cm square openings) placed inside the chamber to increase solids capture. An in-tank pump supplied water to the settling chamber and flow rates were set to 600 LPH. Waste from both filters was disposed of weekly.

Aeration was provided to each high tunnel by a 2.5-HP regenerative blower. A 7.6-cm diameter PVC pipe carried air the length of the tunnel that was stepped down to a 5-cm line at each tank. The 5-cm line carried air to a PVC manifold that delivered air to ten evenly dispersed 22.8-cm diameter rubber-faced air diffusers with small holes punctured in the rubber.

The substrate was a polyethylene safety fence that was suspended from the upper steel cables of each tank and attached to the air manifold at the bottom. The substrate mesh size was 9.6 × 4.3cm, which allowed free movement of shrimp and water through the material. Four 2.6 × 1-m sheets of substrate were hung, evenly spaced, across the

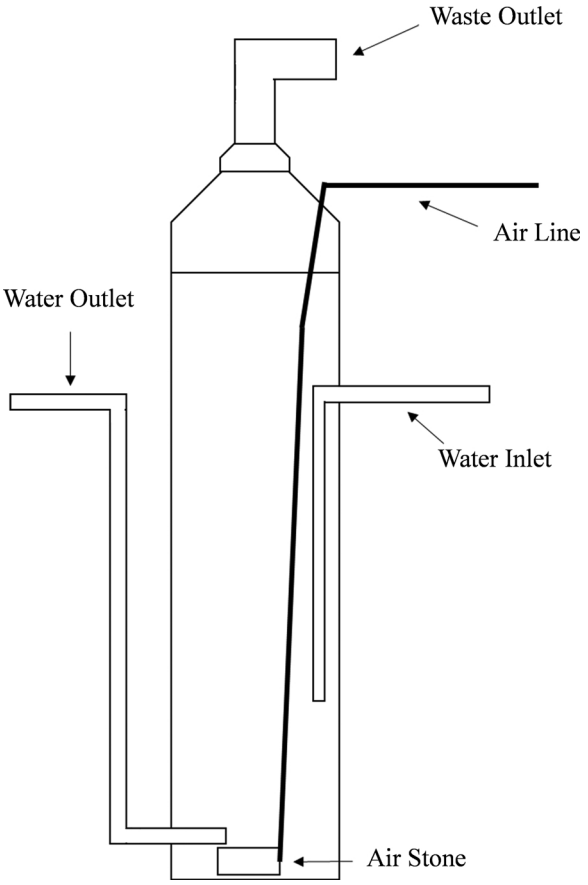

Fig. 1. The design of the PVC foam fractionators used for this project.

width of the tank and a single 5.5 × 1-m section hung down the length of the tank near the center. The substrate added 4.2 m^2 of additional surface area in each tank, which was an increase of 13.4% when accounting for the tank sides, bottom, and aeration equipment in the water.

2.2. Experimental design

The experiment contained four treatments with four replicate tanks each. The treatments were high-density stocking with substrate (HDS), high-density stocking with no substrate (HDNS), low-density stocking with substrate (LDS), and low-density stocking with no substrate (LDNS). One replicate from each treatment was randomized to each of the four tunnels to block for variability between tunnels. The high-density tanks were stocked at 200 shrimp/m^3, for a total of 2180 shrimp per tank. The low-density tanks were stocked at 100 shrimp/m^3, for a total of 1090 shrimp per tank.

