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Metagenomic Characterization of Biofloc Microbial Communities for Enhanced Nutrient Recycling and Fish Health

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ABSTRACT

Biofloc Technology (BFT) represents a paradigm shift in sustainable aquaculture, operating through zero-water-exchange systems where dense microbial aggregates perform continuous in situ bioremediation of toxic nitrogenous wastes while providing supplemental nutrition to cultured species. This review synthesizes current metagenomic insights into the structural architecture, functional metabolic capacities, and host-immunomodulatory mechanics of biofloc-associated bacterial communities. High-throughput sequencing methodologies, particularly whole-genome shotgun metagenomics, have revolutionized our understanding of floc microbiomes by enabling species-level taxonomic resolution, reconstruction of Metagenome-Assembled Genomes (MAGs), and direct quantification of biogeochemical functional genes. Metagenomic profiling consistently identifies *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* as core phyla, with community succession dynamically regulated by carbon-to-nitrogen (C/N) ratio manipulation, dissolved oxygen gradients, and total suspended solids. Functional gene analysis reveals interconnected nitrogen transformation pathways heterotrophic assimilation (*amoA*), nitrification (*nxrA*), denitrification (*narG*, *nirK/S*, *nosZ*), anaerobic ammonium oxidation (*hzsA/B*), and dissimilatory nitrate reduction to ammonium (*nrfA*) operating concurrently within spatially structured biofloc microenvironments. Concurrently, phosphorus (*ppk*, *phoD*) and sulfur

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(*sox* multienzyme) cycling pathways maintain broader system stoichiometry. Critically, biofloc microbiomes enhance host immunity through quorum quenching enzymes (AHL-lactonases) that suppress pathogen virulence, continuous probiotic provisioning, and immunostimulation via microbe-associated molecular patterns. Despite these benefits, challenges persist including high biological oxygen demand, total suspended solids management, and the potential for bioflocs to harbor latent opportunistic pathogens and antibiotic resistance genes. Future integration of metatranscriptomics, metabolomics, and machine learning with portable sequencing platforms promises real-time, predictive microbiome management, optimizing system stability, reducing energy consumption, and advancing BFT as a cornerstone of sustainable aquaculture intensification.

Keywords— Biofloc Technology, Metagenomics, Nitrogen Cycling, Aquaculture Sustainability, Microbiome, Quorum Quenching

1. Introduction

1.1 Aquaculture Sustainability and the Biofloc Paradigm

The rapid escalation of global aquaculture production is indispensable for meeting human dietary protein demands and reinforcing food security. However, conventional intensive aquaculture systems face severe sustainability constraints, primarily driven by high water consumption, massive discharges of nutrient-rich effluent into recipient coastal and freshwater bodies, and an inherent vulnerability to catastrophic pathogen outbreaks (Mohd & Mushtaq, 2025). Traditional flow-through and open-pond operational models often lead to environmental eutrophication due to unconsumed feed and excretory waste accumulation, which in turn degrades surrounding aquatic ecosystems. To address these ecological and economic limitations, Biofloc Technology (BFT) has emerged as an innovative, sustainable closed-system paradigm based on zero or minimal water exchange (Porkka, 2021).

The fundamental operational mechanism of BFT involves the retention and proliferation of dense, suspended microbial aggregates termed bioflocs directly within the culture water column. These heterogeneous microbial consortia perform continuous *in situ* bioremediation by assimilating hazardous excretory metabolic bi-products, primarily total ammonia nitrogen (TAN, which exists in equilibrium between toxic un-ionized ammonia, NH_3, and ionized ammonium, NH_4^+), alongside nitrite (NO_2^--N) and organic particulate matter (Sheng et al., 2026). By converting dissolved inorganic pollutants into edible microbial cell biomass, biofloc systems effectively transform toxic waste into a supplementary, high-protein food resource. Consequently, BFT enhances water-use efficiency, optimizes feed conversion, minimizes biosecurity risks from external intake water, and lowers overall operational expenditures (Fayed et al., 2026).

