Next-Generation Aquaculture Systems Integrating Microbial Engineering, Biofloc Technology, and Circular Resource Utilization

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ABSTRACT

With aquaculture expanding quickly all around the world, there is a need to pursue the intensification of aquaculture sustainably, aligning with improved technologies and better use of land and water resources. This study compares a novel Integrated Microbial Engineering System (IMES), which involves the use of biofloc technology (BFT), targeted microbial consortia inoculations and circular resource utilization, with a conventional recirculating aquaculture system (RAS) and standard BFT in a 90-day growout trial of Pacific white shrimp *L. vannamei* at a density of 300 shrimp/m³. IMES vastly exceeded both of the control systems with respect to all major performance measures. Specific growth rate in IMES reached 4.18 ± 0.13%/day compared with 3.24 ± 0.10%/day in BFT and 2.11 ± 0.14%/day in Control ($p < 0.05$). Feed conversion ratio was lowest in IMES (1.18 ± 0.04) and survival reached 91.2 ± 1.5%. The ammonia levels of TAN were kept below 0.4 mg/l during the entire trial of IMES. The MiSeq 16S rRNA amplicon sequence analysis showed a unique nitrifier-enriched microbial community present in IMES (dominated mostly by Proteobacteria (38.1%) and Bacteroidetes (28.3%)). The NRE was 71.3% and WRE was 94.6% for IMES, indicating very good improvements in the implementation of the Circular Economy in aquaculture. The results shown here indicate that the IMES approach is feasible and scalable for next generation of sustainable intensive aquaculture.

Keywords: Biofloc technology; *Litopenaeus vannamei*; Microbial engineering; Circular economy; Nitrogen cycling; Recirculating aquaculture systems; Sustainable aquaculture.

How to cite this article: Dwivedi A, Sharma JG. Next-Generation Aquaculture Systems Integrating Microbial Engineering, Biofloc Technology, and Circular Resource Utilization. Int J Drug Deliv Technol. 2026;16(51s): 1884-1892. DOI: 10.25258/ijddt.16.51s.152

Source of support: Nil.

Conflict of interest: None.

1. Introduction

Aquaculture is the fastest growing food production system in the world and has become the major source for aquatic food for human consumption, even as compared to fishery from the wild. The production in 2020 was well over 87.5 million tonnes and has been steadily increasing at an annual rate of about 5.3%, fueled by the over-50% average seafood demand per capita, which has been found to be on the decline, and dwindling wild fish stocks and increased consumer awareness of the health benefits of high-quality aquatic protein (FAO, 2022). To feed the expected human population of ten billion people in 2050, aquaculture production needs to increase by almost two-fold in the next 30 years to meet the 1.3% requirement of proteins (Tacon & Metian, 2008). This requirement puts a strain on freshwater resources, coastal ecosystems and on the global nitrogen cycle as well—in an unprecedented way, especially since intensive aquaculture production is a nutrient-intensive process.

Owing to the high amounts of dissolved inorganic nitrogen, which includes the nitrogen forms ammonia and nitrate, as well as suspended organic solids and unutilised feed and unexcretion waste (Crab et al., 2007), the conventional flow-through and semi-intensive aquaculture systems in ponds generate huge numbers of effluents. These effluents when discharged to receiving water bodies cause eutrophication, trigger HABs and create hypoxic dead zones for coastal waters. The economic and ecological damages from aquaculture nutrient pollution have encouraged more and more stringent regulation in many of the major aquaculture producing countries, and this resulted in the development and adoption of water treatment and recirculation technologies which disconnect nutrient pollution from its discharge in the environment (Hargreaves, 2006). Biotechnology for aquaculture (BFT) and integrated microbial management, along with recirculating aquaculture systems (RAS), are the three most promising technological pathways for sustainable intensification in this case.

Recirculating aquaculture systems were developed in response to the need to treat water quality and manage discharge in intensive production which involve filters remove waste and microorganisms from the culture water from a closed loop system that can allow multiple times as much water to be used as in a flow through system. The focus of RAS operation is the moving bed biofilm reactor (MBBR), or submerged biofilter (Hargreaves, 2006), which contains autotrophic nitrifying bacteria that can convert toxic ammonia to nitrite, and then to fairly benign nitrate. In spite of these benefits, traditional RAS systems require significant capital outlay for the filtration system, produce concentrated sludge streams in need of secondary processing and may suffer from excessive rate of volatilization of nitrification reactions due to high organic loads from more intensive shrimp culture. The operational energy costs which are mainly the cost of aeration and pump work further restrict the economic viability of introducing RAS in the commercial systems and smallholder and medium scale producers are unable to adopt the RAS technology when designing state of the art aquaculture in the developing aquaculture economic systems.

