

Enhancement of Stabilization/Solidification Method with Bioaugmentation for Sludge Remediation

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(Received: 27 July 2025; Accepted: 2 March 2026; Published online: 10 May 2026)

ABSTRACT: The Stabilization/Solidification (S/S) method encapsulates heavy metals in sludge, while bioaugmentation can degrade organic contaminants. However, each method is limited in its ability to address both contaminants simultaneously. This study aims to enhance the S/S method by integrating bioaugmentation, using either immediate or pre-inoculation of bio-agents. Ordinary Portland Cement (OPC) served as the binder in the S/S process, with the bacteria *Sphingobacterium spiritivorum* and the fungi *Aspergillus brasiliensis* acting as bio-agents. Before testing, chemical oxygen demand (COD) and heavy metals in sludge and cement were measured. COD and heavy metal concentrations' leaching behavior were tested using TCLP after 14 and 28 days. The result shows 23.39% of organic contaminants were in the fibreboard sludge. The TOC results for Portland cement were negligible. All heavy metal elements (Cr, Cu, Zn, As, and Pb) in the Portland cement and fibreboard sludge exceeded the standard USEPA limit. COD reduction was evaluated for immediate versus pre-inoculation of fungi and bacteria over 14 and 28 days. Pre-inoculation consistently yielded lower COD levels than immediate inoculation, with fungi pre-inoculation achieving the greatest reduction (579 mg/L at 14 days; 544.7 mg/L at 28 days) and bacteria pre-inoculation showing moderate improvement (579.3 mg/L at 14 days; 570.3 mg/L at 28 days). Concentrations of heavy metal reductions followed a similar trend: after 28 days, pre-inoculated samples contained 0.01 mg/L (Cr), 0.22 mg/L (Cu), 0.06 mg/L (Zn), and 0 mg/L for both As and Pb, whereas immediate-inoculated samples showed 0.04 mg/L (Cr), 0.22 mg/L (Cu), 0.01 mg/L (Zn), and 0 mg/L for As and Pb. Statistical comparison shows significant differences between immediate-inoculation and pre-inoculation, but not between the bio-agents used in this study. To improve the findings, a co-culture method that combines both bio-agents is recommended, offering a viable strategy for industrial sludge management, with future studies needed to scale up the method for practical, sustainable waste treatment applications.

ABSTRAK: Kaedah Pengukuhan dan Penstabilan (S/S) bertujuan mengkapsulkan logam berat dalam enap cemar, manakala bioaugmentasi digunakan bagi mengurai bahan pencemar organik. Walau bagaimanapun, setiap kaedah mempunyai keterbatasan dalam menangani kedua-dua jenis pencemar secara serentak. Kajian ini bertujuan menambah baik kaedah S/S dengan menggabungkan bioaugmentasi, sama ada melalui inokulasi segera atau pra-inokulasi mikrob. Simen Portland Biasa digunakan sebagai agen pengikat dalam proses S/S, manakala bakteria *Sphingobacterium spiritivorum* dan kulat *Aspergillus brasiliensis* bertindak sebagai agen bio. Sebelum kajian dijalankan, Permintaan Oksigen Kimia (COD) dan logam berat dalam enap cemar serta simen ditentukan. Tingkah laku larut lesap COD dan kepekatan logam

berat diuji menggunakan kaedah TCLP selepas 14 dan 28 hari. Dapatan kajian menunjukkan sebanyak 23.39% bahan pencemar organik terdapat dalam enap cemar papan gentian. Kandungan TOC dalam simen Portland didapati sangat rendah dan tidak memberi kesan ketara. Semua unsur logam berat (Cr, Cu, Zn, As dan Pb) dalam Simen Portland Biasa dan enap cemar papan gentian melebihi had piawaian yang ditetapkan oleh USEPA. Pengurangan COD dinilai bagi inokulasi segera berbanding prainokulasi dengan kulat dan bakteria selama 14 dan 28 hari. Prainokulasi secara konsisten menghasilkan tahap COD lebih rendah berbanding inokulasi segera, dan prainokulasi kulat menunjukkan pengurangan paling ketara (579 mg/L pada 14 hari; 544.7 mg/L pada 28 hari) manakala prainokulasi bakteria menunjukkan peningkatan sederhana (579.3 mg/L pada 14 hari; 570.3 mg/L pada 28 hari). Kepekatan pengurangan logam berat mengikut corak serupa: selepas 28 hari, sampel prainokulasi mengandungi 0.01 mg/L (Cr), 0.22 mg/L (Cu), 0.06 mg/L (Zn), dan 0 mg/L bagi As dan Pb, manakala sampel inokulasi segera menunjukkan 0.04 mg/L (Cr), 0.22 mg/L (Cu), 0.01 mg/L (Zn), dan 0 mg/L bagi As dan Pb. Perbandingan statistik menunjukkan terdapat perbezaan signifikan antara kaedah inokulasi segera dan prainokulasi, namun tiada perbezaan signifikan antara jenis agen bio yang digunakan dalam kajian ini. Bagi meningkatkan keberkesanan rawatan, kaedah ko-kultur menggabungkan kedua-dua bioagen dicadangkan, menawarkan potensi terbaik bagi pengurusan sisa industri. Kajian lanjut diperlukan untuk penskalaan bagi aplikasi praktikal dan rawatan sisa lestari.