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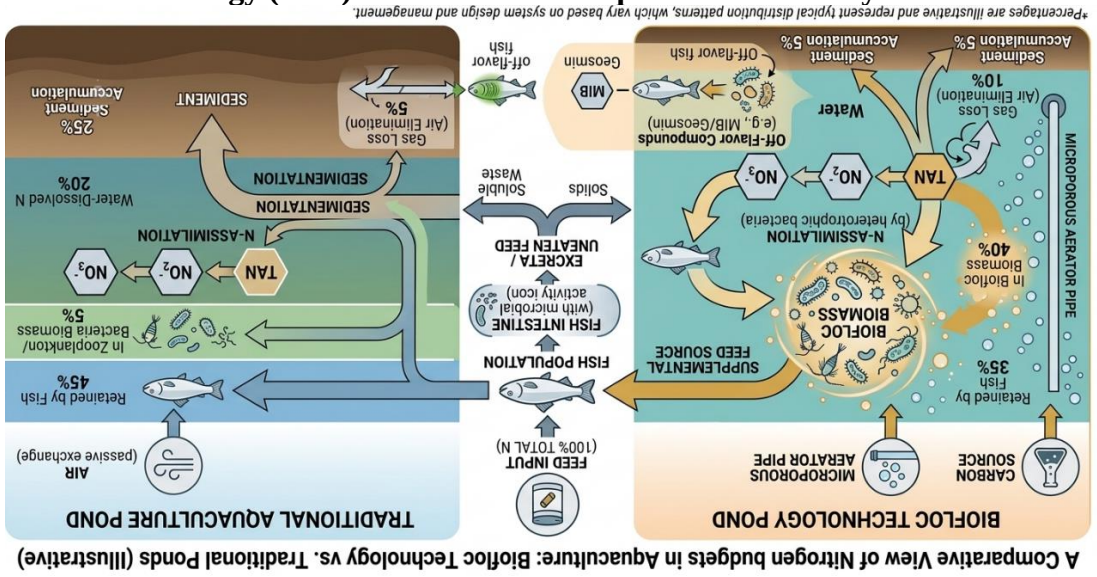
Figure 1: Comparative Nitrogen Mass-Balance and Fate Dynamics Between Biofloc Technology (BFT) and Traditional Aquaculture Pond Systems.



1.2 Principles of Biofloc Technology (BFT) and Carbon-to-Nitrogen (C/N) Ratio Manipulation

The establishment, metabolic trajectory, and structural stability of a biofloc system are primarily governed by the systematic manipulation of the aquatic Carbon-to-Nitrogen (C/N) ratio. Standard commercial aquaculture feeds typically exhibit a low C/N ratio, generally ranging between 7:1 and 10:1, which is insufficient to support complete heterotrophic assimilation of excreted nitrogenous waste (Liu et al., 2023). Under these unsupplemented conditions, autotrophic nitrifying bacteria slowly process ammonia, resulting in potential transient spikes of toxic intermediate nitrite. Elevating the system's operational C/N ratio to an optimal range of 10:1 to 20:1 achieved through the strategic addition of exogenous organic carbon sources such as molasses, plant starch, cellulose, or tapioca flour dramatically shifts the metabolic dynamics of the aquatic microbiome (Soliman, 2016).

This artificial elevation of available organic carbon stimulates heterotrophic bacteria to outcompete slow-growing autotrophic nitrifiers for dissolved inorganic nitrogen. Heterotrophic microorganisms rapidly assimilate dissolved ammonium together with organic carbon substrates to produce single-cell microbial protein. As bacterial cell densities increase, extracellular polymeric substances (EPS) synthesized by key microbial strains induce macro-aggregation, binding heterotrophic bacteria, microalgae, fungi, protozoa, rotifers, diatoms, and detrital particles into suspended bioflocs (Michaud, 2007).

Operational maintenance of biofloc systems requires routine physical profiling of these suspended solids using Imhoff settling cones, where volumetric precipitation (measured in mL/L after 15 minutes of settling) serves as an empirical indicator of microbial aggregate concentration and system health. However, host species tolerance to

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suspended solids dictates BFT applicability. Robust filter-feeding or benthic species such as the Japanese eel (*Anguilla japonica*), Nile tilapia (*Oreochromis niloticus*), carps, and Pacific white shrimp (*Penaeus vannamei*) demonstrate elevated growth rates, reduced feed conversion ratios (FCR), and increased survival when reared in biofloc environments (Schveitzer et al., 2024). Conversely, clear-water adapted teleosts and crustaceans, such as channel catfish, hybrid striped bass, and giant freshwater prawns, exhibit physiological stress and branchial clogging under elevated biofloc solids, highlighting the necessity of species-specific system tuning (Plumb & Hanson, 2010).

2. Metagenomic Approaches for Characterizing Biofloc Microbiomes

2.1 High-Throughput Sequencing Methodologies: 16S rRNA Amplicon vs. Shotgun Metagenomics

Elucidating the intricate structural architecture and metabolic capacities of the floc-associated bacterial (FAB) community requires high-resolution culture-independent analytical techniques. Traditional culture-based methods fail to isolate the vast majority of aquatic microorganisms, while early molecular techniques like Denaturing Gradient Gel Electrophoresis (DGGE) lack the depth required to capture low-abundance functional taxa. The implementation of High-Throughput Sequencing (HTS) has fundamentally overcome these constraints, offering deep insights into biofloc microbial ecology (Rajeev et al., 2024).