Biofloc technology was derived from "applied understanding of activated sludge microbiology" and provided an “all new concept to consider for in situ nitrogen management”. BFT systems maintain a high C:N ratio (typically in the 12:1 - 20:1 range) in the culture water by adding an external carbon source, like molasses, sucrose or tapioca starch, which promotes the growth of heterotrophic bacteria that use one of the two carbon sources to convert inorganic N directly into microbial biomass rather than through conventional autotrophic nitrification per unit volume at significantly higher rates (Avnimelech, 1999; De Schryver et al., 2008). The organisms that grow on the microbial floc are 25–50% crude protein (CP) coupled with lipids, vitamins, minerals and bioactive compounds that provide water purification and additional feed for the cultured organisms at once, thus decreasing the need for purchasing ready-made pellet feed to lower the feed conversion ratio (FCR) (Ekasari et al., 2010; Kuhn et al., 2010). BFT for the production of Pacific white shrimp (Litopenaeus vannamei) has been thoroughly tested and validated at the lab and commercial level, as Wasielesky et al. 2006 showed that super-intensive production is a viable option by using densities of 150-250 shrimp/m² under the zero exchange suspended microbial floc system. Subsequent studies have corroborated that BFT can be implemented in order to decrease feed costs by 10-25%, improvements in innate immune responses from the probiotics contribution, and in well-managed systems, the continuous production cycles can be achieved for 90-120 days without the necessity of water exchange (Crab et al., 2012; Ballester et al., 2010).

Yet, there are also known issues with the operation of traditional BFT systems that limits their effectiveness and expandability for commercial use. Total suspended solids (TSS) can be a problem in zero exchange systems and are often allowed to build-up beyond target levels (i.e. > 400mL/L biofloc volume (BFV)) without being controlled, which can result in anaerobic microenvironments in floc aggregates with the generation of toxic H_2S and the process metabolism switching from desired nitrogen assimilation routes (Schveitzer et al., 2013). Poor or zero establishment of a stable nitrifying community in new BFT systems can be a great mortality threat at high stocking densities with the risk of high, acute nitrite levels during the maturation phase (Krummenauer et al., 2011). In addition, as BFT community succession is passive, the final composition of the communities can vary due to the specific indigenous microbiota of each water source as well as the feed and intestinal flora of each of the animals. Together they color a spotty picture suggesting that further streamlining and intentional control of microbial community structure and function are needed in the next-generation of BFT-based systems.

Deliberate design, seeding, and operating specific microbial consortia with targeted ecological functions in aquaculture systems is termed microbial engineering, and have proven to be a promising complementary solution to these BFT limitations. Aerobic nitrifying bacteria such as Nitrosomonas spp. and Nitrospira spp. can be added as enriched cultures to speed up the development of adequate ammonia oxidation capacity in the important early phase that can cause startup nitrite spikes, significantly decreasing the length of time and severity of the spikes (Ray et al., 2010). At the same time, opportunity for opportunistic pathogens to break out in the shrimp culture ponds could be mitigated with the use of quorum-sensing-disrupting bacteria and broad-spectrum competitive exclusion agents, which include members of the genus Lactobacillus and Bacillus species known to be antagonistic to Vibrio harveyi, representing a step toward mitigating antimicrobial resistance problems in shrimp aquaculture (Defoirdt et al., 2004; Moriarty, 1997). However, Browdy et al. (2012) and Emerenciano et al. (2013) have posited the theoretical and empirical foundations needed for engineered microbial consortia in BFT systems, claiming that defined starter cultures of key functional guilds is the next step in BFT development beyond empirical C:N ratio control.

Rounding of the 3 supporting pillars of the architecture necessary to move intensive aquaculture towards a truly circular economy model is circular resource utilization, which includes efficient reuse and valorization of nutrients from settled sludge, efficient recycling of excess biofloc biomass to be used as partial protein source for fish feed, and optimization of water reuse with integrated solid matter separation and nutrient recovery. Kuhn et al., (2010) found that biofloc dried biomass has protein digestibility ranging between 82 and 88%, which is comparable to that of soy protein concentrate and revealed that a 25% protein substitution of dried biofloc biomass in L. vannamei did not affect the growth performance of the organisms. Luo et al. (2013) demonstrated the possibility to treat aquaculture sludge with high nitrogen content (NHN) as a raw material to generate inorganic nitrogen as a hydroponic fertilizer, thus enabling the nitrogen loop to be closed between aquaculture and plant culture. Zhao et al. (2012) have achieved very low exchange BFT production of Marsupenaeus japonicus with a recovery in sludge of 6.8% DW nitrogen and 1.9% DW phosphorus which are ironed with good quality organic fertilizer.

Though there is a strong rationale both theoretically and experimentally for combining BFT, microbial engineering and circular resource management in a single system, no present study has been done to assess these three in a single controlled experimental system. Therefore, an Integrated Microbial Engineering System (IMES) was designed and assessed in a 90-day growout study compared to standard BFT and conventional RAS based on water quality parameters, growth, microbial community profile and nutrient cycling efficiency using L. vannamei as the species.