2.3. Animal husbandry

The shrimp were shipped to Kentucky State University as post-larvae from Shrimp Improvement Systems (Islamorada, FL, USA). The shrimp were reared in an indoor, 20-m^3 nursery tank for 55 days until stocking in the tanks used in the experiment. Varying crumble sizes of Zeigler Brothers Raceway Plus Diet were fed during the nursery stage (Zeigler Brothers, Inc., Gardners, PA, USA), which contained 50% protein, 15% fat, 1% fiber, 10% moisture, and 7.5% ash. Before stocking into the experimental tanks, the shrimp were transitioned onto Zeigler Hyper-intensive Shrimp 35, a 2.5-mm diet with 35% protein, 7% fat, 2% fiber, 12% moisture, and 15% ash (manufacturer's reporting) and were fed this diet throughout the experiment. After the nursery phase, the shrimp were stocked into the experimental tanks at an average of 7.15 g/shrimp and grown for 120 days. All tanks were provided the same amount of feed per shrimp, regardless of treatment (i.e., high density tanks received twice the amount of feed compared to low density tanks). Each tank was hand twice daily at approximately 1200 and 1600 h. Feed amounts were calculated based on a baseline estimated feed conversion rate (FCR) of 1.5:1 and a growth rate of 1.5 g/week; observations of feed consumption and water quality factors, especially temperature, were also used to adjust feed rations. Individual shrimp weights were determined every two weeks to monitor growth rates. At harvest, 100 shrimp per tank were weighed individually; the bulk weight of all shrimp harvested from each tank was also determined. This information, along with feed amounts, was used to calculate survival, FCR, mean weight, and growth rates.

2.4. Water quality

Temperature, dissolved oxygen (DO), pH, and salinity were measured twice daily at approximately 0830 and 1600 h using a YSI ProDSS MultiMeter (Yellow Springs, Inc Yellow Springs, Ohio, USA). Total ammonia-nitrogen (TAN), nitrite-N (NO_2), nitrate-N (NO_3), and turbidity were measured weekly. TAN, NO_2, and NO_3 were measured using Hach methods 8155, 8507, and 8039, respectively, using a Hach DR6000 Spectrophotometer (Hach Company, Loveland, CO, USA). Turbidity was measured using a Hach 2100q Turbidimeter and reported in nephelometric turbidity units (NTU).

2.5. System maintenance

The water used for this experiment had been used for a 142-day tilapia study that examined two fish densities (50 and 75 fish per m^3) in biofloc systems conducted in the high tunnel tanks the previous year. Prior to the current experiment, the water was homogenized among the high tunnel tanks and redistributed to all tanks evenly. Salinity for all tanks was adjusted to 15 ppt using Crystal Sea Bioassay Laboratory Formula (Marine Enterprises International, Baltimore, MD, USA). Dissolved oxygen levels were maintained above 5.7 mg/l by adjusting the airflow to each tank as needed. All high tunnels were managed to maintain water temperature between 27.5 and 28.5 °C by raising or lowering the sides and windows on the tunnel to retain or release heat. For all tanks, water lost to evaporation was replaced with rainwater that was collected from the tops of the high tunnels and stored in large tanks below ground level. Decreases in pH were corrected with the addition of sodium bicarbonate to maintain a target pH of 7.8. Solids (biofloc) levels were managed by operating the settling chambers and foam fractionators as needed; the target range of turbidity was between 50 and 100 NTU (Ray et al., 2010, 2011; Schveitzer et al., 2013).

2.6. Isotope and elemental analyses

Shrimp, feed, and biofloc material were sampled for isotope analysis at harvest from all 16 tanks. Five shrimp samples from each tank were dried at 60 °C, ground, digested with 10% HCl solution to remove inorganic carbon, and rinsed with water (Bunn et al., 1995). Biofloc samples were collected by centrifuging water samples collected directly from the tanks. All samples were dried at 60 °C, ground finely, and sent to the University of Arkansas Stable Isotope Laboratory (Fayetteville, AR, USA) for isotope analysis. Samples were combusted in an elemental analyzer and the resulting gas was analyzed using a Delta Plus Mass Spectrometer (ThermoFisher Scientific, Waltham, MA, USA). C and N isotope concentrations were used to calculate $\delta^{13}C$ and $\delta^{15}N$ values as:

$$\delta = \left(\frac{Rsample}{Rstandard} - 1\right)x1000$$

where R is the ratio of heavy/light isotopes ($^{13}C/^{12}C$ or $^{15}N/^{14}N$).

Analyses of final water samples for dissolved nitrate and the elements Na, Mg, P, K, Ca, Cr, Fe, Ni, Cu, As, Sr, Cd, Ba, Hg, and Pb were performed at the University of Georgia's Laboratory for Environmental Analysis. The elements were measured using inductively coupled plasma-mass spectrometry (ICP-MS) and nitrate was measured using ion chromatography.

