EEET ECOLOGICAL ENGINEERING & ENVIRONMENTAL TECHNOLOGY

Ecological Engineering & Environmental Technology, 2026, 27(2), 71–88
https://doi.org/10.12912/27197050/215909
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Received: 2025.11.25
Accepted: 2025.12.29
Published: 2026.02.07

Bio carrier integrated multistage and anaerobic-anoxic-oxic systems for enhanced organic and nutrient removal from municipal wastewater: A pilot scale study

Manish Kumar[1], Dayanand Sharma[1], Rakesh Choudhary[2*], Ajay Kumar[2]

[1] Department of Civil Engineering, Sharda University, Greater Noida 201310 (U.P.), India
[2] Department of Civil Engineering, National Institute of Technology Delhi, New Delhi 110036, India
* Corresponding author's e-mail: environmentrakesh@gmail.com

ABSTRACT

Traditional wastewater treatment processes, such as activated sludge and trickling filters, generally face short-term dynamic operational instability and low removal efficiencies of nutrients along with high energy consumption. Dealing with these problems, integrated biological systems which maintain stable performance under the changing load should be developed. The objective of this study was to design a bio-carrier-contained A2O (anaerobic-anoxic-oxic) based advanced process train for improved removal efficiency of organic matter and nutrient from municipal wastewater. Two pilot scale plant configurations, Setup-1 (three stage aerobic configuration with 30% fill of carrier) and Setup-2 (A2O process in which carriers were present in the aerobic zone), were fed at a flow rate of 100 L day^{-1} and hydraulic retention time (HRT) was set to 6–8 h. The progressive acclimation and stabilization of biofilm was reached for Setup-1, where COD removal increased from 73.7 to over 85%, BOD removal rose from 86.7% to above 92% and TSS reduction achieved 90%. The system was operated at neutral pH (7.2–7.8) and with steady-state biofilm densities of 0.9–1.45 g m^{-2}. Developed biofilms on the bio-carrier surface showed maximum dry weight production under lower operational temperature conditions (approximately 20 °C). In Setup-2, the removal of COD and BOD were higher than 79 and 87% respectively, whereas TSS removal was greater than 92%. Nutrient removal efficiency was considerable, NH_4^+-N and TN removal efficiencies were near 80% and 75%, respectively, while TP removal efficiency was approximately 66%. Surface biomass density was up to 4.72 g m^{-2} indicating later microbial colonization. The bio-carrier-assisted multistage configuration is proved in this study to be an effective, environmentally friendly and economically viable process for advanced treatment of wastewater and nutrient control.

Keywords: biofilm, biomass, colonization, denitrification, microbial communities, moving bed biofilm reactors (MBBR), nitrification, SDG 6 (clean water and sanitation).

INTRODUCTION

The growing demand for efficient wastewater treatment alternatives has generated around the globe interest in processes that could combat organic pollutants and nutrients under different conditions of treatment. Conventional biological treatment processes such as activated sludge process, trickling filters and membrane bioreactors have been the workhorses for many years in wastewater treatment. Nevertheless, numerous limitations impede their commercial exploitation at a large-scale including high energy consumption, higher land demand, hydraulic instability and poor nutrient removal (Gupta et al., 2023; Regmi et al., 2011). These constraints have become more evident under increasingly variable influent loads and stringent effluent discharge norms. Recent advances in predictive water-quality modelling have shown that ensemble machine-learning approaches combined with explainable artificial intelligence can significantly improve the assessment and forecasting accuracy needed for modern wastewater-management systems (Choudhary et al., 2025a). In addition, the growing need to comply with stricter discharge requirements and aging wastewater systems overloaded with pollutants have compelled investment in this industry (Alitaleshi et al., 2024; Sharma et al., 2024). For instance, fuzzy logic–based life cycle assessment frameworks have been successfully applied to capture uncertainty, multi-criteria trade-offs, and sustainability performance in complex urban waste management systems, providing valuable insights for environmentally sound infrastructure planning (Choudhary et al., 2025b). Wastewater treatment is a basic need for environmental engineering and avoids the discharge of pollution loaded effluents to natural water bodies, affecting ecological health on one side and public health (including material cycles: e.g., hydro-geochemical interchanges) on the other. Water treatment is conventionally classified into four stages: preliminary, primary and secondary, and tertiary treatments that separate certain classes of materials and any dangerous wastes discharged into receiving systems (Madan et al., 2022). Herein, secondary biological treatment is important as dissolved and colloidal organic matter is biologically transformable through microbial metabolism and simultaneously nutrient transformation and removal also advance. The biological treatment technology has been developed through more intensive research in microbial processes and reactor engineering. Classic water treatment systems are the activated sludge, trickling filters and MBRs that regulate microbial dynamics through aeration, mixing and controlled hydraulic regimes (Jagaba et al., 2024a). However, due to environmental threats including micropollutant contamination, nutrient over-enrichment and water scarcity (Gzar et al., 2021), the conventional systems have been renovated or replaced by advanced configurations such as filled bed bioreactors (FBBRs), sequencing batch reactors (SBRs), constructed wetlands (CWs) and advanced oxidation processes (AOPs) (Di Trapani et al. 2008). One of the primary benefits of these new systems is their potential to alleviate challenges in centralized wastewater management faced by developing nations, increasing treatment coverage levels in areas with limited access to resources. However, high and steady removal of BOD, COD and nutrients (N&P) has continued to be a challenge in conventional as well as advanced systems due to process instability, insufficient biomass retention, substrate shock loading and loss of functional microbial guilds under fluctuating conditions.

The encouraging prospects of biofilm technologies are being pointed out in recent research works, showing their capacity to be viable alternatives to the classic suspended-growth systems. Biofilm reactors, that include trickling filters, rotating biological contactors (RBC), moving bed biofilm reactors (MBBR) and integrated fixed-film activated sludge (IFAS) are characterized by high cell density with enhanced solids retention times (SRTs) and excellent response to fluctuation in loading (Falletti et al., 2014). Particularly MBBR is gaining interest for being a compact, modular system of flexible operation (Dias et al. 2018). In MBBR and hybridized processes such as nitrification/denitrification and other biological advanced wastewater treatment technologies, the bio-carrier (engineered support media designed) to enhance microbial attachment for biofilm development is essential for technological enhancement. The high specific surface area offered by these carriers promotes biofilm formation and efficient microbial activity at high organic and hydraulic loads (Zhuang et al., 2019). Biofilm is structurally and ecologically complex with a diversity of metabolic activities during both nitrification, denitrification, phosphorus removal and biodegradation of xenobiotic compounds; therefore, biofilm-based systems are uniquely versatile for use in municipal and industrial wastewater treatment (Dereli et al., 2021). However, despite the extensive application of MBBR and IFAS technologies, there remains limited understanding of how carrier placement, redox zoning, hydraulic regimes and microbial community succession jointly influence biofilm stability and nutrient removal performance. This knowledge gap motivates the present work.

The uniqueness of the work is in the incorporation of a bio-carrier-based process train that combines both the synergistic effects of suspended and attached biological systems in a unified fashion. In contrast to conventional MBBR and IFAS systems, the new concept represents a well-structured multi-stage reactor system, in which biomass carriers are specifically placed for anaerobic, anoxic and aerobic stages to engineer stepwise carbon oxidation, nitrification, denitrification and biological phosphorus removal (EBPR). The system conducts the nitrification, denitrification and phosphorus removal simultaneously in compact construction with reduced operational energy demand. The system also comprises a technique for the selection and manipulation of bio-carrier materials on a physical, chemical and biological basis to enhance biofilm formation, microbial biodiversity and operational stability. This study links microbial ecology and process performance under different hydrodynamic conditions, indicating a causative relationship between bio-carrier design, ecological adaptation, and treatment capacity. In addition, the proposed process explores the synergistic combination effects of suspended and biofilm microorganisms, which has been seldom reported in practice. Furthermore, practical application of HBR in treating wastewater is emphasized and the novelty lies in addressing the practical issues of HRT, carrier fill ratio and aeration control. This research is expected to promote the advancement of biological process intensification in wastewater treatment towards enhancing global sustainability objectives including energy saving, sludge minimization, and small footprint for adoption into current treatment plants.