2. Literature Review

The systematic development of the scientific foundation of biofloc technology in aquaculture was initiated by Avnimelech (1999), who showed that the metabolic direction of microbial community in the culture water from ammonia production to ammonia assimilation in tilapia production ponds could be achieved by manipulating the carbon-to-nitrogen ratio. Heterotrophic bacteria helped immobilize dissolved inorganic nitrogen (DIN) into bacterial biomass, thus keeping the TAN concentration below 1.0 mg/L (at high stocking rates) by supplementing pond water with carbonaceous substrates to achieve the ideal C:N ratio between 10:1 and 20:1. This basic observation put the C:N ratio at the heart of the operational control in BFT management and mechanistic research by De Schryver et al. (2008) clarified the biochemical stoichiometry of this process, showing that it was able to remove approximately N = 8,07g of ammonia nitrogen per 100g of added glucose nitrogen from the culture water – meaning that the amount of nitrogen that is removed in this process can be much higher than the one for conventional autotrophic nitrifying biofilters, given that the same 'amount' of biofilter is used. Crab et al. (2007) compared the nitrogen removal behaviour in BFT, CWR, algal systems, and denitrification reactors on a systematic basis and suggested that the BFT systems provide the maximum nitrogen removal capacity/volume among all these systems with an added benefit of nitrogenous proteins production by the microflora.

The first step towards adapting BFT principles in commercial shrimp farming was taken by Wasielesky et al. (2006), who established that growth of Litopenaeus vannamei shrimp could be achieved at super-intensity levels (up to 250 shrimp/m2) using an experimental ZE microbial floc culture system, and that natural production by the biofloc community supplies a significant proportion (15-20%) of the nutritional requirements for shrimp growth. Later studies by Ballester et al. (2010) later reiterated that supplementation of dietary protein, in BFT, could be reduced from traditional 35-40% to 25-30%, without affecting growth performance, due to the contribution of consumed biofloc biomass in providing the supplementary protein. Ju et al. (2008) took another step towards nutritional characterization of biofloc with the fatty acid and phospholipid profile analysis of biofloc grown under various C:N ratios: those sustained with molasses and those sustained with sucrose and tapioca starch. They found that fatty acid and phospholipid profile of biofloc vary greatly depending on the type of carbon source used for C:N ratio management with the biofloc maintained with molasses had significantly better polyunsaturated fatty acid (PUFA) profiles compared to those fed with sucrose and tapioca starch. These nutritional benefits were further expanded by Ekasari et al. (2010) who found that biofloc cultured at intermediate salinity (15 ppt) has a better essential amino acid profile than biofloc cultured in freshwater, which is directly related to species that can be cultured at intermediate level salinity like L. vannamei. In addition, Samocha et al. (2007) indicated that molasses with the rich carbohydrate fraction and trace mineral content provided stabilize and protein-rich BFT aggregates in different operating conditions, which made it an ideal choice to use for carbon supplement in commercial BFT applications.

The dynamics of the microbial community changes in BFT is one of the most complex phenomena in engineered biological systems involving a consortia of bacteria, archaea, microalgae, protozoa, ciliates, nematodes, and rotifers in a density dependent structured equilibrium within the matrix of floc aggregates (Moriarty, 1997). One of the first comprehensive, culture-independent characterizations of the microbial communities in minimum exchange intensive aquaculture systems was done by Ray et al. (2010) based on 16S rRNA gene clone library analysis. They found Proteobacteria to be the major and predominant phylum throughout all production phases, which harboured the key nitrifying and heterotrophic decomposer guilds, Alphaproteobacteria, and Gammaproteobacteria, respectively, with the remaining functional guilds Firmicutes and Bacteroidetes found as secondary groups in the floc matrix involved in the enzymatic hydrolysis of complex polysaccharides and proteins. Importantly, Ray and co-authors (2010) showed that the composition of microbial communities had a significant response to active management of the TSS, via periodic removal of sludge, throughout a POTW, again highlighting the importance of physical system management to maintain desirable functional microbial communities. The outcome of these community-level discoveries provided the empirical foundation for the work to follow involving efforts to generate more predictable and reproducible performance profiles of engineered BFT communities.

The pathogen suppression aspect of the BFT microbial ecology has increasingly attracted scientific research because of the need to find alternative solutions to antibiotic prophylaxis in the industry. Defoirdt et al. (2004) proposed that bacterial quorum sensing – a mechanism of intercellular communication, regulated by the density of bacteria and mediated by the acyl-homoserine lactone molecules – is very important and underestimated for the dynamics of diseases in shrimp farming systems. It was suggested that quorum quenchers could be deliberately added, either in the form of quorum-sensing-disrupting bacteria or strains that produced quorum-quenching enzymes, in order to quench virulence of pathogens, while not exerting selection pressure for conventional antibiotic resistance. Emerenciano et al. (2013) scoured the literature on probiotics in BFT systems and compiled the evidence, including that the inclusion of Bacillus spp. and/or Lactobacillus spp. strains in the biofloc community by regular inoculation is able to competitively exclude opportunistic species belonging to Vibriaceae, stimulate activity of phagocytic cells in the host immune system, and support feed digestibility due to production of exogenous enzymes. This has spurred the creation of a new range of probiotics consortia for BFT farms with high densities; indeed, the significance of the impact of pathogens is greatest at the feed end, as is the urge for the application of 'just in case' antibiotics.