2.7. Data management and statistical analyses

Shrimp production metrics, water quality data, and isotopic data were analyzed using Statistix 10 and R software. Any non-parametric data were transformed to meet the assumption of the tests. An α-value of 0.05 was used to determine whether there were significant differences due to density or substrate. A two-way analysis of variance (ANOVA) was used for final data collected at the end of the study, including isotope data and shrimp production data. A two-way repeated measures (RM) ANOVA was used for all water quality data collected weekly or daily. The mean morning and afternoon readings were combined for all daily water quality parameters (temperature, DO, pH, salinity).

3. Results

No significant interactions were detected between substrate and density for any variable measured. Substrate did not significantly impact any water quality, shrimp production, or isotope dynamics metric measured. Total ammonia nitrogen (Fig. 2) and average daily salinity concentrations were not significantly different among treatments regardless of density (Tables 1 and 2). Average daily temperature (Fig. 3) and turbidity (Fig. 4) were significantly higher in both high density treatments while average daily dissolved oxygen (Fig. 5) and average daily pH (Fig. 6) were significantly lower in high density treatments. Nitrite (Fig. 7) concentrations were significantly higher in both high-density treatments over the course of the study. Final nitrate (Fig. 7) concentrations were significantly higher in high density treatments. Final nitrate concentrations dropped significantly in low density treatments or were stable in high density treatments when compared to initial nitrate concentrations.

Density significantly impacted shrimp performance. Average final weight per individual, growth rate, and FCR were all negatively impacted by high density (Table 3). Overall shrimp harvest was significantly higher in the high-density treatments than in the low-density treatments.

Isotope levels in shrimp tissues were not significantly affected by density or substrate (Table 4). For biofloc, δN^{15} levels were significantly greater in high density treatments than in low density treatments, however there was no significant differences in percent N. There were no differences in δC levels, however the percentage of carbon found in low density treatments was significantly higher than that of the high-density treatments.

Treatments with substrate had significantly higher levels of dissolved Mg and P than treatments without substrate (Table 5). Treatments without substrate had significantly higher levels of Ba than treatments with substrate. No significant differences between treatments were found for any other elements measured.

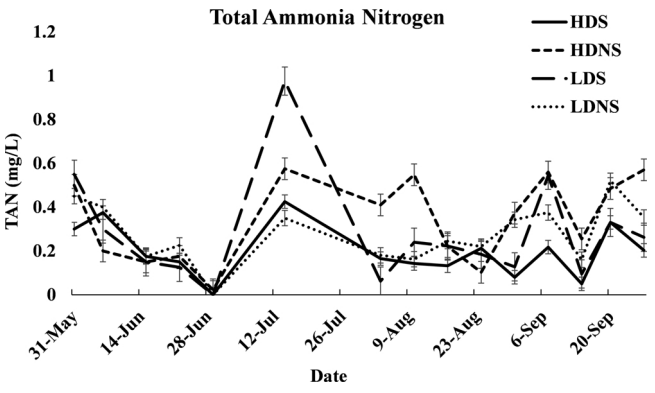

Fig. 2. Mean total ammonia nitrogen concentration for each treatment over the study.

4. Discussion

Density significantly impacted several shrimp production and water quality metrics as well as δN^{15} and percent C values in biofloc material, while substrate impacted turbidity and the concentration of three dissolved elements. Higher turbidity levels are likely indicative of increased microbial abundance in high density treatments, which probably augmented water column respiration. The low pH and dissolved oxygen levels in high density treatments are indicative of this increased respiration, along with greater levels of shrimp respiration. Such findings have been noted in other density studies in which microbes depleted oxygen and added carbon dioxide to the water column, driving down pH (Martin et al., 1998; Prangnell et al., 2016).

The increased turbidity in high density treatments may have also influenced thermal dynamics of the systems, causing increased solar radiation absorption as a result of darker water, which could lead to higher water temperature (Paaijmans et al., 2008). Although mean treatment temperatures may not appear substantially different in Fig. 3, the RM ANOVA detects even subtle differences that occur repeatedly in a data set. Water temperature was maintained within acceptable levels for L. *vannamei* survival without the use of artificial heat sources; however, the large daily fluctuations in temperature may have impacted shrimp performance (Wyban et al., 1995). Although heating costs can be one of the main expenses in RAS operations that produce tropical species like L. *vannamei* (Masser et al., 1999; Ebeling and Timmons, 2012), outdoor systems are inherently less stable than indoor systems. Some studies using indoor systems with high amounts of environmental controls have exceeded growth rates of 2 g/week, something shrimp producers should consider when designing their systems (Fleckenstein et al., 2019).