To strengthen the novelty, this study specifically contributes by providing a comparative evaluation of two pilot-scale configurations, namely the three-stage aerobic system and the A2O process with carriers, by presenting detailed kinetics of surface and volumetric biofilm accumulation in the range of 0.03 to 4.72 g m^{-2} which is rarely quantified in hybrid systems, and by establishing a clear mechanistic linkage between hydrodynamic conditions, microbial succession patterns and the resulting nutrient removal performance. The general objective of this work is to create an integrated bio-carrier process train for the simultaneous enhanced removal of biochemical oxygen demand (BOD), chemical oxygen demand (COD) and nutrients, which include nitrogen and phosphorous in municipal and industrial wastewaters. The objective of this study is to get an advanced multistage process configuration in which the anaerobic, anoxic and aerobic zones are provided with bio-carriers to achieve the best degradation efficiency for pollutants by the cooperative microbial interaction among various microorganisms. Moreover, the study aims to investigate the optimal operational parameters such as hydraulic retention time (HRT), organic loading rate (OLR), and aeration conditions that significantly influence hydrodynamics performance and operational stability of the bioreactor. Pilot-scale experiments and comparisons with conventional treatment technologies will be used to assess the treatment efficiency, energy requirements, and applicability of individual unit configuration. Furthermore, the structural and functional properties of biofilms formed on carriers shall be studied by light and electron microscopy and molecular biology methods in order to link microbial ecology with removal efficiencies. The findings will be a scientifically grounded and engineering-relevant platform for the optimization of operation, and it will also identify a practical, sustainable yet economical process to transform conventional wastewater treatment facilities in view of increasingly stringent discharge demands. This work contributes to advancing the utilization of integrated bio-carrier-based technology as a technologically advanced method toward sustainable and stable wastewater treatment performance in practice.

MATERIALS AND METHODOLOGY

The experimental study was carried out at the Delhi Jal Board, sewage treatment plant (STP) 45 MGD Phase Four, Delhi, India, where pilot-scale systems were established. To provide a clear visual understanding of the study location, pilot-scale treatment units, wastewater flow points, and analytical procedures adopted in this investigation, a comprehensive photographic overview is presented in Figure 1.

Although compact in appearance, the pilot reactors were equipped with multiple sampling ports and monitoring points, enabling continuous performance assessment under real STP operating conditions. These visual representations support the experimental design and sampling strategy discussed below and establish a direct link between the field-scale pilot operation and the laboratory-based analytical measurements used for performance evaluation. Two parallel pilot-scale biological treatment units with the same capacity were developed to study the start-up characteristics and performance of bio-carrier-based BNR processes in a controlled field-laboratory hybrid environment. Each treatment train comprised three sequentially arranged bioreactors and a secondary clarifier. Reactor configuration was composed of the first and second reactors with internal dimensions 150 × 150 × 500 mm, the third reactor with internal dimensions 200 × 200 × 500 mm, and the secondary clarifier with internal dimensions of 250 × 250 × 500 mm,

Figure 1. Overview of the study location, pilot-scale wastewater treatment systems, and analytical workflow: (A) geographic location of the pilot-scale treatment facility in Delhi, India; (B) pilot-scale reactor units showing compartmentalization, bio-carrier media placement, and wastewater flow pathways; (C) wastewater at different operational stages (influent, aeration, and treated effluent); (D) laboratory-based physicochemical and nutrient analysis of collected wastewater samples

designed to process a daily flow rate of approximately 100 liters of settled wastewater (refer Figure 2 and Figure 3). All tanks were constructed from stainless steel sheets to allow visual observation of biofilm growth, flow patterns, and aeration performance. Although the pilot reactors appear as compact stainless-steel units in Figure 1, each reactor was equipped with multiple strategically placed sampling ports and monitoring points (influent, intermediate and effluent positions). These access points enabled systematic collection of operational data required for generating the detailed time-series profiles of COD, BOD, TSS and nutrient parameters presented in the Results section. Thus, the numerical results reported in this study are directly derived from continuous field-based sampling rather than from laboratory simulations. A simplified schematic of the internal arrangement and flow sequence is shown in Figure 1 and Figure 2. First settled sewage was pumped continuously through the system at a flow rate of approximately 100 L day^{-1} via a peristaltic pump, giving an overall HRT of 6–8 hours for the treatment train combined. Oxygen was supplied by medium-bubble diffusers located at the bottom of aerobic zones to allow homogenization and efficient oxygen transfer. The DO concentration was kept at 2.0 ± 0.2 mg L^{-1} and monitored by a portable DO meter (Model HQ40d, HACH Corporation, USA). During operation, DO never dropped below the threshold value, signifying stable and sustained aerobic conditions.

Airflow was controlled with a manifold and rotameter assembly to ensure aeration intensity was consistent in all reactors. All the reactors were run at room temperature (25–30 °C). For hydraulic stabilization, two vertical baffles were installed: one at the inlet to dissipate influent flow energy and the other at the outlet to prevent short-circuiting and ensure uniform sedimentation within the secondary clarifier. Effluent and internal recirculation samples were withdrawn intermittently for performance evaluation to support mass balance assessments and system optimization.

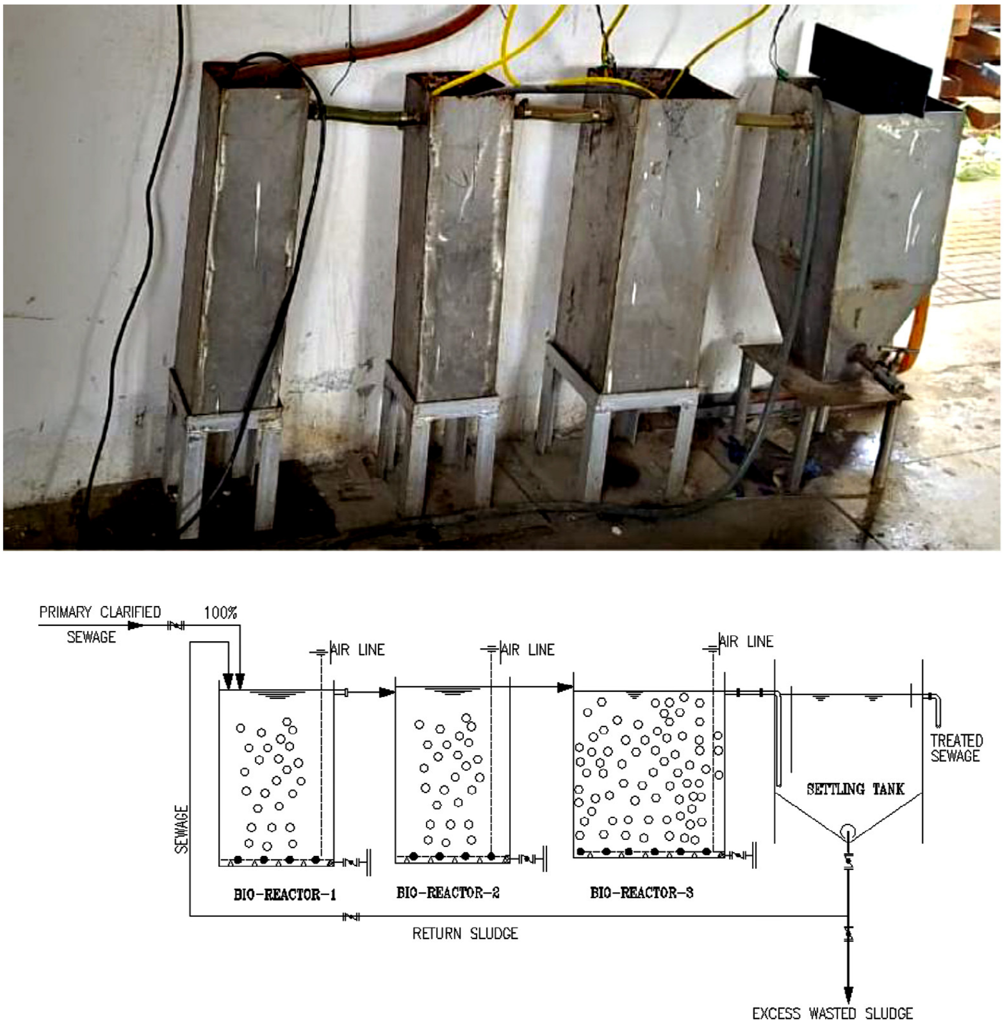

Figure 2. Pilot-scale reactor configuration and physical layout of the experimental wastewater treatment system used for Setup-1 (three-stage aerobic configuration) and Setup-2 (A2O process)