The stability of biofloc system when operated in commercial conditions is an ongoing research and the management of biofloc volume and avoiding excessive TSS accumulation are being investigated. To evaluate the relationship between biofloc volume (BFV) with the efficiency of nitrogen removal (NR) in L. vannamei tanks, Schveitzer et al. (2013) systematically evaluated and revealed that there is a quadratic relationship of maxima between BFV and NR efficiency at 200–250 mL/L of BFV in imhoff cone settled measurements. When suspended phytoplankton was above a certain threshold (~400 mL/L), anaerobic microenvironments were formed within the interior of dense floc aggregates increasing community metabolism to sulfate reducing and producing hydrogen sulfide at levels very toxic to shrimp. In a similar way, Krummenauer et al. (2011) reported that stocking rates above 300 shrimp/m³ were sensitive as far as the maintenance of water quality was concerned, especially in a commercial operation in southern Brazil, as overstocking resulted in the deterioration of water quality. The combined results of these studies emphasized the importance of applying holistic sludge management practices like the conical sedimentation clarifier (CSRC) used in the IMES configuration examined in the current study to realize different sludge management objectives, including maintaining the volume of biofloc in the OW and recycling the settled biofloc as high nutrient feed for IMES.

The circular economy aspect of integrated aquaculture has been promoted by a series of complementary investigations which show together the feasibility of near zero discharge production with the possibility of closed nutrient recycling. Kuhn et al. (2010) determined that dried biofloc biomass produced from the biological treatment of fish effluent has a protein digestibility between 82 and 88% which is similar to the digestibility of soy protein concentrate and could be used to substitute up to 25% of the fish protein in L. vannamei formulations without negatively affecting growth parameters, feed conversion ratio, or survival rates. The economic effect of this result is very considerable, as for intensive shrimp farming systems 50-70% of the total running cost is the feed. It was found from a study conducted by Luo et al. (2013) that the sludge which is high in N produced by intensive BFT culture can be made use of in hydroponic crop production after the treatment in sequencing batch PBRs through nitrogen source conversion by suitably manipulating the C:N ratio, thus proving the technical feasibility of using such sludge in integrated aquaculture-hydroponics circular culture system. When the volume of biofloc is managed by sedimentation and selective removing, they can be recovered from the same water treatment system with a percentage that is acceptable for application into the rearing water without water exchange due to BFT operation, according to Zhao et el. (2012) the results indicated that for zero exchange BFT system of M. japonicus, the recovered sludge contained 6.8% TN on DW and 1.9% TP on DW which are close to the specification of high grade commercial organic fertilizer, and the counts of obtained sediments were not significant difference from the 120 days without exchange water treatment system. In her support, Azim & Little (2008) observed similar growth and welfare parameters for Nile tilapia cultured in indoor BFT tanks using lower amounts of commercial proteins, which further reinforces the ability to nutritionally substitutions biofloc biomass for the cultured species.

In a detailed review, Browdy et al. (2012b) concisely and eloquently summarized how these research foci fit together: that the evolution of BFT systems from an empirical approach to one in which microbial communities are being married deliberately is, in part, the logical progression of BFT system development, and also the most likely means towards achieving the consistency and scalability commercial aquaculture requires. They suggested that undefined starter cultures of functional guilds, which consist of autotrophic nitrifying bacteria, functional aggregator (heterotrophic) bacteria, and probiotic bacteria that have proven to suppress pathogenic bacteria, be used to support system maturation and to quickly reduce the risk of system start-up failure and performance variability that currently prevents the potential of BFT implementation on a larger scale. Further proof of complementary evidence was provided by Hari et al. (2004) using semi-intensive shrimp ponds, under a concept of pond culture to engineered BFT to full integrated IMES framework, where introduction of a minimal amount of carbohydrates to the pond water, as a subtle manipulation to increase the pond C:N ratio in order to achieve apparent improvements in N use efficiency and shrimp growth. The combined research from the literature examined in this review provides strong rationale through mechanisms, experimental, and applied studies to support the development of “microbial engineering biofloc technology” combined with circular resource utilization toward a single and coherent system design in aquaculture namely the objective of the present study.

3. Materials and Methods

3.1 Experimental Design and System Configuration

The experiment was a completely randomized design (CRD) with three treatments having three replicates each (n = 9 tanks) conducted at the Aquaculture Research Station of University of Agriculture Faisalabad, Pakistan over 90 days (August – November 2024). The three treatments were: (i) control RAS with drum filter (K1 Kaldnes media, AnoxKaldnes, Norway) recirculated with 2 tank volumes per hour, (ii) BFT with no exchange, and (iii) IMES, BFT with a 6-h cycle nutrient recycled conical sedimentation clarifier (CSC) and a defined microbial consortium. The size of tanks was 3,000L, circular, made of fiberglass and each of them was equipped with 1.5-kW regenerative blower which supply ambient air at 20L/min/kg of estimated biomass by 12 diffusers of 10-cm.