Elevated nitrite and nitrate concentrations found in the high-density treatments are relatively common in biofloc shrimp systems and have been found in other density studies (Wyban et al., 1987; Esparza-Leal et al., 2010). In both high- and low-density treatments, nitrate levels were similar or significantly lower than the levels found at the start of the study. This could be, in part, because these systems were exposed to natural sunlight, supporting a predominantly photoautotrophic biofloc with an abundant algal community. Algae extract nitrogen, including nitrate, from the water to build cellular proteins (Ebeling et al., 2006). In wastewater treatment, high-rate algal ponds (HRAPs) are heavily mixed, high-algal biomass, open raceways used for nutrient removal (Park et al., 2011). These HRAP systems allow portions of the algae to be harvested, removing nitrogen from the system over time. Nitrogen removal rates in HRAP systems are up to 2.5 mg/l per day. In this study, the estimated amount of nitrogen removal from the shrimp production systems in this study was around 1.8 mg/l per day, accounting for initial nitrogen in the water, N content of feed added, and N content of the shrimp removed. According to Craggs et al. (2012), HRAP systems typically have water quality (turbidity, pH, chlorophyll *A* concentration) similar to those in the current study (Kring et al., 2019). In addition, several studies examining the effects of supplemental light on indoor shrimp systems have noted a large decrease in nitrate accumulation due to increased levels of light and suggest the growth and removal of algae from the systems as the cause (Baloi et al., 2013; Fleckenstein et al., 2019).

Although there was not a specific denitrification filter on these systems, it is also possible that some denitrification occurred in the settling chambers at the interface of water and settled solids, a process

Table 1
Water quality parameters measured twice per day. Data are presented as mean ± standard error. Different superscript letters within rows denote significant differences between treatments. *P*-values are presented for the effects of density, substrate, and interactions between these factors.

	High density		Low density		*P*-values		
	HDS	HDNS	LDS	LDNS	Den	Sub	Int
Temperature (°C)	26.5^{a} ± 0.1	26.5^{a} ± 0.1	26.2^{b} ± 0.1	26.3^{b} ± 0.1	0.05	0.59	0.62
Dissolved Oxygen (mg/l)	7.0^{a} ± 0.0	7.0^{a} ± 0.0	7.3^{b} ± 0.0	7.3^{b} ± 0.0	0.00	0.21	0.72
Salinity (ppt)	17.5 ± 0.0	17.6 ± 0.0	17.5 ± 0.0	17.9 ± 0.0	0.54	0.54	0.57
pH	8.1^{a} ± 0.0	8.1^{a} ± 0.0	8.2^{b} ± 0.0	8.2^{b} ± 0.0	0.00	0.95	0.54

HDS: High Density with Substrate, HDNS: High Density No Substrate, LDS: Low Density with Substrate, LDNS: Low Density No Substrate, Den: Density, Sub: Substrate, Int: Interaction.

described by Ray et al. (2010). The settling chambers in this study had a water retention time of around one hour, likely creating an anerobic environment ideal for denitrification (Van Rijn et al., 2006). The netting inside the settling chambers added additional surface area for the microbial community which may have amplified the denitrification process. Since nitrate is a major limiting factor in water reuse (Ebeling and Timmons, 2012), the consistent removal of nitrate in these high tunnel-based biofloc systems may offer shrimp producers an advantage because they can reuse water for extended periods of time, thereby reducing water and salt costs as well as reducing waste discharge (Browdy et al., 1995; Whetstone et al., 2000).