Process configurations and operational strategy

Figure 3 shows the biological treatment system (Setup-1), fabricated to examine the performance of a multistage bio-carrier-assisted aeration process for municipal sewage treatment. Influent comprising concentrated clarified water from a running STP system was passed through a three-staged continuous-flow reactor and then into a secondary settling tank. Each aeration compartment was filled with bio-carrier media at 30% to provide adequate area for microbial adhesion, biofilm formation and improved oxygen diffusion. The mode of operation for Setup-1 was based on the integrated suspended–attached growth modality within which free-floating activated sludge and attached biofilm communities were symbiotically maintained. The aeration tank series created plug-flow hydraulics, which provided controlled substrate degradation and turnover of the microbial community between Stages 1–3. The first reactor primarily treated high-strength organics through rapid heterotrophic oxidation, while the two subsequent reactors supported nitrification, stabilization, and polishing of effluent. For biological stability, about 50% of the clarified effluent from secondary sedimentation was internally recycled to the first aeration zone. This recirculation enhanced hydraulic buffering, improved mixing and increased contact between substrate and biomass. The concentration of mixed liquor suspended solids (MLSS) in the aerobic reactor was maintained at 2500–3000 mg/L, while return sludge line MLSS values were held at 8000–10.000 mg/L, indicating good biomass thickening and recapture. Intermittent sludge wasting maintained an SRT of 10–14 days such that microbial growth and decay rates could be balanced without the development of excessive biomass, which might otherwise inhibit oxygen transport. Figure 3 illustrates the detailed process flow configurations of both pilot-scale treatment systems, including Setup-1 (three-stage aerobic configuration) and Setup-2 (anaerobic–anoxic–oxic process with bio-carrier media in the aerobic zone). The first unit is the anaerobic reactor (mixing only), where phosphorus release and organic matter hydrolysis take place under strictly anaerobic conditions using primary clarified influent from the existing STP plant. The flow then enters the anoxic reactor (mixing only) for denitrification, during which nitrates recycled from the aerobic reactor are reduced to nitrogen gas. The anoxic zone effluent is fed into the aerobic reactor, which in the current installation contains 30% bio-carrier media (Media-M1) to promote biomass retention and nitrification through biofilm formation. Continuous oxygen is fed to maintain acceptable DO levels for nitrifiers. The secondary clarifier removes separated biomass, producing the final treated effluent (refer Figure 2). The settled sludge is partially returned to the anaerobic reactor as return sludge to stabilize microbial activity, while excess sludge is periodically wasted to maintain solids balance. Nitrates necessary for efficient denitrification are also supplied through an internal sludge recycle loop connecting the aerobic and anoxic reactors. All reactors operated with continuous DO and oxidation-reduction potential (ORP) monitoring to establish distinct redox conditions across zones.

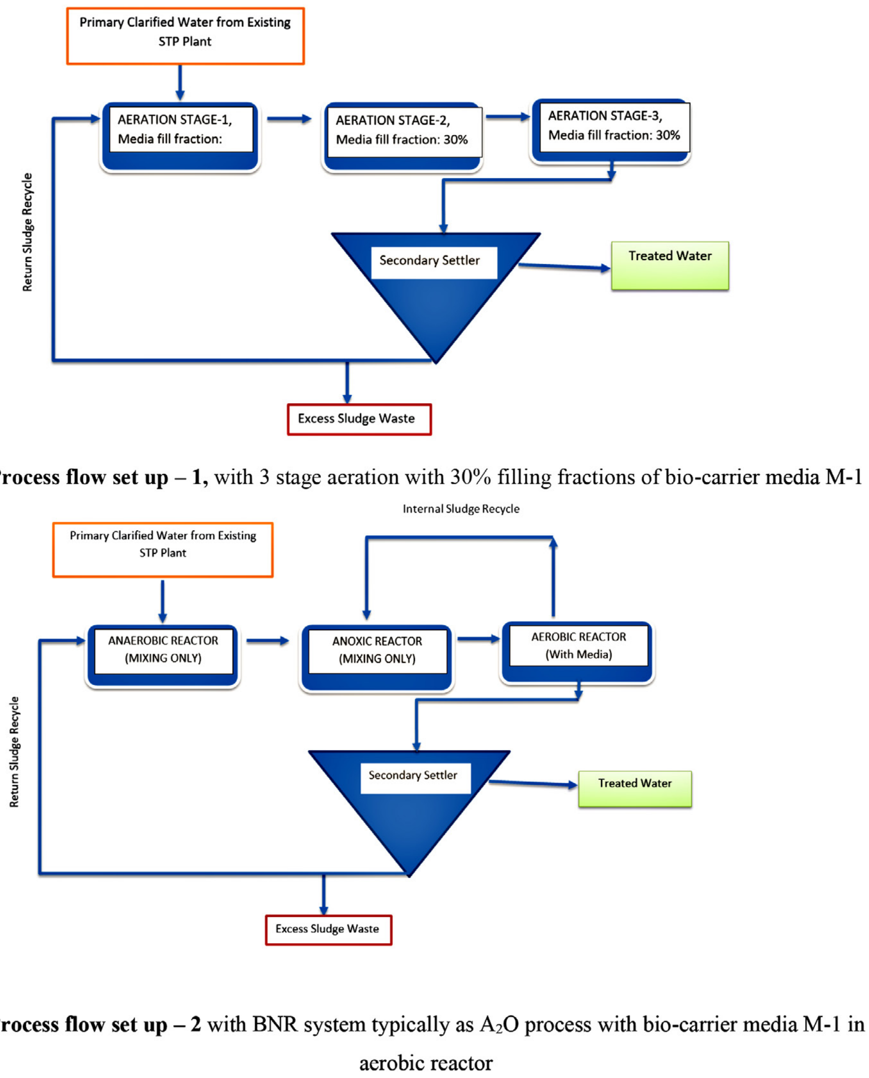

Figure 3. Process flow configurations of the pilot-scale treatment systems: (a) Setup-1, three-stage aerobic reactor with 30% bio-carrier filling; (b) Setup-2, anaerobic–anoxic–oxic (A2O) system with bio-carrier media in the aerobic reactor

The operational variables, except for recycle ratio (R), MLSS, and SRT, were maintained as those in Setup-1. The influent was flowed continuously, while effluent samples were collected daily at steady state where less than 10% variation in BOD, COD, and nutrient removal levels was observed for seven successive days. Both pilot configurations (Setup-1 and Setup-2) were continuously operated for a total duration of 45 days under flow-through conditions to evaluate start-up behaviour, biofilm formation characteristics and treatment performance. The initial 10 to 15 days corresponded to the acclimation and transition phase, during which microbial attachment, EPS secretion and hydraulic stabilization gradually developed. Days 16–30 represented the active growth and maturation phase, marked by progressive increases in COD, BOD and TSS removal as well as biofilm accumulation on the carriers. Steady-state conditions were defined operationally when daily effluent COD, BOD and nutrient concentrations showed less than 10% variation over seven consecutive days. All time-series plots presented in Figures 3–7 originate from measurements collected throughout this 45-day monitoring period, during which influent and effluent samples were analysed at prescribed intervals. This operational timeline establishes a direct and transparent link between reactor functioning, sampling events and the numerical results reported in the study.

Sampling and analytical procedures

Influent and effluent samples were taken daily in both the startup and steady-state periods to evaluate the efficiency of each bio-carrier system. The physico-chemical and nutrient parameters were determined according to the Standard Methods for the Examination of Water and Wastewater (APHA 2005; Yadav et al., 2022). The influent and effluent pH was measured using a calibrated digital pH meter. The concentration of DO within each reactor was monitored with a hand-held DO meter and adjusted as required in order to maintain consistent aerobic conditions. The BOD_5 value, which is indicative of the amount of biodegradable organic load in the wastewater, was measured by the standard five-day incubation at 20 °C using sealed BOD bottles. COD was determined by the closed-reflux dichromate procedure, in which organic and inorganic matter is oxidized under acidic conditions, and the absorbance was measured at 600 nm. The concentration of total suspended solids (TSS) was determined gravimetrically by passing a known volume of wastewater through a pre-weighed glass microfiber filter, drying at 105 °C to constant weight and expressing the result as the mass of dry solids retained. Ammonium-nitrogen (NH_4^+–N) was tested by Nessler's method, in which ammonium reacts with Nessler's reagent to form a coloured complex measured at 425 nm using a UV–Vis spectrophotometer. Total nitrogen (TN) was determined by alkaline persulfate digestion followed by ultraviolet detection of nitrate. Total phosphorus (TP) was analysed by persulfate digestion and subsequent colour formation based on the ascorbic acid method with absorbance measured at 880 nm. Three repetitions were performed for each analysis to reduce experimental error. The sampling frequency ranged from daily to every other day depending on the parameter. For each setup, grab samples were collected from the influent line, at the outlet of each bioreactor compartment, and from the final clarifier to develop the time-series trends shown in Figures 4, 6 and 7. COD, BOD, TSS and pH were analysed on a daily basis, whereas nutrient parameters (NH_4^+–N, TN and TP) were measured three times per week due to their longer analytical processing time and lower expected daily variation. All parameters were analysed in triplicate, and the mean values were used for plotting. These procedures ensured a systematic and consistent dataset supporting the detailed graphical profiles presented in the Results section. The removal rates of each pollutant were determined using the formula

$$\eta = \frac{(C\_in - C\_out)}{C\_in} \times 100 \tag{1}$$

where: η – Removal efficiency (%), C_in – Influent concentration, C_out – Effluent concentration.