3.2 Stocking, Feeding, and Microbial Consortium Preparation

Pathogen-free Litopenaeus vannamei post-larvae (PL_{15}) (0.52 ± 0.04 g mean initial weight) were stocked at 15 ± 1 ppt salinity (Red Sea Salt, Red Sea Aquatics, Germany) at a stocking density of 300 shrimp/m^3. All systems were fed four times daily according to the Zeigler Shrimp Grow diet (35% crude protein, 7% lipid; Zeigler Bros. Inc., USA), the protein level of which was reduced to 30% from day 31 onward for the BFT and IMES tanks, based on 10% biomass sampling and weekly re-correction. Crude molasses (48% sucrose equivalent) was used to maintain a C:N = 15:1 using daily TAN measurements in BFT and IMES tanks which was supplied three times a day. The IMES microbial consortium was composed of same optical density ($OD_{600} = 1.0$) amounts of enriched nitrifying bacteria (serial passage on ATCC Medium 2265 for 30 days), Bacillus subtilis CGMCC 11437, and Lactobacillus plantarum DSM 20174, which were inoculated at each with 10⁶ CFU/mL on day 0, 15 and 30.

3.3 Water Quality, Growth Performance, and Microbial Community Analysis

Daily measurements of temperature, dissolved oxygen, pH and salinity (YSI ProDSS) were taken. Weekly measurements for TAN (indophenol-blue, APHA 4500-NH3), NO2-N (sulfanilamide, diazotisation), NO3-N (ion chromatography, Metrohm 861) and TSS (GF/C filter, dried at 105ºC for 24 hours) were conducted. Shrimp were batch weighed 15 days after each other using 100 shrimp per tank and an Ohaus Pioneer balance (100 - ±0.01 g). Growth indices calculated are based on SGR (%/day) = 100 × (ln FBW − ln IBW)/days, FCR = total dry feed / wet weight gain, and PER = wet weight gain / protein fed. Proximate composition of whole body was followed according to AOAC (2016) on freeze-dried tissue. To analyze the microbial community, water samples from days 0, 45 and 90 were centrifuged (5,000 × g, 15 min, 4°C) and stored at −80°C for the extraction of DNA (PowerWater Kit, Qiagen), which was amplified (primers 341F/806R) and sequenced on an illumina MiSeq (2 × 300 bp; BGI/SZ). Data analysis was performed using QIIME2 v2024.2 (DADA2; SILVA 138.1) and alpha and beta diversity was then calculated in R v4.3.1 (phyloseq, vegan). Retention, water reuse and sludge recovery efficiency were quantified using nitrogen and phosphorus mass balances. One way ANOVA with Tukey HSD post hoc tests ($\alpha = 0.05$) were used for analysis of data (in IBM SPSS v28, GraphPad Prism v9).

4. Results

4.1 Water Quality

The temperature (28.4-28.6°C) and salinity (15.0-15.1 ppt) were not significantly different between treatments and were stable during the trial (Table 1). Total ammonia nitrogen (TAN) steadily rose in the Control system, with TAN levels reaching 5.82 ± 0.31 mg/l after 90 days and approaching 24-h LC_{50} for L. vannamei; the TAN in the BFT system averaged 0.81 ± 0.08 mg/l and the TAN in the IMES remained below 24-h LC_{50} for L. vannamei throughout the experiment ($p < 0.001$); Figure 1A). Nitrite-nitrogen was significantly lower in IMES (0.16 ± 0.02 mg/L) than in BFT (0.43 ± 0.05 mg/L) and Control (1.98 ± 0.18 mg/L) (Figure 1B; $p < 0.001$). pH was kept within the acceptable range for L. vannamei (7.3–8.0) for all the systems, and the highest values were observed in the IMES system with values of 7.63 ± 0.04, which correlated with the partial recovery of alkalinity due to the denitrification activity in the engineered consortium (Figure 1C). Dissolved oxygen kept at higher concentration throughout in all treatments and in the IMES treatment, the greatest mean value (6.81 ± 0.18 mg/l) was recorded which is an indicator of lesser biological oxygen demand per unit volume (Figure 1D). The active role of the biofloc that was managed in BFT was reflected in the TSS values that were significantly different from IMES where BFT with biofloc showed a value of 412.3 ± 38.6 mg/L while IMES demonstrated a value of 248.7 ± 22.4 mg/L.