Targeted marker gene sequencing, which amplifies specific hypervariable regions (most commonly V3–V4) of the bacterial 16S ribosomal RNA (rRNA) gene, provides comprehensive profiling of community taxonomic structure, richness, and diversity metrics. Although 16S rRNA amplicon sequencing remains cost-effective for screening microbial successions across environmental gradients, it exhibits inherent technical constraints (Fadeev et al., 2021). PCR amplification biases can distort true cellular relative abundances, while sequence conservation often limits taxonomic resolution to the genus level. Furthermore, 16S amplicon profiling cannot directly identify functional genes, relying instead on predictive bioinformatic algorithms that infer metabolic potential from reference databases (Schloss et al., 2011).

Whole-Genome Shotgun (WGS) metagenomics bypasses PCR target gene amplification by directly fragmenting and sequencing total environmental DNA extracted from biofloc matrices. Shotgun metagenomics provides species- and strain-level taxonomic resolution, pinpoints antibiotic resistance genes (ARGs), and uncovers the full repertoire of functional genes present within the microbiome (Grice & Segre, 2012). By assembling overlapping metagenomic reads into contiguous fragments (contigs) and binning them into Metagenome-Assembled Genomes (MAGs), researchers can reconstruct discrete metabolic pathways and assign uncultivated taxa to specific biogeochemical roles (Mirete et al., 2025).

Table 1: Comparative Methodological Specifications of 16S rRNA Amplicon and Shotgun Metagenomic Sequencing

Sequencing Parameter	16S rRNA Amplicon Sequencing	Shotgun Metagenomic Sequencing
Primary Target	Hypervariable regions of 16S rRNA gene	Total environmental genomic DNA

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Taxonomic Resolution	Genus level (rarely species level)	Species and strain level; MAG recovery
Functional Profiling	Predictive inference (e.g., PICRUSt2, Tax4Fun)	Direct detection and quantification of functional genes
Host DNA Interference	Minimal (mitigated by bacterial-specific primers)	Significant in host-associated mucosal samples
PCR Amplification Bias	Moderate to High (primer binding variance)	Minimal (unbiased random fragmentation)
Relative Analytical Cost	Low to Moderate	Moderate to High

2.2 Metatranscriptomics and Advanced Long-Read Sequencing Technologies

While shotgun metagenomics resolves the static genomic *potential* of the biofloc microbiome, metatranscriptomics isolates and sequences total environmental messenger RNA (mRNA) to profile real-time functional gene expression. Metatranscriptomic profiling captures dynamic metabolic shifts in response to environmental perturbations, such as sudden fluctuations in dissolved oxygen, carbon addition events, or thermal stress. Quantifying actual transcript abundance allows researchers to confirm whether specific metabolic pathways such as denitrification or stress-response mechanisms are actively transcribed under operational conditions (Meenakshisundaram et al., 2021).

Third-generation long-read sequencing technologies, including Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio HiFi), have further advanced metagenomic capabilities. Long-read platforms generate continuous sequencing reads spanning several kilobases to tens of kilobases, successfully spanning complex repetitive genomic regions that typically cause short-read assemblies to fragment (Oehler et al., 2023). Long-read metagenomics substantially improves the assembly of intact, high-quality MAGs from environmental matrices and resolves structural genome variations. When combined with short-read data in hybrid assembly workflows, these technologies reduce host DNA interference and yield detailed maps of complex microbial ecosystems (Mirete et al., 2025)

2.3 Bioinformatics Pipelines and Functional Profiling Workflows

The extraction of biological insights from biofloc metagenomic datasets depends on robust bioinformatics pipelines. The analytical workflow initiates with high-purity total genomic DNA extraction, using specialized physical and chemical lysis protocols optimized for complex environmental matrices containing high organic loads and polysaccharide-rich extracellular matrices. Following DNA extraction and high-throughput sequencing, raw reads undergo stringent quality control, adapter trimming, and filtering to remove low-quality bases and host genomic contamination (Rajeev et al.,2024).

Downstream bioinformatic processing follows two primary branches based on the sequencing approach. For 16S rRNA amplicon data, error-correction algorithms (such as DADA2 or Deblur) construct Amplicon Sequence Variants (ASVs), which are

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taxonomically annotated against curated reference databases including SILVA, Greengenes, or RDP using software pipelines like QIIME2 or Mothur. For shotgun metagenomic data, clean reads are assembled *de novo* into contigs using metagenomic assemblers (such as MEGAHIT or SPAdes), followed by gene prediction software (like Prodigal) (Regueira-Iglesias et al., 2023). Predicted coding sequences are subsequently aligned against functional annotation databases including the Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), and Carbohydrate-Active EnZymes (CAZy) to reconstruct biogeochemical pathways, quantify functional gene copy abundances, and track metabolic dynamics across the culture cycle (Salamov, & Solovyevand, 2011).