The growth kinetics of biofilm and its relationship with system stability were studied by monitoring the development profile of attached biomass on the bio-carrier media over time. At predetermined intervals, media samples were taken out and gently washed to remove loosely bound solids, dried at 105 °C to constant weight, and subsequently ignited at 550 °C to determine VSS. The weight difference between the two measurements was regarded as the biologically

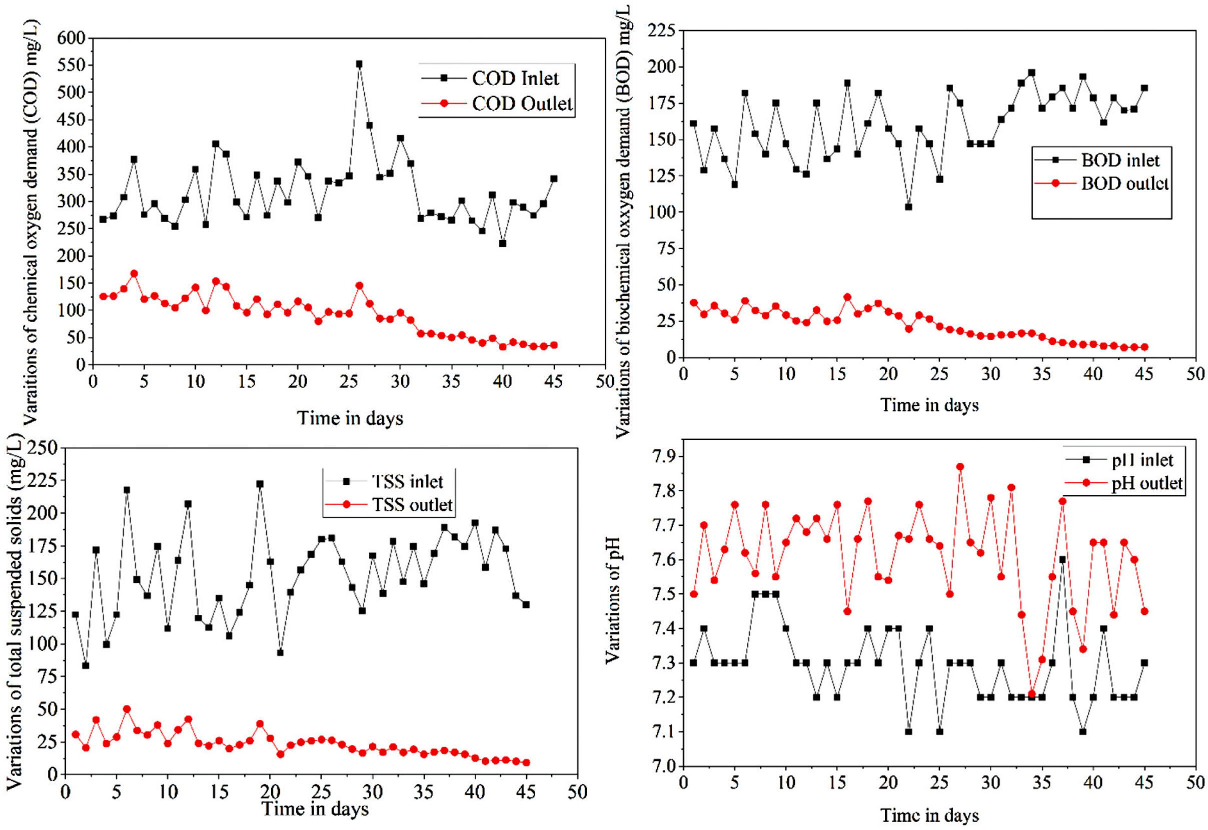

Figure 4. Inlet and outlet water quality parameters for process flow Scheme-1: COD, BOD, and TSS and pH

active biomass adhered to the carrier surface. The attached biomass concentration was expressed as milligrams of VSS per square centimetre of carrier surface area, and the volumetric biofilm growth rate was expressed as milligrams of VSS per litre of reactor volume per day. For monitoring biofilm development trends presented in Figures 4 and 7, representative carrier elements were withdrawn at predefined operational intervals (Days 5, 10, 15, 20, 25, 30, 35, 40 and 45). At each interval, three carrier pieces were sampled from the aerobic zone to ensure spatial representation along the reactor depth. Each carrier was gently rinsed to remove loosely bound solids before VSS determination. The reported biomass values represent the mean of triplicate measurements and were used to derive both surface (g m^{-2}) and volumetric (kg m^{-3}) biofilm accumulation rates. This systematic sampling protocol provides the basis for the temporal biomass growth curves shown in the Results section. The stability of the process was determined by statistical analysis using one-way ANOVA at a confidence level of 95% ($p < 0.05$) to compare differences between reactor setups.

RESULTS AND DISCUSSION

Variations in pH, COD, BOD, and TSS in the setup 1

Influent COD values of Setup-1 varied from 222.6 to 552.3 mg/L, and effluent concentrations were reduced to 33.1–167.7 mg/L, leading to an average removal efficiency of around 73.7%, which progressively increased with system maturation to approximately 80.8–85.7% (refer Figure 4). This upward trend reflects the gradual establishment, acclimatization and stabilization of the biofilm community over the operational period. The pattern is consistent with hybrid MBBR systems in which removal efficiencies tend to increase steadily as microbial consortia colonize carrier surfaces and develop extracellular polymeric substance (EPS) matrices that enhance substrate diffusion and adsorption (Ødegaard, 2006).

The good oxidative ability of Setup-1 is also consistent with the results of (Zhuang et al., 2019), reporting ~90.9% COD removal in a hybrid moving-bed biofilm–membrane reactor at similar hydraulic retention times (HRT = 6–8 h) and aeration schedules. Similarly, (Dias et al.,

2018) found COD removals of around 89–92% in MBBR systems treating coking wastewater with 50% carrier filling ratios. These results demonstrate that the three-phase aeration design of Setup-1 (30% media and 50% internal recycle) is capable of delivering sufficient contact time and oxygen-utilisation efficiency comparable with advanced hybrid systems.

The gradual increase from ~74% to >80% COD removal at the later stage corresponds to microbial succession occurring within the reactor, wherein heterotrophs dominate the initial phase while nitrifiers and slowly growing oligotrophic species establish later. This sequence is recognised to increase the metabolic flexibility of the community and exert positive effects on overall system robustness (McQuarrie and Boltz, 2011).

The influent BOD ranged from 103.6 to 196 mg/L, and effluent values were as low as 6.9–41.6 mg/L, with an average BOD removal greater than 86.7%, which increased to >92% after 20 days (refer Figure 4). These values indicate efficient carbonaceous biodegradation and strong organic stabilisation. In general, biofilm reactors have been reported to produce better BOD removal efficiency than suspended-growth reactors because they contain both attached and suspended biomass. Biofilms offer a stable micro-environment with coexistence of aerobic and anoxic microniches necessary to sustain simultaneous nitrification and denitrification (Alitaleshi et al., 2024).

Similar systems have been observed to achieve BOD removal between 85% and 95% under favourable conditions. For instance, a hybrid-carrier MBBR treating domestic wastewater removed ~88% BOD at lower HRTs, indicating the resilience of attached-growth systems (Gzar et al., 2021). The current findings indicate that the biological kinetics and mass-transfer characteristics of Setup-1 are competitive with the best-performing bio-carrier systems reported so far.

