SEQUENTIAL ANAEROBIC–AEROBIC DHS (DOWN-FLOW HANGING SPONGE) FOR ANTHRAQUINONE DYE WASTEWATER DECOLORIZATION INVOLVING MANGANESE-OXIDIZING BACTERIA

KHỬ MÀU NƯỚC THẢI NHUỘM ANTHRAQUINON BẰNG HỆ THỐNG DHS KỴ KHÍ - HIẾU KHÍ NỐI TIẾP VỚI SỰ THAM GIA CỦA VI KHUẨN OXY HÓA MANGAN

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Abstract - This study evaluated the performance of a sequential anaerobic–aerobic Down-flow Hanging Sponge (DHS) system with manganese-oxidizing bacteria (MnOB) as a low-cost biological treatment for dye wastewater. Synthetic wastewater containing Alizarin Red S (100 mg/L) was fed into two reactors in series with a hydraulic retention time of 12 hours. In the anaerobic phase, about 70% of COD, TOC were removed, indicating partial dye degradation together with other substrates. Decolorization reached 75–80% with concomitant Mn(II) formation, reflecting the involvement of both chemical and biological pathways, with the latter predominating as confirmed by the abiotic MnO_2 control. In the aerobic phase, MnOB indirectly enhanced decolorization by oxidizing Mn(II) to regenerate bio-MnO_2, raising overall efficiency above 95%. Residual COD (~50 mg/L) and TOC (~20 mg/L) showed incomplete degradation, emphasizing the need to optimize the aerobic stage or integrate advanced oxidation for complete treatment.

Key words - Decolorization; Anthraquinone dye; Down-flow Hanging Sponge; Manganese-oxidizing bacteria (MnOB); Anaerobic–aerobic treatment

Tóm tắt - Nghiên cứu đánh giá hiệu quả hệ thống giá thể treo nhỏ giọt (DHS) kỵ khí – hiếu khí có vi khuẩn oxy hóa mangan (MnOB) như một giải pháp sinh học chi phí thấp để xử lý nước thải nhuộm. Nước thải giả lập chứa Alizarin Red S (100 mg/L) được cấp vào hai bể phản ứng nối tiếp với thời gian lưu 12 giờ. Ở pha kỵ khí, khoảng 70% COD, TOC đã được loại bỏ, bao gồm một phần thuốc nhuộm cùng với các cơ chất hữu cơ khác. Hiệu suất khử màu đạt 75–80% kèm theo sự hình thành Mn(II), phản ánh sự phối hợp giữa cơ chế hoá học và sinh học, trong đó cơ chế sinh học chiếm ưu thế theo kết quả đối chứng phi sinh học với MnO_2. Ở pha hiếu khí, MnOB gián tiếp hỗ trợ khử màu bằng cách oxy hóa Mn(II) tạo bio-MnO_2, giúp hiệu quả khử màu tổng thể của hệ vượt 95%. COD (~50 mg/L) và TOC (~20 mg/L) tồn dư nhận thấy cần tối ưu thêm giai đoạn hiếu khí hoặc kết hợp công nghệ oxi hóa bậc cao để phân huỷ hoàn toàn thuốc nhuộm.

Từ khóa - Khử màu; Thuốc nhuộm anthraquinon; Hệ thống giá thể treo nhỏ giọt (DHS); Vi khuẩn oxy hóa Mangan (MnOB); Xử lý kỵ khí – hiếu khí

1. Introduction

The textile industry is a significant source of water pollution, discharging large volumes of wastewater containing synthetic dyes. These compounds typically possess complex, stable aromatic structures, rendering them recalcitrant to natural degradation. Their persistence in aquatic ecosystems not only causes aesthetic issues by altering water color but also poses considerable toxicological and carcinogenic risks, threatening both aquatic life and human health [1]. Consequently, the development of effective and sustainable technologies for treating dye-laden wastewater is an urgent environmental priority.