Lower turbidity and significantly higher P and Mg concentrations in the treatments with substrate may point to lower algal abundance. Algae use P from the water for growth, sunlight for energy, and Mg as a component of chlorophyll. Substrates likely blocked some light, which consequently reduced algal abundance and allowed relatively higher P and Mg concentrations in the water (Finkle and Appleman, 1953; Delorenzo et al., 2012; Guo et al., 2013). The cause of the decreased Ba levels in systems with substrate is unclear; however, Ba is normally found in marine environments as barium sulfate and is likely not toxic to penaeid shrimp and had little impact on this study (Daugherty, 1951).

The increased carbon percentages in biofloc from low density treatments may be a result of algae accumulating carbon since algae tend to generate more carbohydrates than bacteria do (Ebeling et al., 2006).The higher biofloc δN^{15} values in high density treatments may be due to a shift in biofloc composition that corresponds with increasing density or could be a result of the biofloc system processing more N due to higher feed inputs. Biofloc systems are composed of organisms across a number of phyla (Avnimelech, 2009; Xu and Pan, 2014). The variety of organisms present in such biofloc particles may form several trophic levels. At each subsequent trophic level, the biofloc becomes isotopically heavier due to fractionation. Although increased δN^{15} levels were not found in shrimp tissues in this study, this isotopic enrichment in biofloc may have implications in studies where shrimp rely more on biofloc as a food source.

Although final individual shrimp weight, growth rate, and survival were all significantly lower in high density treatments, overall biomass production increased significantly, which is similar to the findings of other shrimp density studies (Moss and Moss, 2004; Esparza-Leal et al.,

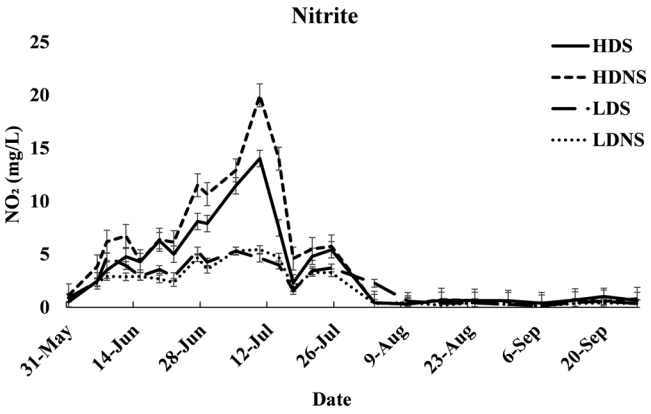

Fig. 3. Mean temperature for each treatment over the study.

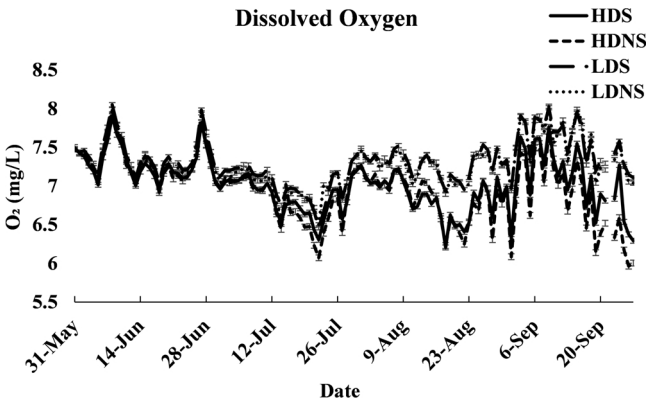

Fig. 4. Mean turbidity for each treatment over the study.

Table 2
Water quality parameters measured weekly. Data are presented as mean ± standard error. All measurements in mg/l unless otherwise noted. Values with different superscripted letters within rows denote significant differences between treatments. *P*-values are presented for the effects of density, substrate, and interactions between these factors.

	High density		Low density		*P*-values		
	HDS	HDNS	LDS	LDNS	Den	Sub	Int
TAN	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.09	0.85	0.09
Nitrite	3.9^{a} ± 0.1	5.2^{a} ± 0.1	2.5^{b} ± 0.0	2.2^{b} ± 0.0	0.02	0.54	0.34
Initial Nitrate-N	162.3 ± 8.8	175.4 ± 10.1	175.8 ± 10.6	177.6 ± 10.8	0.95	0.98	0.97
Final Nitrate-N	168.5^{a} ± 10.9	165.2^{a} ± 7.2	91.9^{b} ± 13.0	94.5^{b} ± 10.7	0.00	0.98	0.86
Turbidity (NTU)	116.3^{a} ± 10.7	129.3^{a} ± 14.4	104.4^{b} ± 8.2	108.2^{b} ± 9.5	0.05	0.21	0.39

HDS: High Density with Substrate, HDNS: High Density No Substrate, LDS: Low Density with Substrate, LDNS: Low Density No Substrate, Den: Density, Sub: Substrate, Int: Interaction.