The influent TSS varied from 83.25 to 222.3 mg/L, and effluent concentrations were reduced to 9.2–50.2 mg/L, with an average removal of around 85.4%. In the mature operational phase, the efficiency enhanced to ~90%, attributed to improved flocculation, stronger biofilm–floc interactions, and better clarifier settling performance (see Figure 4). TSS removal in hybrid biofilm reactors relies on biological floc formation as well as mechanical interception of fine solids by carrier surfaces (Hassan, 2020). The floc structures settle effectively in the clarifier as microfloc clusters bound by EPS, providing excellent effluent clarity. (Wang et al., 2022) reported TSS removal above 80% in hybrid MBBRs treating ceramic industry effluent, supporting the performance trends observed in this study. Accordingly, the configuration of Setup-1 resulted in solids retention comparable to well-designed industrial pilots.

The influent pH of Setup-1 fluctuated between 7.1 and 7.6, whereas the treated effluent ranged from 7.21 to 7.87 (average ≈ 7.56), confirming that the system was well-buffered and maintained a stable biological environment (refer Figure 4). The slight rise in effluent pH is mainly due to CO_2 desorption coupled with the nitrification process, which produces hydroxyl ions and consumes carbonic acid, thereby shifting the equilibrium toward mildly alkaline conditions (Jagaba et al., 2024; Sharma et al., 2024). Since the enzymatic activity of heterotrophs and nitrifiers, especially Nitrosomonas and Nitrobacter, operates optimally between pH 7.0 and 8.0 (Xu et al., 2025), this near-neutral to slightly alkaline range is essential to maintain efficient biological reactions.

The observed pH range is a key factor governing balanced nitrification and denitrification reactions in hybrid or moving-bed biofilm systems, as supported by previous studies. A review by (Jagaba et al., 2024) noted that near-neutral pH combined with adequate dissolved oxygen (DO > 2 mg/L) promotes stable COD removal and nitrification rates. As a result, the pH dynamics in Setup-1 demonstrate that redox conditions, alkalinity balance, and buffering capacity were consistently maintained through the reactor design incorporating controlled recirculation, moderate solids retention time (SRT), and bio-carrier structure.

Surface and volumetric biomass growth rates in process Setup-1

Time profiles of biofilm development trends in Setup-1 (Figure 5) demonstrate a well-defined sequential colonization pattern common for hybrid biofilm suspended growth systems. Biomass development, expressed through surface biofilm density (g m^{-2}) and volumetric biomass concentration (kg m^{-3}), presented a typical sigmoidal growth curve with distinct attached, growing, and detached phases. This progression reflects the classical biofilm life cycle, beginning with initial cell adhesion, followed by accelerated EPS-supported accumulation and

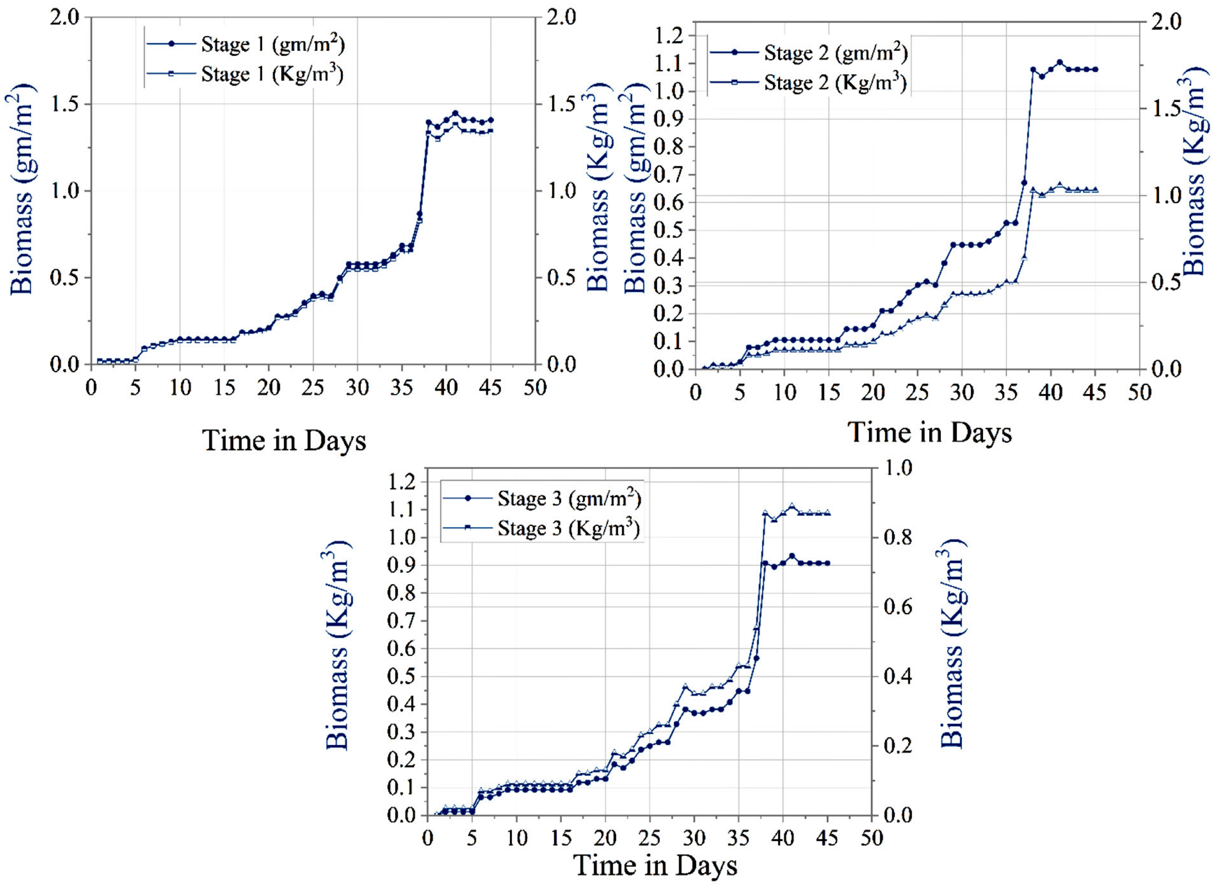

Figure 5. Assessment of surface and volumetric biomass growth rates in process Setup-1

concluding with shear-induced detachment under stable hydrodynamic conditions.

For the initial 10 days, no measurable biomass accumulation (< 0.1 g m^{-2}) was observed across all stages, which represents the lag or conditioning phase of biofilm formation. Microbial attachment to the carrier surfaces was initially governed by reversible physical forces such as van der Waals and electrostatic interactions, followed by early EPS secretion that transitions the attachment from reversible to irreversible. This stage mirrors the observations of Dereli, Clifford, and Casey (2021), who also reported minimal surface growth during the early weeks of MBBR operation due to microbial acclimatization, surface wetting and slow development of adhesion proteins. The slow increase in biomass is attributable to relatively low nutrient consumption and a limited substrate affinity (low k_s), characteristics typical of non-mature and diffusion-limited early biofilms (Falletti et al., 2014).

From days 10–30, a clear exponential growth phase was observed in all stages, with Stage 1 showing the most pronounced increase from 0.1 to 0.8 g m^{-2} (surface) and from 0.05 to 0.9 kg m^{-3} (volumetric). This phase corresponds to extensive substrate availability and EPS-driven cohesion that facilitates rapid layerwise biomass accumulation. The higher accumulation in Stage 1 is attributed to the increased organic loading near the inlet, which supports the development of a dense heterotrophic community. Stages 2 and 3 exhibited similar but slightly lower gradients due to declining substrate concentrations along the treatment train. This spatial variation is consistent with the findings of Jagaba et al. (2024), who documented 30–40% higher biofilm volumes in the upstream compartments of hybrid systems. The concurrent increase in SAB and VAB reflects a balanced progression of attached and suspended biomass, indicative of effective hydrodynamic mixing and stable biomass retention (Regmi et al. 2011) (Table 1).