3. Structure and Taxonomic Diversity of Biofloc Bacterial Communities

3.1 Core Floc-Associated Bacterial (FAB) Taxa

Metagenomic characterization reveals that biofloc aggregates host highly structured, multi-kingdom microbial consortia. The bacterial core of the floc-associated bacterial (FAB) community is consistently dominated by four major phyla: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*, alongside localized populations of *Planctomycetes* and *Verrucomicrobia*. The class *Proteobacteria* encompassing *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* typically constitutes the largest functional fraction, driving both heterotrophic organic matter turnover and autotrophic nitrogen transformations (Gutierrez-Perez et al., 2022).

Within these broad taxonomic groups, specific microbial genera fulfill defined ecological roles within the biofloc aggregate. Heterotrophic organic waste degraders and nitrogen assimilators are represented by robust, enzyme-secreting genera such as *Bacillus*, *Pseudomonas*, *Comamonas*, and *Alkaliphilus*. These bacteria synthesize extracellular hydrolytic enzymes including proteases, amylases, and cellulases that decompose complex organic feed residues and fecal matter while assimilating dissolved ammonium into single-cell protein (Rajeev, & Cho, 2024). Concurrently, autotrophic nitrifying populations, such as *Nitrosomonas* (ammonia-oxidizing bacteria, AOB) and *Nitrospira* (nitrite-oxidizing bacteria, NOB), inhabit localized regions within the aggregate matrix, converting toxic inorganic nitrogen species into nitrate (Almstrand et al., 2013).

Photosynthetic and purple non-sulfur bacteria, particularly members of the family *Rhodobacteraceae*, play vital roles in carbon cycling, trace nutrient biosequestration, and sulfur oxidation within biofloc environments. Additionally, predatory bacteria such as *Bdellovibrio* act as natural population regulators within the floc ecosystem by obligately parasitizing Gram-negative bacteria, thereby limiting the overgrowth of specific strains (Rashid et al., 2024). Conversely, opportunistic pathogenic genera including *Vibrio* and *Enterobacter* are frequently detected as minor components of the core biofloc microbiome. Under stable water quality conditions and high heterotrophic bacterial densities, these potential pathogens are maintained at low, non-pathogenic levels through competitive exclusion and metabolic suppression (Wang et al., 2025).

3.2 Environmental and Operational Drivers of Community Succession

The compositional structure of the biofloc microbiome undergoes continuous ecological succession across the aquaculture culture cycle. This temporal succession is primarily regulated by operational parameters, including the type and frequency of

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organic carbon supplementation, dissolved oxygen (DO) concentration, total suspended solids, pH, and system salinity (Chen et al., 2024). Carbon source selection directly shapes community composition; simple carbohydrates like sucrose and molasses favor fast-growing heterotrophic *Gammaproteobacteria* and *Firmicutes*, whereas complex plant polysaccharides select for *Alphaproteobacteria* and *Bacteroidetes* equipped with specialized carbohydrate-active enzymes capable of breaking down recalcitrant polymers (Muthoka et al., 2025).

As bioflocs mature, physical aggregate growth alters internal chemical microenvironments. In early operational phases, small biofloc particles remain thoroughly oxygenated, favoring obligate aerobes and autotrophic nitrifiers. However, as microbial aggregation progresses and floc particle diameters exceed 100 to 300 μm, internal oxygen diffusion limitations create micro-anoxic inner core zones within the aggregate (Yang et al., 2026). This spatial separation creates micro-niches that allow facultative and obligate anaerobes such as denitrifying strains and fermentative bacteria to thrive in the center of the aggregate, while obligate aerobes remain active on the oxygenated outer surface. This micro-spatial structuring allows aerobic nitrification and anaerobic denitrification to proceed concurrently within the same culture vessel (Xiong et al., 2022)

4. Metagenomic Insights into Biogeochemical Nutrient Recycling Pathways

4.1 Nitrogen Metabolism: Heterotrophic Assimilation, Nitrification, and Denitrification

Nitrogen recycling in biofloc systems operates via three primary, interconnected metabolic pathways: heterotrophic nitrogen assimilation, autotrophic nitrification, and denitrification. Heterotrophic assimilation represents the fastest TAN removal pathway in BFT systems operating under elevated C/N ratios. Heterotrophic bacteria utilize dissolved ammonium directly as a nitrogen source for cellular protein synthesis, consuming organic carbon as an energy source. Stoichiometrically, the conversion of inorganic nitrogen into bacterial biomass prevents the accumulation of toxic nitrite intermediates, rapidly clearing TAN from the culture medium (Khanjani et al., 2025).

Autotrophic nitrification occurs parallel to heterotrophic assimilation, particularly in systems operating under moderate carbon addition rates. Nitrification proceeds via a two-step aerobic reaction: ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) first oxidize ammonium (NH_4^+) to hydroxylamine (NH_2OH), which is subsequently converted to nitrite (NO_2^-). Nitrite-oxidizing bacteria (NOB) then oxidize nitrite to nitrate (NO_3^-) (Ward, 2008).