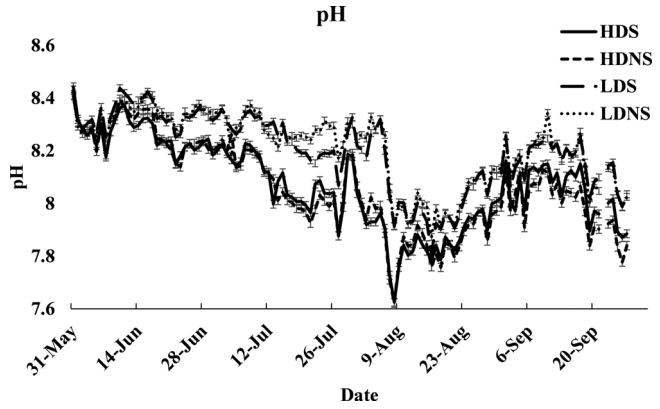

Fig. 5. Mean dissolved oxygen concentration for each treatment over the study.

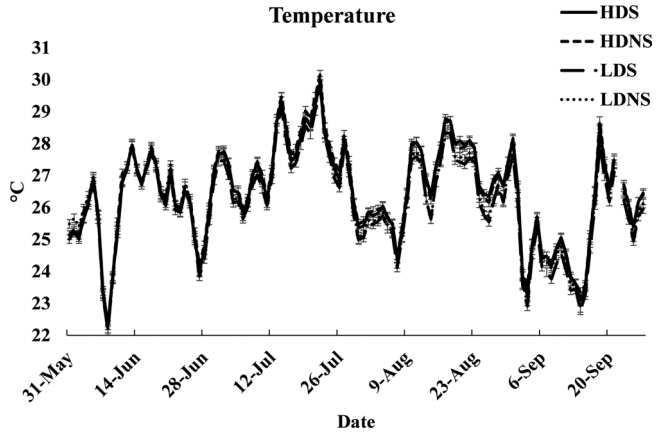

Fig. 6. Mean pH in each treatment over the course of the study.

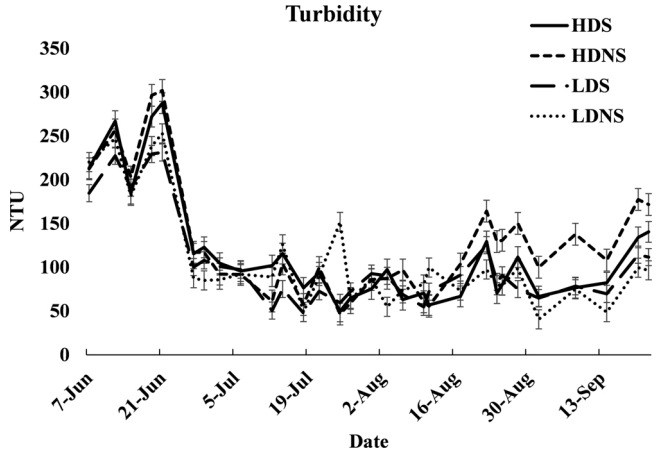

Fig. 7. Mean nitrite-N concentration in each treatment over the course of the study.

2010). The greatly increased biomass production makes a strong case for the use of the higher shrimp density. Overall, shrimp produced at high density were approximately 6% smaller than those grown at low density. At a mean weight of 24.8 g, the shrimp from high density treatments would still be considered large or jumbo shrimp for the purpose of marketing in the US. These size categories fetch a relatively high price in the market.