Biofilm mass achieved a quasi–steady state after day 35 with minimal net biomass accumulation, indicating that the system had transitioned from active growth to a maintenance phase. Corresponding surface mass densities at the end of

Table 1. Overall performance summary of treatment system process for setup 1

Parameter	Inlet range (mg/L)	Outlet range (mg/L)	Max → Avg → Min (Last 20 Days)	Last 10 Days	Last 15 Days	Last 20 Days	Average removal efficiency / Range
pH (Inlet)	7.1–7.6	–	7.6 → 7.30 → 7.1	7.27	7.26	7.26	Stable (neutral–slightly alkaline)
pH (Outlet)	–	7.21–7.87	7.87 → 7.62 → 7.21	7.56	7.53	7.57	Stable
COD (mg/L)	222.6–552.3	33.1–167.7	552.3→317.18→222.6 → 167.7→91.84→33.1	284.55→40.78	286.77→47.32	320.25→61.65	~73.7% (80.75–85.67% over last 20 days)
BOD (mg/L)	103.6–196	6.9–41.6	196→159.7→103.6 → 41.6→22.33→6.9	177.45→8.61	177.76→11.00	173.39→12.41	~86.7% (92.84–95.15% over last 20 days)
TSS (mg/L)	83.25–222.3	9.2–50.2	222.3→152.94→83.25 → 50.2→23.4→9.2	169.25→13.31	165.15→14.91	162.84→16.53	~85.4% (89.84–92.14% over last 20 days)
Biomass Stage 1 (gm/m² / kg/m³)	–	–	1.4474 / 1.38 → 0.50 / 0.48 → 0.0132 / 0.02	1.28 / 1.22	1.06 / 1.01	0.92 / 0.88	–
Biomass Stage 2 (gm/m² / kg/m³)	–	–	1.1053 / 1.06 → 0.38 / 0.37 → 0 / 0	0.99 / 0.94	0.82 / 0.78	0.71 / 0.68	–
Biomass Stage 3 (gm/m² / kg/m³)	–	–	0.9342 / 0.89 → 0.33 / 0.31 → 0 / 0	0.83 / 0.80	0.69 / 0.66	0.60 / 0.57	–

the growth period were 1.45, 1.10 and 0.93 g m^{-2} for Stages 1–3, with volumetric concentrations of 1.38, 1.06 and 0.89 kg m^{-3}, respectively. The gradual downward slope observed thereafter is attributable to the substrate-gradient reduction effect, in which diminishing carbon availability constrains biomass growth toward the downstream compartments, consistent with the findings of Prasad et al. (2021) and Sharma et al. (2024). Small oscillations after day 38 suggest intermittent sloughing events, representing the expected trade-off between shear-induced detachment and new biomass formation (Gupta et al., 2023). The pattern of decreasing biomass (Stage 1 → Stage 3) is consistent with functional zonation along the treatment train. Stage 1 preferentially supported the heterotrophic oxidation of readily degradable COD, Stage 2 sustained mixed heterotrophic nitrifying populations, and Stage 3 facilitated residual nitrification and polishing, mirroring the ecological stratification described by Bassin et al. (2016) and Madan, Madan, and Hussain (2022). The steady-state biomass range of 0.9–1.45 g m^{-2} agrees with reported aerobic MBBR values of 0.8–1.6 g m^{-2} (Prasad et al., 2023; Ødegaard et al., 2000). COD and BOD removals consistently exceeding 80% further support the stability and metabolic activity of the mature biofilm. The applied SRT (10–14 days) promoted a dynamic equilibrium between growth and detachment, aligning with recommended SRT ranges that prevent diffusion limitations and maintain biofilm resilience (Di Trapani et al. 2008). Overall, Setup-1 achieved biological maturity at approximately day 35, and its design configuration (30% carrier fill and 50% recirculation) maintained robust biofilm development, effective load distribution, and sustained high organic removal efficiencies.

Variations of pH, COD, BOD and TSS in the Setup-2

Process Setup-2 performance assessment during the 45-day retention period revealed consistently high organic and solids removal efficiencies, in line with the performance reported for other hybrid biological treatment systems in the literature. The effluent pH remained slightly but consistently higher than the influent (7.23–7.87), a buffering pattern consistent with observations by Xu et al. (2025) who attributed pH stabilization in anaerobic–anoxic–aerobic systems to ammonia uptake, bicarbonate equilibrium reactions and enhanced nitrifying/heterotrophic activity. The maintenance of near-neutral pH reflects efficient successive biological transformations and minimal microbial stress, supporting stable reactor operation. As shown in Figure 6, COD decreased markedly from a mean influent concentration of 330 mg/L to 100 mg/L in the effluent, indicating that both soluble and particulate organic fractions were effectively degraded. A COD removal efficiency of approximately 70% aligns well with values reported for MBBR configurations incorporating anoxic and anaerobic zones, such as the 65–80% range documented for municipal

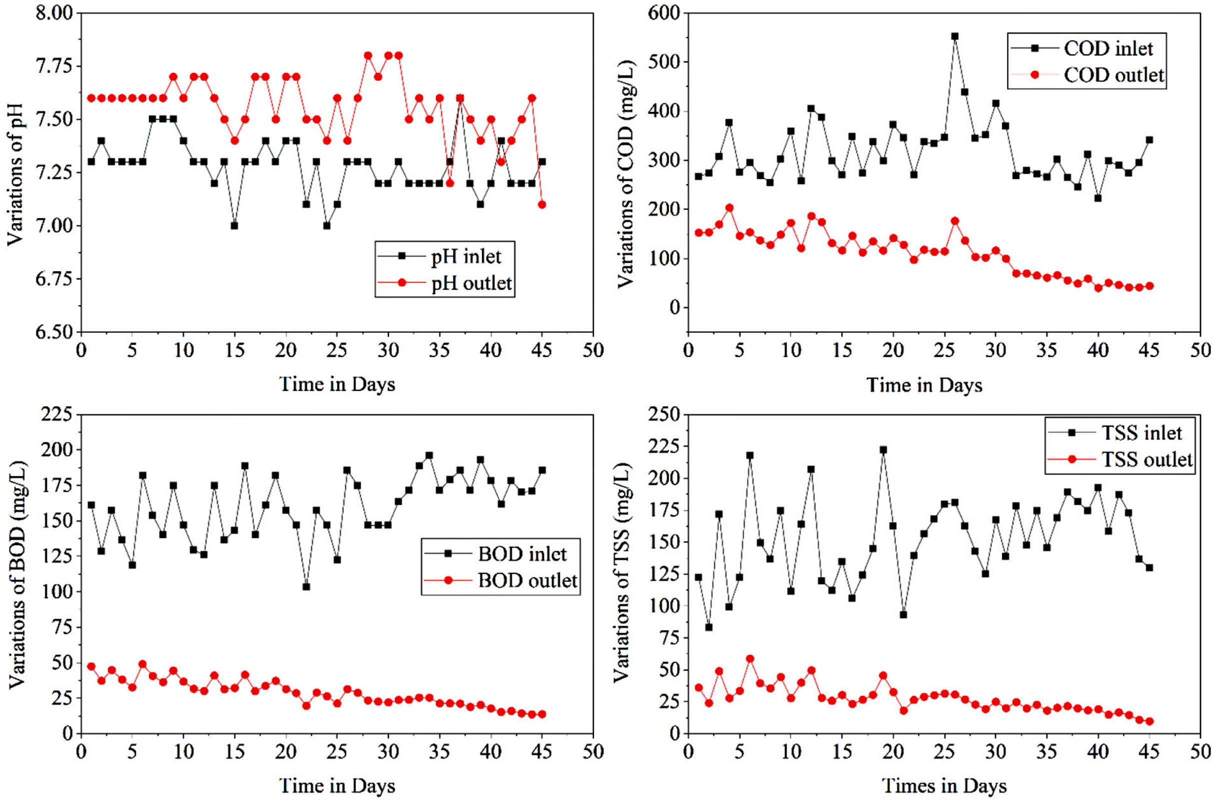

Figure 6. Inlet and outlet water quality parameters for process Setup-2: pH, COD, BOD, and TSS

wastewater by Jagaba et al. (2024). The maximum influent COD of 362.6 mg/L (Day 18), followed by a decline to 140.56 mg/L, illustrates the resilience and load-buffering capacity of the biofilm-supported aerobic reactor when subjected to fluctuating organic loads. The continued downward trend toward the end of the operational period, culminating in the lowest outlet COD of 38.66 mg/L on Day 45, indicates that the biological system had reached a stable acclimation and biomass equilibrium phase.

The BOD removal performance was also above 90%, with concentrations reduced from approximately 96 mg/L to 20 mg/L on average. The minimum measured value (8.18 mg/L on Day 37) and the final-day removal (95.9 to 8.38 mg/L) confirm high carbonaceous biodegradation efficiency and strong metabolic activity of the biomass. Studies by Zhuang et al. (2019) have shown that biological conversion and oxygen transfer improve in hybrid systems employing attached-growth carriers, owing to higher specific surface area, stable microbial retention and enhanced oxygen distribution. The BOD removal efficiencies achieved here are higher than those typically observed in conventional activated sludge processes (85%), suggesting that the carrier-assisted aerobic zone provides an additional performance gain by sustaining both attached and suspended consortia.