Conventional treatment methods are predominantly based on physicochemical mechanisms such as coagulation-flocculation, activated carbon adsorption, membrane filtration, and advanced oxidation processes (AOPs). While these approaches can achieve a certain degree of decolorization, they are often constrained by high operational costs, substantial energy consumption, and the generation of chemical sludge, which presents secondary disposal challenges [2]. These drawbacks have spurred growing interest in biological methods as a cost-effective and environmentally benign alternative. Numerous studies have demonstrated the efficacy of using bacteria, enzymes, or algae to remove dyes from wastewater [3]. However, the performance of these biological systems can be inconsistent, and the complex underlying interaction mechanisms are not yet fully elucidated, warranting further investigation.

Manganese-oxidizing bacteria (MnOB) can catalyze the oxidation of soluble Mn(II) to biogenic manganese oxides (bio-MnO_x) under aerobic conditions. Both chemically synthesized MnO_x and biogenic bio-MnO_x have garnered attention for their unique structures and physicochemical properties. Specifically, bio-MnO_x formed through MnOB activity exhibit a high negative surface charge density derived from their crystal structure, resulting in strong adsorption affinity and a large capacity for various organic compounds and metal ions [4], [5]. When integrated with an anaerobic stage capable of reductively cleaving chromophoric bonds, a subsequent aerobic phase containing MnOB promises a synergistic treatment mechanism through a biogeochemical manganese cycle, thereby enhancing the degradation of color and non-biodegradable organic matter. While numerous studies have demonstrated the effectiveness of sequential anaerobic-aerobic systems for dye treatment [6],

[7], [8], these systems have primarily relied on conventional microorganisms and activated sludge, with limited research focusing on the specific role of MnOB and the in-situ formation of bio-MnO_2.

This study, therefore, develops and evaluates a sequential anaerobic-aerobic Down-flow Hanging Sponge (DHS) system for treating wastewater containing an anthraquinone dye. The specific objectives were: (1) to evaluate the system's overall removal efficiency for color and organic matter (COD, TOC); (2) to compare the relative contributions of the chemical reduction by MnO_2 versus the biological decolorization mechanism in the anaerobic phase; and (3) to assess the role of MnOB in regenerating bio-MnO_2 and its subsequent impact on the overall decolorization efficiency in the aerobic phase.

2. Materials and Methods

2.1. Chemicals and Synthetic Wastewater

The anthraquinone dye Alizarin Red S (ARS) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Commercial manganese dioxide (MnO_2, Kishida Chemical Co., Ltd., Osaka, Japan) was used as the oxidant; it is a fine black–brown powder with a specified purity of not less than 90%.

Synthetic wastewater was prepared by dissolving ARS and a carbon source in deionized water. The carbon source initially consisted of K-medium (yeast extract and peptone) and was later supplemented with sucrose. The solution also contained a phosphate buffer (6.02 mg/L KH_2PO_4 and 60.2 mg/L Na_2HPO_4), essential nutrients, iron, and trace elements for microbial support. To prevent microbial contamination, the nutrient medium was sterilized by autoclaving (120 °C, 20 min). Prior to feeding, all solutions were purged with N_2 gas to minimize premature oxidation of organic components.

2.2. Experimental Setup and Operation

2.2.1. DHS set up

The treatment system consisted of two acrylic Down-flow Hanging Sponge (DHS) reactors connected in an anaerobic-aerobic series, each with an effective volume of 2.0 L and an internal diameter of 50 mm. Each reactor contained 20 polyurethane sponge cubes (2 × 2 × 2 cm), providing a total carrier volume of 0.16 L (8% of the reactor volume), a ratio within the typical 5–20% range reported for DHS systems [9], ensuring sufficient surface area for biofilm attachment while preventing clogging. A schematic diagram of the DHS setup is shown in Figure 1.

To establish the microbial communities, the sponge carriers for each reactor were inoculated prior to operation using a pre-soaking method. Two sludge types served as the inocula: digested granular sludge from a UASB process (MLSS = 3,300 mg/L, pH = 7.2) for the anaerobic stage, and activated sludge from a municipal WWTP (MLSS = 2,690 mg/L, pH = 5.9) for the aerobic stage. Prior to inoculation, each sludge type was mixed with a pre-treated MnO_2 suspension. The suspension (100 g/L) was pre-saturated with 1 g/L Mn(II) for 1 h, repeated three times to ensure saturation of adsorption sites. The sponge chains were then soaked in 1 L of their respective sludge-MnO_2 mixtures and occasionally hand-kneaded to enhance microbial attachment. Afterward, the anaerobic- and aerobic-seeded sponge chains were hung in their respective reactors. This procedure is a conventional DHS start-up practice designed to promote robust microbial colonization and shorten the acclimation period [10], [11]. The wastewater feed (synthetic K-medium supplemented with dye) was initiated only after the inoculated sponges were in place; no liquid sludge was introduced with the influent.