Table 1. Water Quality Parameters (Mean ± SE) Across Experimental Systems Over the 90-Day Trial

Parameter	Control (RAS)	BFT	IMES
Temperature (°C)	28.4 ± 0.3	28.6 ± 0.4	28.5 ± 0.3
Salinity (ppt)	15.1 ± 0.2	15.0 ± 0.3	15.1 ± 0.2
pH	7.48 ± 0.06 ^{a}	7.56 ± 0.05 ^{ab}	7.63 ± 0.04 ^{b}
DO (mg/L)	6.56 ± 0.19 ^{a}	6.32 ± 0.21 ^{b}	6.81 ± 0.18 ^{a}
TAN (mg/L)	5.82 ± 0.31 ^{a}	0.81 ± 0.08 ^{b}	0.36 ± 0.04 ^{c}
NO_2-N (mg/L)	1.98 ± 0.18 ^{a}	0.43 ± 0.05 ^{b}	0.16 ± 0.02 ^{c}
NO_3-N (mg/L)	8.42 ± 0.62 ^{a}	4.18 ± 0.31 ^{b}	2.34 ± 0.24 ^{c}

TSS (mg/L)	24.6 ± 3.1 ^{a}	412.3 ± 38.6 ^{b}	248.7 ± 22.4 ^{c}
BFV (mL/L)	N/A	186.4 ± 14.2 ^{a}	148.6 ± 12.8 ^{b}

^{abc} Different superscripts in the same row indicate significant differences (Tukey HSD, $p < 0.05$). TAN = total ammonia nitrogen; BFV = biofloc volume; N/A = not applicable.

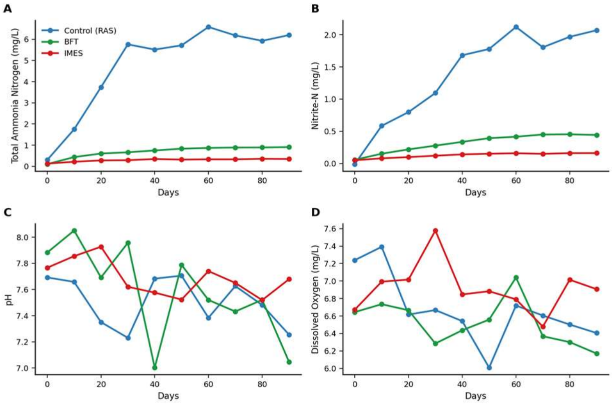

Figure 1. Water quality parameters across Control RAS, BFT, and IMES over 90 days: (A) TAN (mg/L); (B) NO_2-N (mg/L); (C) pH; (D) DO (mg/L). Means ± SE, n = 3. Prepared using GraphPad Prism v9.

4.2 Growth Performance and Feed Utilization (Table 2; Figure 2)

IMES produced significantly higher final body weight (20.3 ± 0.62 g) than BFT (16.8 ± 0.48 g) and Control (12.4 ± 0.52 g) after 90 days ($p < 0.001$; Table 2). SGR in IMES (4.18 ± 0.13%/day) was 29.0% greater than BFT and 98.1% greater than Control. FCR was lowest in IMES (1.18 ± 0.04) versus BFT (1.42 ± 0.05) and Control (1.85 ± 0.08), representing 17.0% and 36.2% improvements respectively ($p < 0.001$; Figure 2B). Protein efficiency ratio was highest in IMES (2.61 ± 0.14) relative to BFT (2.24 ± 0.11) and Control (1.68 ± 0.09). Figure 2C shows that the survival rate in IMES (91.2 ± 1.5%) was significantly higher compared to that of BFT (83.6 ± 1.8%) and Control (72.3 ± 2.2%; $p<0.01$), with Control mortality rates being attributable to three acute ammonia toxicity events (TAN > 4.5mg/L for more than 24 hours) throughout the experimental period from weeks 4 – 7.

Table 2. Growth Performance and Feed Utilization of L. vannamei Across Experimental Systems After 90 Days

Parameter	Control (RAS)	BFT	IMES
Initial Body Weight (g)	0.52 ± 0.04	0.53 ± 0.03	0.52 ± 0.04
Final Body Weight (g)	12.4 ± 0.52 ^{a}	16.8 ± 0.48 ^{b}	20.3 ± 0.62 ^{c}
Weight Gain (g)	11.88 ± 0.48 ^{a}	16.27 ± 0.45 ^{b}	19.78 ± 0.60 ^{c}
SGR (%/day)	2.11 ± 0.14 ^{a}	3.24 ± 0.10 ^{b}	4.18 ± 0.13 ^{c}
FCR	1.85 ± 0.08 ^{a}	1.42 ± 0.05 ^{b}	1.18 ± 0.04 ^{c}
PER	1.68 ± 0.09 ^{a}	2.24 ± 0.11 ^{b}	2.61 ± 0.14 ^{c}
Survival Rate (%)	72.3 ± 2.2 ^{a}	83.6 ± 1.8 ^{b}	91.2 ± 1.5 ^{c}
Final Biomass (kg/m³)	3.39 ± 0.21 ^{a}	4.72 ± 0.18 ^{b}	6.23 ± 0.24 ^{c}

SGR = specific growth rate; FCR = feed conversion ratio; PER = protein efficiency ratio. ^{abc} Different superscripts indicate significant differences (Tukey HSD, $p < 0.05$).