Suspended solids (TSS) also declined steadily from 167 mg/L to a minimum of 9.3 mg/L, with effluent concentrations averaging 25 mg/L. This trend is consistent with Dias et al. (2018), who demonstrated improved flocculation, biofloc density and settleability in systems downstream of biofilm reactors. Efficient TSS removal is critical not only for effluent clarity but also for mitigating solids-related stress in subsequent stages and minimizing particulate loading on receiving waters. Overall, the integrated anaerobic–anoxic–aerobic configuration combined with media-supported aeration facilitated stable organic load reduction, improved biomass settleability and maintained high, uninterrupted biological activity throughout continuous operation. With these removal efficiencies, the system is well positioned to meet municipal discharge requirements and is suitable for decentralized and on-site domestic wastewater treatment. Continued monitoring of nutrient parameters (NH_4-N, TN and TP) remains essential for compliance with increasingly stringent effluent standards and for supporting global objectives related to resource recovery and sustainable wastewater management.

Variations of ammoniacal nitrogen, total nitrogen and total phosphorous in the Setup-2

The nitrogen and phosphorus removal performance in Setup-2 provides clear evidence of the synergistic functioning of the anaerobic, anoxic and aerobic zones, each enabling specific biochemical transformations that collectively drive nutrient elimination. NH_4^+-N concentrations showed a pronounced decreasing trend over the 45-day operational period. Effluent NH_4^+-N decreased from initial concentrations of 25–30 mg/L to <5 mg/L by Day 35 and ultimately stabilized at 2–4 mg/L. This consistent decline indicates effective nitrification in the aerobic media reactor, where ammonia was oxidized to nitrite and nitrate by classical nitrifiers such as Nitrosomonas and Nitrobacter. These results corroborate findings by McQuarrie and Boltz (2011) and Alitaleshi, Daghbandan, and Pendashteh (2024), who reported >85% ammonia removal in hybrid biofilm systems under similar process conditions.

TN also exhibited substantial reduction, declining from ~25–30 mg/L to <10 mg/L by the end of the study. The more pronounced decline after Day 25 indicates the establishment of stable simultaneous nitrification and denitrification (SND) communities. The anoxic zone effectively converted accumulated nitrate to nitrogen gas, reducing overall TN loading prior to discharge. These observations align with Gzar, Al-Rekabi, and Shuhaieb (2021), who demonstrated that internal sludge recycling enhances carbon availability and nitrate exposure, thereby improving heterotrophic denitrification efficiency.

An initial transient increase in NH_4^+-N and TN during the early operational days reflects the acclimation delay of nitrifying and denitrifying communities, which are known to be sensitive to environmental fluctuations and possess relatively long generation times. Once acclimated, nutrient removal improved markedly, suggesting that the system reached biological and functional stability, a pattern consistent with start-up behaviour reported for MBBR and other hybrid systems (Wang et al., 2022).

Total phosphorus (TP) exhibited a similar declining pattern, decreasing from 3.5–4.5 mg/L at the influent to 2.0–2.8 mg/L in the effluent, with minima of 1.6 mg/L (Figure 7). The

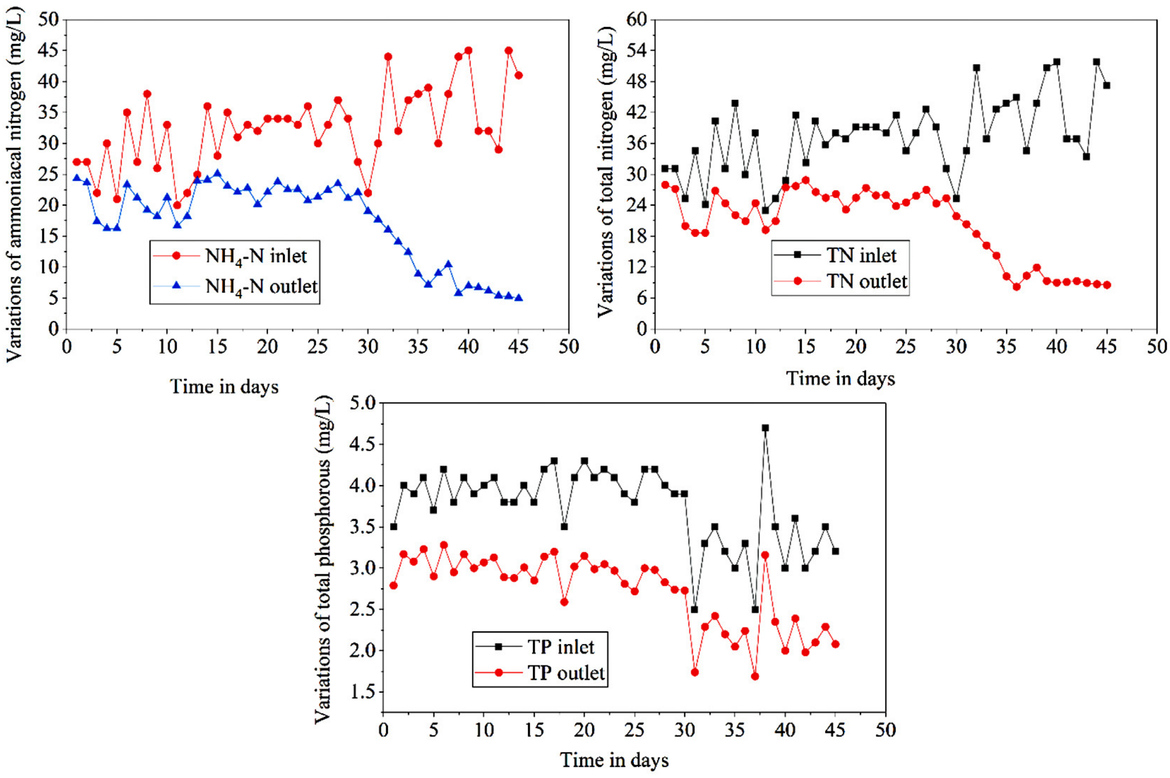

Figure 7. Inlet and outlet water quality parameters for process Setup-2 NH4-N, TN, and TP

alternating anaerobic and aerobic conditions created an environment conducive to enhanced biological phosphorus removal (EBPR), in which polyphosphate-accumulating organisms (PAOs) release phosphorus under anaerobic conditions and subsequently uptake excess phosphorus during aerobic phases. These trends are consistent with Dereli et al., (2021), who reported 30–45% TP removal in hybrid biofilm–suspended systems due to improved biomass retention and strengthened PAO activity. Overall, the nutrient removal performance of Process Setup-2 demonstrates highly effective biological nutrient transformation, with NH_4^+-N, TN and TP levels remaining within the limits of secondary municipal discharge standards. The synergistic interplay of ammonification, nitrification, denitrification and phosphorus uptake confirms the soundness of the design strategy that integrates distinct redox zones with biofilm-supportive media. This configuration strengthens microbial stability, enhances nutrient conversion pathways and ensures operational robustness under continuous flow conditions.

Taken together, these results demonstrate the system's strong capability to reduce eutrophication potential in receiving waters, thereby reinforcing its suitability for decentralized and municipal wastewater treatment applications, particularly in settings where stringent nitrogen compliance is required. As summarized in Table 2, the pilot-scale system exhibited stable and efficient performance across all monitored parameters. pH levels consistently remained within the optimal biological range (7.0–7.87), supporting uninterrupted microbial activity. Organic pollutants such as COD, BOD and TSS were effectively removed, with peak efficiencies exceeding 84%, 90% and 93%, respectively, during the final 10–20 days of operation.

Nutrient removal also showed steady improvement, with NH_4-N and total nitrogen achieving removal efficiencies of up to 80% and 75%, respectively. Total phosphorus removal reached 66%, indicating partial but significant biological uptake, with potential for further enhancement under optimized EBPR conditions. Biomass concentrations increased gradually throughout the operational period, peaking at 4.17 g/m² (surface) and 3.97 kg/m³ (volumetric), reflecting robust microbial colonization and clear progression toward reactor maturity (refer Table 2). Collectively, these findings confirm the system's operational reliability, high treatment performance and readiness for scale-up or targeted optimization in full-scale applications.

Surface and volumetric biomass growth rates in process flow Setup-2

Temporal changes in biomass concentration over the 45-day operational period indicate a distinct and sequential development of the microbial communities responsible for wastewater treatment in Setup-2. Biomass levels were very low during the initial phase (days 1–5), ranging between 0.0263 and 0.0658 g/m² (0.03–0.07 kg/m³), which is characteristic of a microbial lag and acclimation stage. This early behaviour reflects limited attachment, low metabolic activity and the time required for microorganisms to adapt to new hydrodynamic and substrate conditions. This observation aligns with previous findings by Falletti et al. (2014), who reported that biofilm-based systems typically undergo a short adaptation period before transitioning into active colonization, particularly under newly imposed operational or loading regimes. Such low initial biomass also suggests that attachment kinetics, EPS initiation and surface conditioning were still progressing during this early stage.