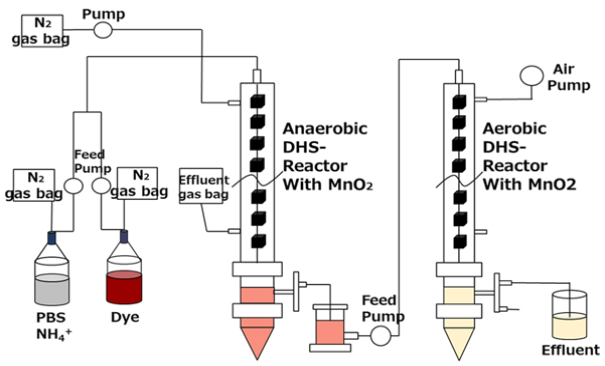

Figure 1. *Schematic diagram of the sequential anaerobic–aerobic DHS system*

2.2.2. DHS operation

The system was operated at 25 °C with a hydraulic retention time (HRT) of 12 h for each reactor (anaerobic and aerobic), resulting in a total system HRT of 24 h. This 12-hour HRT per stage was selected based on two key considerations. First, a longer HRT is recommended in DHS start-up phases to ensure stable biofilm formation and reliable performance [9], [12]. Second, given the low organic loading rate (OLR ≈ 0.4 gCOD/L·d) in our system, this extended HRT was essential for providing sufficient substrate–biomass contact, accommodating the rate-limiting steps of anaerobic dye reduction and aerobic Mn(II) oxidation, and preventing biomass washout. Anaerobic and aerobic conditions were maintained by sparging the first and second reactors with N_2 (1.44 L/day) and air (102.8 L/day), respectively. Initially (Days 1–28), the use of only K-medium (100 mg COD/L) as a carbon source led to significant acid (H^+) generation from nitrification in the aerobic reactor. Therefore, the feed was later replaced with a 1:1 mixture of K-medium and sucrose (50 mg COD/L each) to improve overall pH stability and maintain it within the 6.5–8.0 range.

2.2.3. Abiotic MnO_2 Control Experiment

Prior to initiating the biological treatment study, an abiotic control experiment was conducted to establish a quantitative baseline for the redox degradation pathway. MnO_2 exhibits both strong adsorption capacity and oxidative reactivity [13]. Therefore, the experimental design aimed to saturate adsorption sites, hereby attributing subsequent dye removal predominantly to redox reactions. To accomplish this, a sterilized column packed with 30 g of MnO_2 was fed with a high-concentration Alizarin Red S solution (≈2,000 mg/L) under an HRT of 1 h until removal efficiency stabilized, indicating saturation

of surface adsorption sites. Subsequently, to characterize the redox kinetics, the HRT was progressively increased to 2, 6, and 12 h. Effluent dye concentration, pH, and Mn(II) were systematically monitored throughout the experiment. The absence of biomass ensured that the observed responses reflected only abiotic processes.

2.3. Analytical Methods

Samples were periodically collected from the influent and the effluents of both the anaerobic and aerobic reactors. Prior to analysis, all samples were filtered through a 0.45 µm membrane; those intended for dissolved Mn(II) analysis were subsequently passed through a 0.2 µm filter. Dye concentration was quantified by measuring absorbance at the maximum absorption wavelength of ARS (λmax = 514 nm) using a UV–Vis spectrophotometer. Chemical oxygen demand (COD) was determined by the dichromate digestion method (HACH DRB200), total organic carbon (TOC) was measured using a TOC analyzer (Shimadzu TOC-Vcsh), and dissolved Mn(II) was analyzed with the formaldoxime colorimetric method. The pH was monitored with a standard pH meter.