To prevent nitrate accumulation during long-term zero-exchange operation, denitrification pathways within the anoxic inner cores of bioflocs reduce nitrate to inert nitrogen gas (N_2). Additionally, advanced metagenomic studies have revealed alternative nitrogen transformation pathways within mature bioflocs, including Dissimilatory Nitrate Reduction to Ammonium (DNRA) and Anaerobic Ammonium Oxidation (ANAMMOX), which further expand the operational nitrogen-clearing capacity of the microbiome (Hargreaves, 2013).

4.2 Functional Gene Dynamics

Metagenomic profiling and quantitative PCR (qPCR) assays track nitrogen

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Volume 4, Issue 3 (2026)

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Print ISSN 3007-3189
http://amresearchreview.com/index.php/Journal/about

transformations by monitoring the abundance and expression of specific functional marker genes across the culture cycle (Wu et al., 2022).

The enzymatic conversion of ammonium to hydroxylamine is catalyzed by ammonia monooxygenase, encoded by the *amoA*, *amoB*, and *amoC* gene cluster:

$$NH_4^+ + O_2 + 2H^+ + 2e^- \xrightarrow{amoA/B/C} NH_2OH + H_2O$$

Hydroxylamine oxidoreductase, encoded by *hao*, subsequently converts hydroxylamine to nitrite. Nitrite oxidation to nitrate is mediated by nitrite oxidoreductase, encoded by *nxrA* and *nxrB*. Metagenomic data show high copy numbers of *amoA* and *nxrA* during early system establishment, signaling active autotrophic nitrification (Wright et al., 2020).

Denitrification proceeds through a sequential enzymatic reduction cascade driven by dedicated functional genes:

$$NO_3^- \xrightarrow{narG/napA} NO_2^- \xrightarrow{nirK/nirS} NO \xrightarrow{norB/norC} N_2O \xrightarrow{nosZ} N_2$$

Nitrate reduction to nitrite is catalyzed by membrane-bound (*narG*) or periplasmic (*napA*) nitrate reductases. Periplasmic *napA* operates effectively under aerobic or micro-aerophilic conditions, whereas *narG* functions predominantly under strict anoxia. Nitrite reductase, encoded by either *nirK* (copper-containing) or *nirS* (cytochrome cd_1-containing), drives the rate-limiting reduction of nitrite to nitric oxide (NO) (Spanning et al., 2025). Niche partitioning occurs between *nirK*- and *nirS*-bearing denitrifiers based on carbon availability and nitrate concentrations. Nitric oxide is subsequently reduced to nitrous oxide (N_2O) by nitric oxide reductase (*norB/norC*), and finally to dinitrogen gas (N_2) by nitrous oxide reductase, encoded by *nosZ*. Elevated *nosZ* gene abundance in mature bioflocs ensures complete reduction to N_2, minimizing atmospheric emissions of the greenhouse gas N_2O (Kumar et al., 2020).

ANAMMOX processes are tracked via hydrazine synthase genes (*hzsA*, *hzsB*), which mediate the direct anaerobic coupling of ammonium and nitrite into nitrogen gas. Concurrently, the *nrfA* gene encodes nitrite reductase (cytochrome c_{552}), driving DNRA to retain nitrogen within the system as ammonium under high organic carbon loading (Tang et al., 2023)

4.3 Phosphorus Mobilization and Sulfur Turnover

Shotgun metagenomics reveals that biofloc microbiomes actively cycle phosphorus and sulfur alongside nitrogen compounds. Phosphorus cycling is driven by polyphosphate kinase (*ppk*), which regulates polyphosphate synthesis in Polyphosphate-Accumulating Organisms (PAOs), enabling them to uptake and store excess inorganic orthophosphate within bacterial cells (Meenakshisundaram et al., 2021). Organic phosphorus mineralization is facilitated by genes encoding alkaline phosphatases (*phoD*, *phoX*, *bpp*) and phosphonate transport complexes (*phnK*), which convert bound phytate and phospholipids from unconsumed feed into bioavailable phosphate. Taxa strongly correlated with these phosphorus-transforming pathways include *Candidatus* Aquiluna, *Kordia*, *Rubrimonas*, and *Subsaxibacter* (Hu et al., 2023)

Sulfur transformations within bioflocs are mediated primarily by the *sox* multienzyme system (*soxA*, *soxB*, *soxC*, *soxY*, *soxZ*), which encodes thiosulfate and sulfide oxidation pathways. Reduced sulfur compounds, such as hydrogen sulfide (H_2S), can accumulate in localized anaerobic zones within dense bioflocs or bottom sediments and present

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severe toxicity to culture stock. The presence of *sox* gene clusters in biofloc-associated *Proteobacteria* ensures the rapid oxidation of toxic sulfide into non-toxic sulfate (SO_4^{2-}), preserving overall water quality and host health (Dahl, 2020).