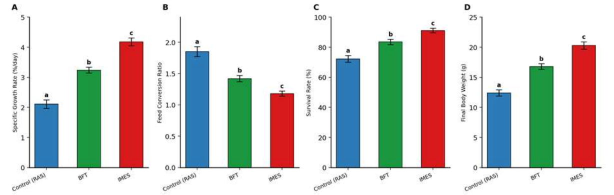

Figure 2. Growth performance of L. vannamei: (A) SGR (%/day); (B) FCR; (C) Survival (%); (D) Final Body Weight (g). Bars = mean ± SE (n = 3). Different letters denote significant differences (Tukey HSD, $p < 0.05$). GraphPad Prism v9.

4.3 Microbial Community Composition

Post-QIIME2, an average of 68432 ± 4218 reads per sample were of high quality and were used for analysis using Illumina MiSeq sequencing platform. Alpha diversity was significantly higher in IMES (Shannon = 4.82 ± 0.18; Chao1 = 1,248 ± 86 OTUs) than in BFT (Shannon = 4.31; Chao1 = 982) and Control (Shannon = 3.98; Chao1 = 812) at day 90 ($p < 0.05$). Proteobacteria were highly abundant in all the media (34.1–40.2%) and the abundance of these bacteria was higher compared with other systems. Bacteroidetes were also higher in IMES (28.3%) than in BFT (25.1%) and Control (22.4%) due to the high hydrolytic activity (Figure 3). The relative abundance of Nitrosomonas (4.82 ± 0.24%) and Nitrospira (3.14 ± 0.57%) at genus level in IMES was significantly higher compared to the others; thus, the nitrifying consortia were established by inoculation ($p < 0.05$). Both Lactobacillus and Bifidobacterium decreased relatively in abundance from day 45 to day 90, with final percentages of 2.1% and 1%, respectively, suggesting that probiotics could be re-inoculated at frequent intervals (every 60 days) in commercial IMES operations.

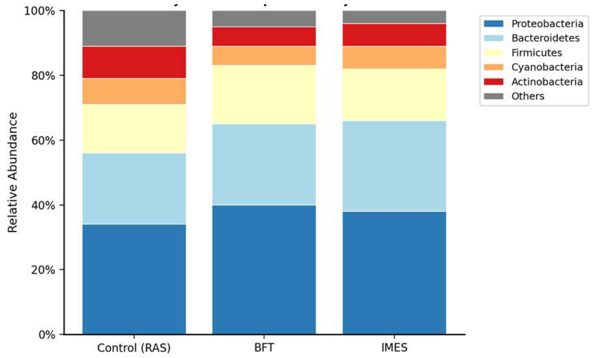

Figure 3. Microbial community composition at phylum level determined by 16S rRNA amplicon sequencing (Illumina MiSeq, V3–V4) at day 90. Relative abundance (%) shown as stacked bars. QIIME2 v2024.2; SILVA 138.1; visualized in R v4.3.1 (phyloseq).

4.4 Nutrient Cycling and Circular Resource Utilization

IMES achieved nitrogen retention efficiency of 71.3 ± 3.2%, significantly exceeding BFT (52.6 ± 2.8%) and Control (28.4 ± 2.6%) ($p < 0.001$; Figure 4). Phosphorus retention followed the same pattern (IMES: 74.2 ± 3.6%; BFT: 55.8 ± 2.9%; Control: 32.1 ± 2.4%). The most success reusing water occurred in IMES, which needed no volume exchange compared to BFT and Control, which needed to exchange about ~12.3% volume per week for nitrate regulation. Sludge reduction reached 68.7 ± 4.2% in IMES versus 41.3 ± 3.8% in BFT and 18.6 ± 2.8% in Control. 6.4 ± 0.4% of PN was detected in the recovered sludge of IMES on a dry matter basis, which indicated that sludge was suitable as an organic fertilizer, and at the same time, it showed that significance integration of the "circular economy" really has been achieved.

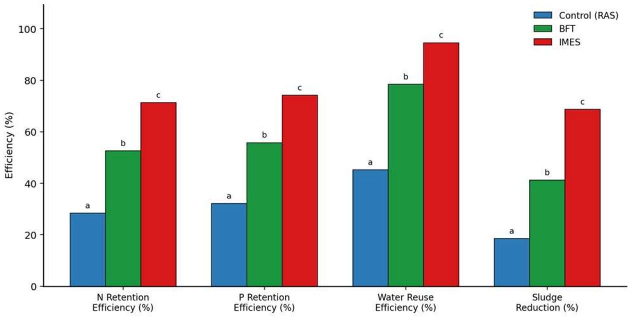

Figure 4. Nutrient cycling and circular resource utilization efficiency (%) across systems. Bars = mean ± SE (n = 3). Different letters denote significant differences within each metric (Tukey HSD, $p < 0.05$). GraphPad Prism v9.