The removal efficiency (%) was calculated as:

$$\text{Removal (\%)} = \frac{C_{in} - C_{eff}}{C_{in}} \times 100 \quad (1)$$

Where, C_{in} and C_{eff} are the influent and effluent concentrations, respectively. All measurements were performed in triplicate, and standard deviations are presented as error bars. Linear regression was used to analyze the relationship between dye removal and Mn(II) production.

3. Results and Discussions

3.1. Chemical Baseline from Abiotic MnO_2 Control

The primary objective of the abiotic control was to establish a quantitative baseline for the redox degradation pathway. However, since MnO_2 also exhibits strong surface adsorption, the experimental design (see Section 2.2.3) aimed to isolate the redox mechanism by first saturating adsorption sites. As anticipated, this resulted in a two-phase removal process: initial adsorption followed by slower, redox-driven degradation [13], [14].

With an initial HRT of 1 h, dye removal reached ~80% on the first day before the efficiency gradually declined and stabilized around Day 26, confirming the saturation of adsorption sites (Figure 2 (a)). From this point onward, removal was governed solely by redox reactions. The system was maintained at 1 h HRT until Day 33, after which the HRT was sequentially increased to 2, 6, and 12 h. Each increase in HRT enhanced dye removal, highlighting the time-dependent kinetics of the oxidative process [15].

During the redox-dominant period (Days 26–57), dye removal exhibited a strong linear correlation with Mn(II) production ($y = 0.525x$, $R^2 = 0.97$; Figure 2(b)). This relationship aligns with the expected 1:2 molar stoichiometry for the oxidative-reductive process:

$$0.5\ C_{14}H_7NaO_7S + MnO_2 + 2H^+ \rightarrow 0.5\ C_{14}H_7NaO_9S + Mn^{2+} + H_2O \quad (2)$$

This stoichiometric agreement provides a robust abiotic baseline, where a molar ratio of 0.5 (dye removed/Mn(II) produced) defines the purely chemical pathway. This baseline is essential for decoupling and quantifying biological contributions in the DHS anaerobic reactor.

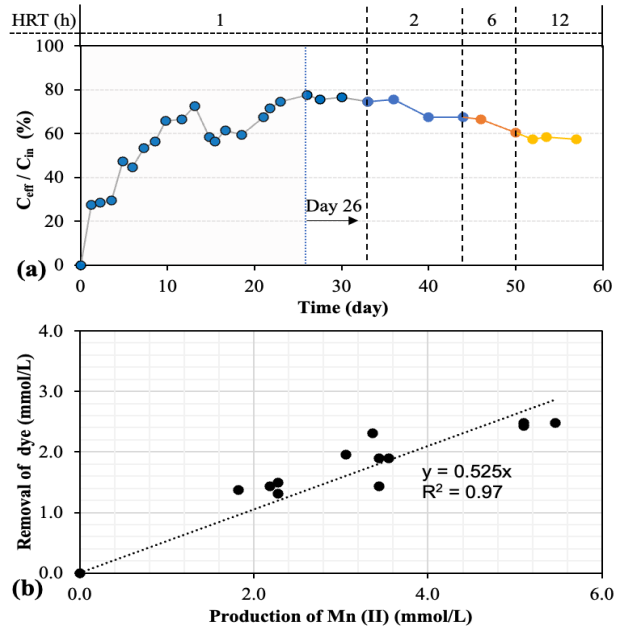

Figure 2. *Performance of the abiotic anaerobic reactor (Days 26–57): (a) Decolorization efficiency over time (Ceff/Cin, %); (b) Relationship between dye removal and Mn(II) production. The dotted line indicates the linear regression (y = 0.525x)*

3.2. Performance of the Sequential Anaerobic – Aerobic System

3.2.1. Overall performance of the combined DHS system

The overall treatment performance of the sequential anaerobic–aerobic DHS system was monitored for nearly 120 days in terms of color, COD, and TOC removal (Figure 3). During the initial 28 days, the system acclimated rapidly, with color removal reaching nearly 100%, reflecting both residual adsorption on MnO_2 and biologically mediated redox reactions. COD and TOC removals stabilized at approximately 60–65% during this start-up period.