Table 2: Key Biogeochemical Functional Genes and Associated Microorganisms Identified via Metagenomics

Biogeochemical Cycle	Target Pathway	Key Functional Genes	Associated Microbial Taxa
Nitrogen	Ammonia Oxidation	*amoA*, *amoB*, *amoC*	*Nitrosomonas*, Nitrosopumilus (AOA)
Nitrogen	Hydroxylamine Oxidation	*hao*	*Nitrosomonas* spp.
Nitrogen	Nitrite Oxidation	*nxrA*, *nxrB*	*Nitrospira*, *Nitrobacter*
Nitrogen	Nitrate Reduction	*narG*, *napA*	*Pseudomonas*, *Bacillus*, *Alkaliphilus*
Nitrogen	Nitrite Reduction	*nirK*, *nirS*	*Azoarcus*, *Rhizobium*, *Pseudogulbenkiania*
Nitrogen	Nitrous Oxide Reduction	*nosZ* (Clades I & II)	*Pseudomonas mosselii*, *Halomonas* spp.
Nitrogen	ANAMMOX	*hzsA*, *hzsB*	*Comamonas*, *Taeseokella*
Nitrogen	DNRA	*nrfA*	*Enterobacteriaceae*, *Bacillus* spp.
Phosphorus	Phosphate Accumulation/Release	*ppk*, *phoD*, *phoX*, *phnK*	*Candidatus* Aquiluna, *Kordia*, *Rubrimonas*
Sulfur	Sulfide/Thiosulfate Oxidation	*sox* multienzyme cluster	*Rhodobacteraceae*, *Paracoccus* spp.

5. Biofloc Microbiome Mechanics in Host Immunity and Pathogen Suppression

5.1 Quorum Quenching and Competitive Exclusion of Pathogens

Biofloc microbial consortia protect cultured aquatic animals through both physical competition and targeted biochemical defense mechanisms. High cell densities of beneficial heterotrophic bacteria outcompete opportunistic pathogens such as *Vibrio parahaemolyticus* (the causative agent of Acute Hepatopancreatic Necrosis Disease, AHPND) and *Aeromonas hydrophila* for essential space and nutrients, suppressing pathogen proliferation within the water column. In addition, biofloc bacteria produce antibacterial secondary metabolites, organic acids, and bacteriocins that directly inhibit pathogen growth (Kumar et al., 2024).

Metagenomic analyses have highlighted Quorum Quenching (QQ) as a major enzymatic mechanism underlying pathogen suppression in BFT systems. Bacterial pathogens regulate virulence gene expression, biofilm formation, and motility through

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Volume 4, Issue 3 (2026)

Online ISSN 3007-3197
Print ISSN 3007-3189
http://amresearchreview.com/index.php/Journal/about

quorum sensing (QS) a cell-density-dependent signaling mechanism mediated by signal molecules such as N-acyl homoserine lactones (AHLs) in Gram-negative bacteria (Eckhard et al., 2026). Beneficial biofloc taxa, including *Halomonas alkaliphila*, *Bacillus velezensis*, and *Bacillus tequilensis*, encode functional AHL-lactonases and acylases. These enzymes cleave the lactone ring or amide bond of AHL molecules, inactivating signaling molecules and halting pathogen virulence gene expression. Because quorum quenching attenuates pathogen virulence without exerting direct bactericidal pressure, it reduces the selective pressure that leads to antibiotic resistance (James et al., 2021).

5.2 Microbial Biomass as In Situ Probiotic and Nutritional Supplementation

Ingestion of biofloc aggregates provides continuous, *in situ* nutritional supplementation for cultured species. Biofloc dry matter consists of 60% to 70% organic material and 30% to 40% inorganic constituents. Microbial biomass within the flocs serves as a complete food matrix, rich in microbial protein, essential lipids, functional carbohydrates, vitamins, and minerals (Ahmad et al., 2017).

Table 3: Proximate Nutritional Composition of Biofloc Aggregate Biomass

Nutritional Constituent	Proximate Quantity (% Dry Matter)	Physiological Function in Cultured Host
Crude Protein	30.5% – 50.0%	Provides bioavailable amino acids; reduces reliance on dietary fishmeal
Crude Lipids	2.6% – 4.8%	Supplies essential fatty acids required for cell membrane synthesis and energy
Crude Fiber	4.0% – 8.4%	Promotes intestinal peristalsis and stimulates endogenous digestive enzyme secretion
Ash / Minerals	7.0% – 39.3%	Delivers macro- and micro-minerals (Ca, Mg, P) for osmoregulation and exoskeleton formation
Caloric Value	18.0 – 29.2 kJ/g	Supports baseline metabolic requirements, enhancing protein-sparing efficiency
Bioactive Compounds	Variable (PHB, EPS, Carotenoids)	Poly-β-hydroxybutyrate (PHB) acts as an internal antibacterial agent and immunostimulant

Continuous consumption of bioflocs lowers feed conversion ratios (FCR) and significantly reduces total feed costs. Furthermore, bioflocs contain exogenous hydrolytic enzymes including proteases, lipases, and cellulases that remain active upon ingestion, assisting host digestion and increasing nutrient digestibility. Bioflocs also harbor bioactive polymers like Poly-β-hydroxybutyrate (PHB), which degrades into short-chain monomers within the digestive tract, inhibiting intestinal pathogens while serving as an energy source for enterocytes (Xu et al., 2016).