5. Discussion

The current work is the first controlled and replicated assessment of such an integrated system combining BFT, targeted microbial engineering and circular resource management and the findings clearly showed the synergy between these three technologies, and the achievement of results significantly better than using any of the technologies alone. This is because the maintenance below the 0.4 mg/L threshold in IMES over a 90-day trial was much lower than the 0.6–1.2 mg/L typically achieved in standard BFT with a similar number of shrimp/m³ (Xu & Pan, 2012), indicating that TAN was converted to biomass by heterotrophic assimilation from the bulk biofloc community and heterotrophic nitrification from the inoculated consortium of Nitrosomonas and Nitrospira. The lower nitrite levels in IMES, however, indicate that the entire nitrification chain (NH_4+ to NO_2- to NO_3-) was completed faster and more strongly in IMES than the standard BFT, in which nitrite is still a known cause for high fish mortalities during the early phase of establishing the autotrophic community (Schveitzer et al., 2013).

Focusing on the FCR, a value of 1.18 was achieved with IMES, which is close to the theoretical dietary minimum for L. vannamei (0.9 – 1.1 when natural food contribution is taken into account) and is at a lower range compared to values typically reported in RAS (1.5 – 2.2) and optimized BFT (1.2 – 1.6) (Wasielesky et al., 2006; Krummenauer et al., 2011). This improvement is a consequence of two factors – biofloc protein, which provides a supplementary nutritional input to fish (estimated between 15 to 20 per cent of total protein consumed by the fish is in line with the dietary protein reduction protocol followed by Ballester et al., 2010) and probiotic effect of Lactobacillus plantarum on the composition of the gut microbiome, digestive enzyme activity and efficiency of absorption of the nutrients (Long et al., 2015). The greater alpha diversity of IMES (Shannon = 4.82; Chao1 = 1,248 OTUs) compared to BFT and Control supports the functional enrichment strategy mentioned by Browdy et al. (2012) to say that there is a series of bioreactions which tend to reinforce or create new microbial diversity in the system, rather than competitively suppressing the indigenous microbial flora. The percentage of Lactobacillus decreased from 2.1% on day 45 to 0.8% on day 90 showing a gradual washout from system, important if the use of lactobacilli is to be maintained as a probiotic in commercial systems every 60 days will be necessary.

IMES has a relatively high water reuse rate (94.6%) compared to those reported by Zhao et al. (2012) who have well-managed ZEBFT with water reuse rate in between 75–85%, which is another game changing paradigm shift compared to the conventional RAS (45.2% as observed in the present study). The TSS reduced to 248.7 mg/L in the IMES conical clarifier, about 40% below the BFT control, keeping the biofloc volume in the space in between 200–250 mL/L as aimed for by Schveitzer et al. (2013) and preventing the development of anaerobic microenvironments, which is the major operational risk of ZE BFT systems at high stocking rates. Also, the nutrient profile of the recovered sludge (6.4% N, 1.8% P dry weight) aligns with that reported by Luo et al. (2013) for BFT sludge, which can be used as organic fertilizer, thus for creating a true circular flow of nutrients among aquaculture production and soil-based crop cultivation. The scope of the present study is restricted by the controlled indoor test conditions which might not account for the wide range of variability of commercial outdoor systems, and there is no formal techno-economic analysis. The next studies to be conducted are priorities: multi-cycle stability studies and commercial scale validation.

6. Conclusion

This study demonstrates that combining targeted microbial engineering, BFT and circular resource utilization in one single aquaculture system design, called the Integrated Microbial Engineering System (IMES), results in synergistic effects across improved water quality, shrimp growth performance, microbial community diversity and nutrient cycling efficiency that individually and collectively outperforms the use of standard BFT or conventional RAS. Litopenaeus vannamei was cultured under IMES with the density of 300 shrimp/m3 that resulted in 4.18%/day specific growth rate, feed conversion ratio 1.18, water reuse efficiency 94.6% and average survival of 91.2% during the 90 days. The two nitrogen removal mechanisms used (bulk heterotrophic assimilation and inoculated autotrophic nitrification) were found to be the main parameters to achieve better water quality and stability. The results of the circular resource utilization obtained indicate a high-nitrogen retaining trend with 71.3% nitrogen retention and recoverable sludge total nitrogen being 6.4% on a dry weight basis, which are the necessary values to make the intensive shrimp production using the technology of near-zero discharge feasible for nutrient recovery for agricultural reuse. The next step in the future consists of assessment of the engineered microbial consortium for multi-cycle stability, formal techno-economic analysis done at commercial scale and cross species evaluation of the IMES framework with Atlantic salmon and Nile tilapia.

Acknowledgements

This research has been funded by the Higher Education Commission (HEC) of Pakistan, Grant No: NRPU-16148. The authors are thankful to Mr. Aamir Shehzad and Ms. Nadia Bashir for technical assistance during experimental trial. BGI Genomics, Shenzhen, China provided Illumina MiSeq sequencing services. The authors have no conflicts of interest to disclose.

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