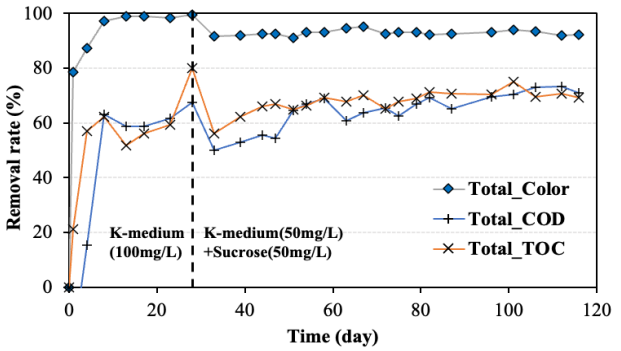

Figure 3. *Time-course overall removal of color, COD, and TOC in the DHS system*

At Day 28, the supplemental carbon regime was modified by halving the K-medium and adding sucrose to improve pH conditions in the aerobic stage. This adjustment caused a transient decline in COD and TOC removals to

around 50%, indicating a temporary shock load. However, the system quickly recovered, achieving a new steady-state efficiency of about 70%. Throughout the entire operation, color removal remained consistently above 95%, demonstrating the robustness of the redox–biological mechanism under varying operational conditions.

Compared with previous reports that often showed moderate–high COD removal but limited decolorization [6], [7], the present system exhibited superior dye removal while COD and TOC removals stabilized at approximately 70%. This indicates that, in addition to the readily biodegradable fractions, a portion of the dye was also degraded, although a considerable fraction persisted in the effluent. This observation is consistent with the statement of Van der Zee and Villaverde [8], who emphasized that decolorization does not necessarily imply complete removal of non-biodegradable organics. These findings highlight the unique advantage of the DHS configuration in sustaining near-complete decolorization while partially reducing the refractory organic load.

3.2.2. Performance of individual anaerobic and aerobic stages

The concentrations of COD, TOC, color, and Mn(II) were monitored at the influent, after the anaerobic phase, and after the aerobic phase throughout the experimental period (Figure 4). These measurements provide insights into the performance of each stage in terms of organic removal, decolorization, and manganese transformation.

a. COD, TOC

The influent was prepared targeting a COD of approximately 200 mg/L and a TOC of approximately 80 mg/L. Actual values were lower at the start (COD 130 mg/L, TOC 50 mg/L). As clarified in Section 2.2.1, no liquid sludge was introduced with the influent, and these initial lower values during the start-up phase are better explained by partial abiotic oxidation of the K-medium when exposed to residual oxygen in the feed bottles, which resulted in lower COD and TOC. From Day 23 onward, N_2 purging of the feed bottles improved influent quality, and by Day 58 both COD and TOC had stabilized near their target levels, as shown in Figures 4(a) and 4(b).

In the anaerobic phase, COD decreased to 50–55 mg/L and TOC to 19–22 mg/L, giving stable removal efficiencies of about 70%. Along with these stable values, the relatively constant COD/TOC ratio (~2.5) indicates that, besides the readily biodegradable substrates, part of the dye was also degraded. In the aerobic reactor, COD and TOC showed little further reduction, leaving residual concentrations in the effluent. These fractions likely originated from non-biodegradable dye-derived compounds, which are more resistant to biodegradation [8], [16]. Previous studies reported that COD removal mainly occurred in the aerobic stage because anaerobic microbes only cleaved dye bonds without complete dye degradation [6]. In contrast, the present study achieved substantial COD and TOC removal already under anaerobic conditions. This difference can be explained by the higher fraction of biodegradable carbon, the well-adapted anaerobic community, and favorable operating conditions such as the long hydraulic retention time (HRT).