During the acceleration phase of the system (days 6–20), biomass increased steadily, reaching 0.5789 g/m² (0.55 kg/m³) by Day 20. This pattern clearly indicates an exponential growth phase, driven by favourable environmental conditions including adequate availability of organic carbon, stable pH and well-regulated hydrodynamic shear that promotes attachment without causing excessive detachment. Exponential biofilm development during this stage is consistent with observations from MBBR-type systems, where support media enhance microbial retention and protect developing communities from washout (Jagaba et al., 2024).

In the maturation phase (days 21–35), biomass further increased to 2.1711 g/m² (2.07 kg/m³), representing a functionally stable and metabolically efficient community capable of high organic and nutrient removal. Small oscillations observed during this period indicate a dynamic equilibrium between microbial growth and endogenous decay, an intrinsic feature of fully acclimated biofilm systems. These findings align with those of Regmi et al. (2011), who demonstrated

Table 2. Comprehensive performance summary of treatment system (Setup-2)

Parameter / Phase	Max	Average	Min	Last 10 days	Last 15 days	Last 20 days	Remarks
pH (Inlet)	7.67	7.32	7.01	7.25	7.25	7.27	–
pH (Outlet)	7.87	7.58	7.23	7.51	7.50	7.55	–
COD (mg/L) – Inlet	362.6	336.89	287.0	325.85	327.00	327.85	–
COD – Outlet	190.54	114.27	38.66	51.31	59.99	68.59	–
COD Removal efficiency (%)	86.54	66.08	47.45	84.25	81.66	79.07	–
BOD (mg/L) – Inlet	101.5	96.56	91.0	96.04	96.56	96.39	–
BOD – Outlet	29.41	18.27	8.18	9.71	10.96	12.13	–
BOD Removal efficiency (%)	91.02	81.07	71.02	89.88	88.64	87.42	–
TSS (mg/L) – Inlet	168.0	145.55	132.0	147.30	145.90	145.10	–
TSS – Outlet	43.61	22.27	9.30	10.07	10.68	11.54	–
TSS Removal efficiency (%)	92.95	84.71	74.05	93.16	92.68	92.04	–
NH_4–N (mg/L) – Inlet	45.0	34.12	18.0	31.00	30.60	33.15	–
NH_4–N – Outlet	37.09	18.85	4.50	6.16	8.29	11.14	–
NH_4–N Removal efficiency (%)	75.00	44.78	17.53	80.13	72.91	66.39	–
Total nitrogen (mg/L) – Inlet	51.75	39.23	20.70	35.65	35.19	38.13	–
Total nitrogen – Outlet	42.65	22.05	7.46	8.77	10.66	13.65	–
TN removal efficiency (%)	63.97	43.80	17.56	75.38	69.70	64.20	–
Total phosphorus (mg/L) – Inlet	8.75	5.55	2.50	7.45	7.40	6.93	–
Total phosphorus – outlet	4.78	3.26	2.21	2.54	2.69	2.73	–
TP Removal efficiency (%)	45.37	41.26	11.60	65.88	63.65	60.59	–
Surface biomass (g/m²)	4.7237	1.56	0.0263	4.17	3.43	2.96	–
Volumetric biomass (kg/m³)	4.49	1.49	0.03	3.97	3.26	2.82	–
Lag phase (Days 1–5)	–	–	–	–	–	0.0263–0.0658 g/m² / 0.03–0.07 kg/m³	Microbial adaptation
Acceleration phase (Days 6–20)	–	–	–	–	–	0.2237–0.5789 g/m² / 0.22–0.55 kg/m³	Active growth
Maturation phase (Days 21–35)	–	–	–	–	–	0.8158–2.1711 g/m² / 0.78–2.07 kg/m³	Stable microbial community
High-density phase (Days 36–45)	–	–	–	–	–	2.8158–4.7237 g/m² / 2.68–4.49 kg/m³	High biomass; potential overload

that biofilm reactors achieve a steady state once nutrient uptake, diffusion and cell turnover processes become balanced.

A significant biomass buildup occurred during the high-density phase (days 36–45), when values peaked at 4.5921 g/m² (4.37 kg/m³) (refer Figure 8). This marked increase likely reflects enhanced EPS production, stronger adhesion forces and full exploitation of available substrate as the system approached complete operational development. While higher biomass densities typically correlate with improved pollutant removal capacity as also supported by Morfopoulos and Samolada (2025) they may also introduce

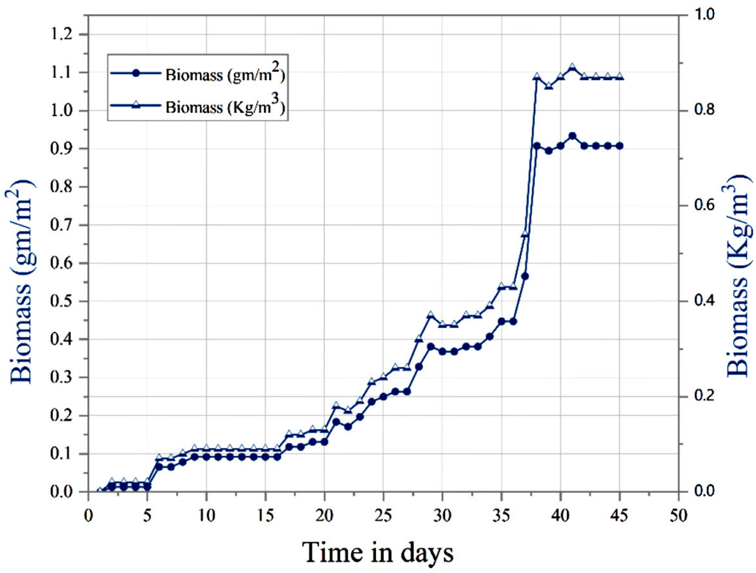

Figure 8. Assessment of surface and volumetric biomass growth rates in process flow setup-2 of aerobic reactor

operational challenges such as media clogging, localized oxygen limitation and increased sludge bulking propensity.

Overall, the continuously increasing biomass trend demonstrates successful microbial acclimation and robust system development, ensuring effective degradation of organic matter and nutrients across operational phases. The progression from lag to acceleration, maturation and high-density stages confirms the effectiveness of integrating anaerobic, anoxic and aerobic microbial activities within the treatment train. However, the elevated biomass levels in later stages underscore the need for careful monitoring of sludge management, aeration efficiency and shear conditions to prevent reactor overloading and to maintain long-term operational stability.

CONCLUSIONS

The present study demonstrates the effectiveness of a bio-carrier-integrated multistage process train for intensified biological wastewater treatment under practical operating conditions. The experimental systems, particularly Setup-1 (three-stage aerobic) and Setup-2 (A2O with aerobic carriers), were designed to promote synergistic suspended-growth and attached-growth biofilm activity, resulting in enhanced organic and nutrient removal. Stable performance was achieved at hydraulic retention times of 6–8 hours with continuous aeration and controlled internal recirculation. Setup-1 achieved mean COD, BOD and TSS removal efficiencies of 85.7%, 92.8% and 90.1%, respectively, indicating strong oxidative capacity and effective solids retention. In Setup-2, COD and BOD removal efficiencies were approximately 79% and 87%, with TSS removal reaching ~92%. Nutrient transformations were also significant: NH_4^+-N and total nitrogen removals reached ~80% and ~75%, while total phosphorus reduction of up to 66% confirmed efficient nitrification–denitrification and biological phosphorus uptake. The biofilm density achieved across mediators (0.03–4.72 g m^{-2}) reflects well-adapted microbial communities and a mature reactor ecology, capable of sustaining high treatment efficiencies. The compact configuration and favorable energy profile make this system an attractive option for small- to medium-scale facilities, with the added advantage of flexible operation and low sludge yield. The integration of anaerobic, anoxic and aerobic zones with carrier-supported biofilms facilitated comprehensive pollutant transformation and provided robust resilience against hydraulic and organic shock loads. Overall, the study demonstrates that retrofitting conventional STPs with a hybrid bio-carrier process offers a technically feasible and economically viable pathway for performance enhancement. The proposed system aligns strongly with sustainable wastewater management and global circular-economy objectives, enabling improved resource recovery and reduced environmental burden.

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