This study resulted in the best survival rates of any experiment at KSU's Center for Sustainable Shrimp Aquaculture Production, including those in indoor, controlled systems. High survival across all treatments and among all 16 tanks, suggests the positive potential for producing marine shrimp in an inland location using high tunnel greenhouses. Regardless of the environmental fluctuations that these systems may experience, shrimp survival remained high. In addition, the FCR was very low in all treatments, ranging from 1.0–1.1. Although the isotopic data do not indicate that shrimp obtained substantial amounts of carbon or nitrogen from the biofloc, this level of feed efficiency warrants further investigation. Feed rations were very carefully adjusted based on a variety of data points, including apparent consumption. By allowing time between feedings during which there was no apparent feed available, this may have given the shrimp a chance to benefit from ingesting the biofloc. There is some evidence that the presence of biofloc may increase enzymatic activity in the digestive tract of shrimp by increasing feed efficiency and lowering FCRs (Becerra-Dorame et al., 2012; Xu and Pan, 2012).

Overall, there were few effects from the addition of substrate, besides effects on elemental dynamics in the water due to algal production and shading. The substrate used in this study was composed of individual sheets with large openings. Although this allowed for increased movement of shrimp and water through the substrate, overall surface area was lower compared to substrates used in other studies (Bratvold and Browdy, 2001; Azim et al., 2002; Anand et al., 2019; Olier et al., 2020). This lower surface area availability reduced space for nitrifying microbes and periphyton production, likely minimizing the impacts of the added substrate. The substrate used in this study (safety fence) is cheap and widely available. The density of this substrate could be increased in shrimp tanks without increasing cost substantially and may result in significant impacts on shrimp production.

Producers are generally aware of the effects of density on water quality and long-term water use; however, the 79% increase in final biomass in high-density treatments versus the low-density treatments in this study seems to offset those shortcomings since production costs would be roughly equivalent regardless of density. Feed management, water quality measurements, and other daily routine procedures were the same regardless of density, which means the systems had similar labor requirements. In addition, aeration was provided equally to all tanks, resulting in similar energy requirements regardless of density, although additional research could determine whether energy use may be reduced with lower shrimp density. Maximizing shrimp density in simplistic high tunnel greenhouses appears to be a feasible production strategy under the conditions of this study.

CRediT authorship contribution statement

Leo J. Fleckenstein: Conceptualization, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Nathan A. Kring:** Investigation, Resources, Writing - original draft. **Thomas W. Tierney:** Formal analysis, Investigation, Resources, Writing - review & editing. **Jill C. Fisk:** Investigation, Resources. **Benjamin C. Lawson:** Investigation, Resources. **Andrew J. Ray:** Conceptualization, Formal analysis, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 3
Shrimp production metrics. Data are presented as mean ± standard error. Values with different superscripted letters within rows denote significant differences between treatments. *P*-values are presented for the effects of density, substrate, and interactions between these factors.

	High density		Low density		*P*-values		
	HDS	HDNS	LDS	LDNS	Den	Sub	Int
Individual wt. (g)	24.5^{a} ± 0.3	25.0^{a} ± 0.4	26.7^{b} ± 0.2	25.9^{b} ± 0.2	0.02	0.99	0.22
Growth Rate (g/wk)	1.0^{a} ± 0.0	1.0^{a} ± 0.0	1.1^{b} ± 0.0	1.1^{b} ± 0.0	0.02	0.99	0.22
Total Harvest (kg)	44.7^{a} ± 0.5	43.4^{a} ± 1.0	25.0^{b} ± 0.2	24.3^{b} ± 0.7	0.00	0.47	0.79
Biomass (kg/m^3)	4.0^{a} ± 0.0	3.9^{a} ± 0.0	2.3^{b} ± 0.0	2.2^{b} ± 0.0	0.00	0.47	0.79
FCR	1.2^{a} ± 0.3	1.3^{a} ± 0.2	1.1^{b} ± 0.2	1.1^{b} ± 0.3	0.02	0.42	0.96
Survival (%)	90.6 ± 5.4	91.8 ± 3.1	91.9 ± 2.4	97.2 ± 5.3	0.43	0.40	0.60

HDS: High Density with Substrate, HDNS: High Density No Substrate, LDS: Low Density with Substrate, LDNS: Low Density No Substrate, Den: Density, Sub: Substrate, Int: Interaction.