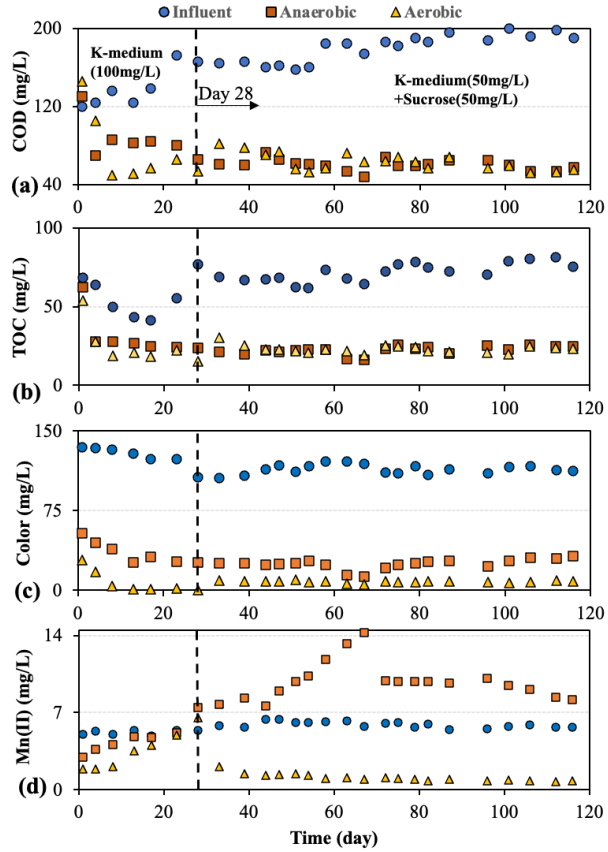

Figure 4. *Influent and effluent concentrations at the anaerobic and aerobic phases: (a) COD, (b) TOC, (c) Color and (d) Mn(II)*

b. Color

In the early stage, influent color values appeared inflated due to insoluble dye particulates. From Day 26 onward, when a 0.45 µm filtration step was applied before measurement, these particulates were removed and the measured influent color decreased slightly, approaching the intended set-point of about 100 mg/L (Figure 4c).

The decolorization was predominantly accomplished in the anaerobic reactor, where the influent dye concentration of approximately 134 mg/L was consistently decreased to a stable level of about 26 mg/L, corresponding to a stage-specific removal efficiency of 75–80%. This agrees with earlier reports where anaerobic microorganisms play a key role in cleaving the characteristic azo or anthraquinone bonds of dyes [6], [17]. The subsequent aerobic stage acted as a crucial polishing step, further reducing the color concentration to a final effluent level of 7–9 mg/L, which is equivalent to 60–70% removal of the remaining load. This resulted in a superior overall efficiency of above 95%. The present results demonstrate both higher and more stable performance compared with previous studies that reported limited color removal [6], [8], [18]. The anaerobic phase served as the main treatment step, while the aerobic reactor provided effective polishing, supported by the system's strong adaptability to operational conditions and a stable microbial community.

c. Mn(II)

The dynamics of dissolved Mn(II) were closely linked to the carbon source. During the first 28 days with only K-medium, Mn(II) concentrations remained minimal (~5 mg/L), suggesting that MnO_2 reduction was limited or the released ions were re-adsorbed (Figure 4d). After sucrose was introduced as a more readily biodegradable substrate, Mn(II) in the anaerobic phase rose sharply, peaking at 14.3 mg/L on Day 67 before stabilizing. This pattern highlights the strong influence of carbon quality on microbial Mn-reduction, as enhanced substrate availability significantly promoted the biological reduction of MnO_2 - a finding consistent with Ma et al. [19]. Together, the results underscore the central role of carbon availability in regulating the manganese redox cycling observed in the system.

After Mn(II) accumulated under anaerobic conditions, the aerobic phase began to remove it from approximately Day 30 onward, indicating that MnOB-driven oxidation was activated. This process converted Mn(II) into bio-MnO_2 and consistently maintained lower effluent concentrations than influent. Consistent with other studies on sequential reduction–oxidation [19], [20], these findings confirm the essential role of MnOB in sustaining the oxidative phase of the manganese cycle.

Overall, the DHS system established a complete and sustainable biological manganese cycle - anaerobic reduction coupled with aerobic oxidation - thereby demonstrating effective control over manganese transformations within the treatment process. This microbial-driven Mn transformation lays the biochemical foundation for the decolorization mechanism.