Figure 2: Integrated Carbon and Nitrogen Biogeochemical Cycling, Poly-β-Hydroxybutyrate (PHB) Biopolymer Synthesis, and Probiotic-

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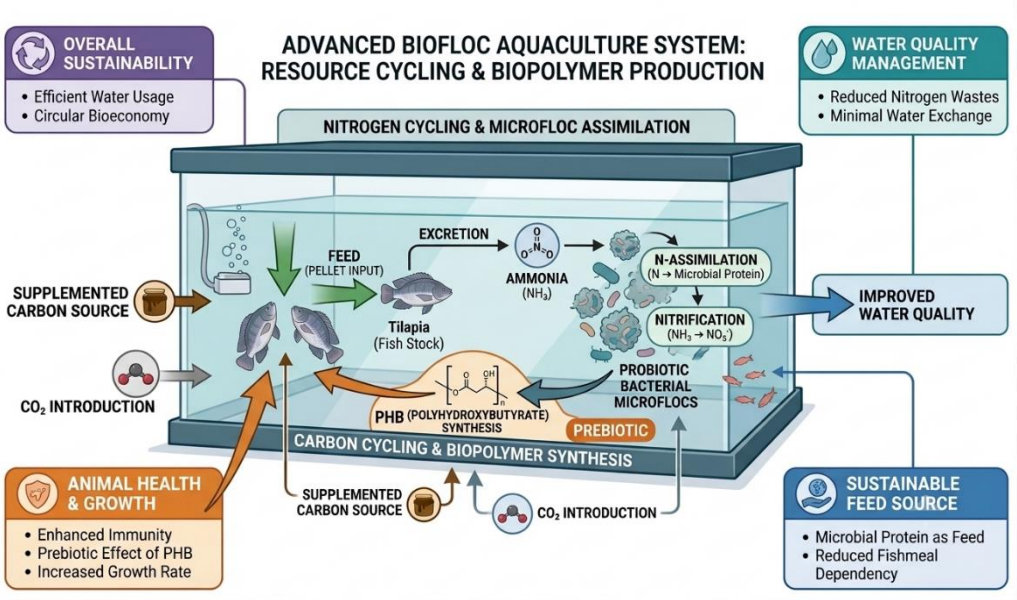
http://amresearchreview.com/index.php/Journal/about

Immunostimulatory Mechanisms in Biofloc Aquaculture.



5.3 Immunomodulation and Modulation of the Fish Intestinal Microbiota

Ingestion of biofloc aggregates significantly alters the host intestinal microbiome structure, promoting colonization by beneficial taxa such as *Bacillus*, *Lactobacillus*, and *Cetobacterium*, while suppressing potential pathogens. Heterotrophic anaerobic bacteria within biofloc cores ferment structural carbohydrates into Short-Chain Fatty Acids (SCFAs) primarily acetate, propionate, and butyrate. SCFAs lower gut luminal pH, creating an acidic environment that suppresses pathogenic bacterial growth while supporting beneficial mucosal microflora (Liu et al., 2023).

Simultaneously, microbe-associated molecular patterns (MAMPs) present on biofloc cell walls such as β-glucans, peptidoglycans, and lipopolysaccharides bind to host pattern recognition receptors (PRRs) on gut epithelial and immune cells (Newman et al., 2013). This interaction stimulates the host non-specific immune system, upregulating serum and mucosal lysozyme activity, superoxide dismutase (SOD), acid phosphatase (ACP), and key pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). As a result, aquatic animals cultured in biofloc systems exhibit elevated immune readiness, superior stress tolerance, and enhanced survival against infectious pathogen challenges (Yan et al., 2023).

6. Synthesis, Challenges, and Future Perspectives

Metagenomic characterization highlights BFT as an integrated biological system, where nutrient cycling, water quality maintenance, pathogen suppression, and host immunomodulation are driven by dynamic microbial interactions. Adding exogenous organic carbon to raise the system C/N ratio promotes heterotrophic bacterial proliferation, which outcompetes autotrophic nitrifiers and rapidly assimilates toxic ammonium into microbial protein (Emerenciano et al., 2017). Metagenomic analyses confirm that mature biofloc aggregates maintain spatial micro-environments, enabling simultaneous autotrophic nitrification (*amoA*, *nxrA*), anoxic denitrification (*narG*,

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http://amresearchreview.com/index.php/Journal/about

nirK/S, *nosZ*), polyphosphate accumulation (*ppk*), and toxic sulfide oxidation (*sox*) within the same culture tank. Ingesting these microbial aggregates delivers continuous probiotic strains, digestive enzymes, essential nutrients, and immunomodulatory compounds directly to the host gut, improving overall animal performance (Zhao et al., 2023).