Table 4
Isotope values and C and N concentrations of biofloc material and shrimp tissues. Data are presented as mean ± standard error. Values with different superscripted letters within rows denote significant differences between treatments. *P*-values are presented for the effects of density, substrate, and interactions between these factors.

		High density		Low density		*P*-values		
		HDS	HDNS	LDS	LDNS	Den	Sub	Int
Biofloc	**δC^{13}**	−28.3 ± 0.2	−29.0 ± 0.1	−29.1 ± 0.2	−29.1 ± 0.1	0.19	0.28	0.27
	δN^{15}	12.4^{a} ± 0.3	12.6^{a} ± 0.2	11.5^{b} ± 0.2	10.6^{b} ± 0.3	0.01	0.50	0.24
	%C	28.0^{a} ± 0.3	26.7^{a} ± 2.0	30.4^{b} ± 1.7	34.6^{b} ± 1.0	0.05	0.61	0.34
	%N	4.1 ± 0.0	3.8 ± 0.3	4.2 ± 0.3	4.8 ± 0.2	0.22	0.74	0.29
Shrimp	**δC^{13}**	−21.1 ± 0.0	−21.0 ± 0.1	−21.1 ± 0.1	−21.0 ± 0.1	0.89	0.30	0.71
	δN^{15}	9.6 ± 0.1	9.6 ± 0.0	9.7 ± 0.0	9.4 ± 0.0	0.63	0.07	0.35
	%C	43.4 ± 0.1	43.2 ± 0.2	43.7 ± 0.1	42.2 ± 0.4	0.48	0.12	0.27
	%N	12.2 ± 0.0	12.2 ± 0.1	12.3 ± 0.0	11.9 ± 0.1	0.47	0.19	0.12

HDS: High Density with Substrate, HDNS: High Density No Substrate, LDS: Low Density with Substrate, LDNS: Low Density No Substrate, Den: Density, Sub: Substrate, Int: Interaction.

Table 5
Dissolved elements in final water samples. Data are presented as mean ± standard error. Values with different superscripted letters within rows denote significant differences between treatments. *P*-values are presented for the effects of density, substrate, and interactions between these factors.

	High density		Low density		*P*-values		
	HDS	HDNS	LDS	LDNS	Den	Sub	Int
Na (ppm)	6158.0 ± 47.5	6202.8 ± 33.3	6154.8 ± 38.5	6049.0 ± 15.3	0.10	0.09	0.16
Mg (ppm)	289.6^{a} ± 3.3	284.5^{b} ± 2.3	289.3^{a} ± 3.1	259.8^{b} ± 1.7	0.10	0.03	0.11
P (ppm)	9.8^{a} ± 1.2	6.1^{b} ± 1.4	10.7^{a} ± 1.4	4.7^{b} ± 1.6	0.73	0.01	0.41
K (ppm)	346.3 ± 8.3	347.0 ± 7.8	342.5 ± 7.5	315.8 ± 9.6	0.11	0.07	0.12
Ca (ppm)	78.8 ± 1.6	85.8 ± 1.6	79.9 ± 1.6	84.0 ± 1.8	0.81	0.12	0.96
Fe (ppm)	1.0 ± 0.0	1.2 ± 0.0	1.0 ± 0.0	1.1 ± 0.1	0.21	0.09	0.54
Sr (ppm)	3.7 ± 0.3	4.8 ± 0.3	3.7 ± 0.3	4.1 ± 0.3	0.55	0.38	0.62
Cd (ppb)	0.5 ± 0.6	1.9 ± 0.6	0.8 ± 0.6	0.7 ± 0.2	0.29	0.51	0.30
Ba (ppb)	29.9^{b} ± 0.6	44.6^{a} ± 0.6	28.7^{b} ± 0.6	43.2^{a} ± 0.6	0.96	0.00	0.77
Hg (ppb)	1.9 ± 2.1	2.1 ± 2.2	2.0 ± 2.4	2.0 ± 0.7	0.27	0.65	0.17

HDS: High Density with Substrate, HDNS: High Density No Substrate, LDS: Low Density with Substrate, LDNS: Low Density No Substrate, Den: Density, Sub: Substrate, Int: Interaction.

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