3.3. Decolorization mechanism mediated by the biological manganese cycle

3.3.1. Anaerobic phase- Mn(II) production

In the anaerobic phase, decolorization proceeded through a combination of biological and chemical pathways. This dual mechanism achieved 75–80% color removal, decreasing from~134 to ~26 mg/L (Figure 4(c)). At the same time, Mn(II) accumulated in the effluent up to ~14.3 mg/L (Figure 4(d)), confirming that MnO_2 reduction occurred in parallel with microbial dye reduction. To quantify the biological contribution, we first established the stoichiometric relationship between chemical decolorization and Mn(II) release in an abiotic control (slope = 0.525; Figure 2b). Using this stoichiometric slope (fixed at 0.5) as a reference, the anaerobic reactor data (Days 28–116) were fitted to yield the regression $y = 0.5x + 0.224$ ($R^2 = 0.67$; Figure 5). This moderate R^2 is consistent with the variability typically observed in complex biological systems [8], [17]. The positive intercept (0.224 mmol/L) quantifies the extent of decolorization attributable to biological activity, independent of the chemical pathway. This indicates that biological processes were the predominant decolorization mechanism, likely involving direct dye reduction with the dye as a terminal electron acceptor [6], [17] or indirect reduction mediated by electron shuttles such as humic substances [8]. Further microbial community analysis is required to elucidate the specific pathways.

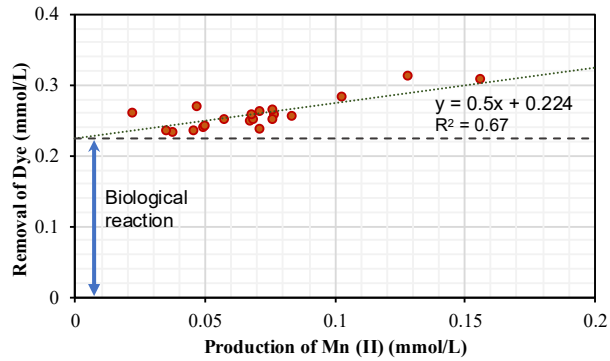

Figure 5. *Decolorization in the anaerobic phase: relationship between dye removal and Mn(II) production (Days 28–116). The dotted line represents the linear regression of the biological reactor data (y = 0.5x + 0.224). The positive y-intercept (0.224) quantifies the dominant biological contribution*

3.3.2. Aerobic phase – MnOB-mediated Mn(II) oxidation

In the aerobic reactor, the activity of MnOB was demonstrated by a sharp decrease in dissolved Mn(II) from ~14.3 mg/L to <2 mg/L, which coincided with a further reduction in color from ~26 mg/L to ~7–9 mg/L (Figure 4 (c), (d)). Crucially, this enhanced decolorization occurred without any significant change in COD or TOC levels (Figure 4a, 4b), indicating a selective oxidative process rather than bulk organic mineralization. This selectivity is attributed to the in-situ regeneration of biogenic manganese oxides (bio-MnO_2), a process catalyzed by MnOB. These biogenic oxides possess superior properties compared to chemical MnO_2, such as larger surface area and higher reactivity [4], [5]. As a result, they can preferentially attack the dye's chromophoric structures while leaving the organic backbone predominantly intact [21], [22]. This mechanism readily explains the observed decoupling of decolorization from COD/TOC removal. Therefore, aerobic decolorization in this system was sustained by the continuous, MnOB-driven regeneration of bio-MnO_2, highlighting the essential indirect role of these bacteria in the manganese redox cycle [19], [20]. While direct dye mineralization by aerobic bacteria is possible [23], [24], it was not a significant pathway in our system. Future work could focus on identifying MnOB strains or operational conditions that couple this efficient decolorization with organic carbon mineralization.

4. Conclusion

This study demonstrated that a sequential anaerobic–aerobic Down-flow Hanging Sponge (DHS) system can achieve nearly complete decolorization (over 95%) of anthraquinone dye wastewater while removing about 70% of COD and TOC. The anaerobic phase was the main treatment stage, reaching 75–80% decolorization and effective COD, TOC removal (~70%), unlike most previous DHS studies where COD reduction occurred mainly in the aerobic phase. Mn(II) formation reflected both chemical and biological pathways, with an important observation of this study being that biological contribution

predominated, as confirmed by an abiotic MnO_2 control. The aerobic phase then played an indirect but essential role: MnOB oxidized Mn(II) to regenerate bio-MnO_2, enabling further oxidation of residual dye. Nevertheless, remaining COD (~50 mg/L) and TOC (~20 mg/L) revealed incomplete degradation. Future work should focus on enhancing aerobic microbial activity or combining DHS with advanced oxidation to achieve complete treatment.

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