Despite these benefits, several operational and biological challenges complicate biofloc management:

First, dense microbial respiration creates high biological oxygen demand (BOD). Uninterrupted, high-capacity mechanical aeration is required to keep flocs in suspension and maintain dissolved oxygen levels. Aeration failures can rapidly cause localized anoxia, aggregate collapse, microbial death, and sudden spikes in toxic TAN (Faußer et al., 2016).

Second, excessive total suspended solids (TSS) accumulation (>500 mg/L) can cause respiratory stress and branchial damage in clear-water adapted species, requiring specialized settling basins or foam fractionators to maintain optimal solids levels (Bitencourt et al., 2025).

Third, metagenomic studies reveal that bioflocs can act as reservoirs for latent opportunistic pathogens (*Vibrio*, *Aeromonas*) and antibiotic resistance genes (ARGs). Environmental stress or water quality deterioration can trigger dysbiosis, allowing opportunistic pathogens within the biofloc matrix to proliferate and cause disease outbreaks (Chouhan et al., 2025).

Fourth, metagenomic data emphasize that probiotic and metabolic functions are highly strain-specific. Broad taxonomic assignments can be misleading, as closely related bacterial strains within the same genus may vary significantly in their virulence, quorum quenching capacity, or metabolic potential (Schmid et al., 2018).

To address these challenges, future biofloc research should integrate multi-omics approaches combining shotgun metagenomics, metatranscriptomics, metabolomics, and single-cell sequencing to map microbial functional dynamics under varied operational conditions. Deploying portable sequencing platforms, such as Oxford Nanopore devices, enables real-time, on-site microbiome profiling in aquaculture facilities (Habib et al., 2026). Early detection of shifts toward dysbiosis or pathogen enrichment allows producers to adjust carbon dosing, aeration, or solids removal proactively. Furthermore, integrating metagenomic datasets with machine learning algorithms will facilitate the development of predictive models that optimize C/N ratios, reduce energy consumption, and ensure long-term system stability (Perzon & Ilan, 2025).

6. Conclusion

Metagenomic characterization has fundamentally transformed our understanding of biofloc systems, revealing them as complex, multi-kingdom microbial ecosystems where nutrient recycling, water quality maintenance, pathogen suppression, and host immunomodulation are inextricably linked through dynamic microbial interactions. The strategic elevation of the carbon-to-nitrogen ratio through exogenous organic carbon supplementation promotes heterotrophic bacterial proliferation, which rapidly assimilates toxic ammonium into microbial protein while outcompeting slower-growing autotrophic nitrifiers. Advanced shotgun metagenomic analyses confirm that

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mature biofloc aggregates maintain spatially structured microenvironments, enabling the simultaneous operation of aerobic nitrification (*amoA*, *nxrA*), anoxic denitrification (*narG*, *nirK/S*, *nosZ*), polyphosphate accumulation (*ppk*), and toxic sulfide oxidation (*sox*) within the same culture vessel. This metabolic redundancy ensures robust nitrogen clearance and system stability across variable operational conditions. The nutritional and immunological benefits of biofloc ingestion are equally well-documented; continuous consumption of microbial aggregates delivers probiotic bacteria, exogenous digestive enzymes, essential nutrients, and immunomodulatory compounds directly to the host gastrointestinal tract. Quorum quenching mechanisms mediated by AHL-lactonases from beneficial taxa such as *Bacillus velezensis* and *Halomonas alkaliphila* provide targeted pathogen suppression without selecting for antibiotic resistance, representing a sustainable disease management strategy aligned with One Health principles. Nevertheless, several challenges must be addressed for broader BFT adoption: high biological oxygen demand necessitating uninterrupted aeration; excessive total suspended solids requiring specialized management; and the potential for bioflocs to serve as reservoirs for opportunistic pathogens and antibiotic resistance genes under dysbiotic conditions. The strain-specific nature of probiotic and metabolic functions further emphasizes that broad taxonomic assignments are insufficient for predicting functional outcomes. Looking forward, the integration of multi-omics approaches combining shotgun metagenomics, metatranscriptomics, metabolomics, and single-cell sequencing with machine learning algorithms and portable sequencing platforms will enable real-time, predictive microbiome management. Such technological convergence will optimize carbon dosing, aeration regimes, and solids removal strategies while minimizing energy consumption and operational costs. By advancing our ability to manipulate and monitor biofloc microbiomes with precision, these innovations will cement BFT as a cornerstone technology for sustainable aquaculture intensification, addressing global food security demands while mitigating the environmental footprint of aquatic food production.

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