KEYWORDS: *Biodegradation, Hazardous Waste, Emerging Pollutant, Remediation*

1. INTRODUCTION

The increasing number of factories in Malaysia has led to an increase in sludge production in industrial wastewater. The amount of waste produced by industrial activities is also variable due to landfill overcapacity and other waste sources. Demand for forest products, including timber for construction, paper, and furniture, has also increased, which corresponds to the rapid population and urbanization growth. In this research, sludge production focuses only on sludge from industrial wastewater treatment plants used in fibreboard production. Fibreboard is a product made from rubber and tropical trees. This scenario has led to an increase in sludge production from wastewater treatment.

The sludge produced in the wastewater treatment facility was obtained from the fibreboard cleaning procedure. The wastewater process completed several steps before the sludge formation. About 500kg of fibreboard sludge was produced by Evergreen Fibreboard Berhad in a month, and the licensed contractor's transportation cost was approximately RM500 per month. After the sludge was delivered to Kualiti Alam Sdn Bhd, a specific procedure was carried out before it was disposed of at the landfill [1]. The Resources Conservation and Recovery Act (RCRA) states that the sludge was categorized as scheduled waste, also known as hazardous waste, requiring specific treatment for safe disposal.

Sludge production contains high concentrations of heavy metals, organic contaminants, and pathogenic microorganisms. A detrimental effect will occur if the sludge is not properly treated. Thus, sludge produced by fibreboard processing may threaten the surrounding environment. The factory is located near the university campus, residential zones, and other developed infrastructure, potentially posing health risks to nearby communities, including respiratory infections and skin problems. As a result, a polluted environment has disturbed the sustainability of life in that area. Besides, sludge production has affected agricultural activities and water quality in nearby locations [2].

The Stabilization/Solidification (S/S) method was used to immobilize the heavy metals. Based on previous studies, the S/S method was less efficient for organic contaminants when

using only cement as the binder [3]. S/S alone resulted in higher COD concentrations, whereas bioaugmentation achieved the lowest COD levels [13]. Moreover, COD levels in the S/S method-only samples increased between 14 and 28 days, indicating limited degradation of organic matter, whereas bioaugmented samples exhibited significant COD reductions over the same period [33]. A high concentration of calcium oxide (CaO) in the cement has made it compatible with heavy metals but not with organic contaminants [4]. The S/S method was less suitable for organic waste due to organic contaminants that prevented binder hydration and typically did not form chemical bonds in binder hydration solutions [5]. A key limitation of the S/S method is its inability to eliminate contaminants, as they remain within the solidified matrix. This has seriously threatened the safety of solidified samples when the binder did not adsorb or react with pollutants, producing an unstable component [6].

Therefore, bioaugmentation was incorporated into the S/S process to mitigate the adverse effects of sludge on human health and the environment and to enhance the removal of organic contaminants. The bioaugmentation degraded the organic contaminants into safe intermediates and end products. The addition of bioaugmentation to the S/S method was inspired by the study conducted by [3], which reported that a bacterial consortium and Portland cement were employed to evaluate the potential for Total Petroleum Hydrocarbons (TPH) degradation within granulated drill cuttings stabilized/solidified through bacterial integration. Then, a compost composed of bacteria and nutrient sources was added to S/S matrices to reduce hydrocarbons in petroleum drill cuttings before disposal [7]. Previous studies have demonstrated that the combination method can degrade both heavy metals and organic contaminants. Thus, the combination of S/S and the bioaugmentation method was conducted in this study to enhance the degrading ability towards heavy metals and organic contaminants.

The bioaugmentation treatment method was shown to have high potential for degrading organic contaminants. Besides, contaminated matrices can be converted into stable, reusable products through biological treatment [8]. However, both groups of contaminants are present in the sludge at high concentrations. Thus, combining both methods is required to ensure that organic contaminants are degraded. This combination treatment method must be integrated to eliminate contaminants over time [7]. Bioaugmentation is a technology that improves biomass by introducing specialized microorganisms into the natural flora. The bioaugmentation method may enhance bioremediation efficiency [9]. Chemical Oxygen Demand (COD) and Total Nitrogen (TN) are commonly degraded during bioaugmentation treatment, and microbes are commonly used as bioagents.

However, previous studies have only demonstrated the effectiveness of the combination method when adding bacteria as bio-agents. Research on the addition of fungi to cementitious systems is limited. In this study, the combination of S/S and bioaugmentation was conducted by inoculating the S/S matrices with microbes (*Sphingobacterium spiritivorum*) and fungi (*Aspergillus brasiliensis*) as bio-agents. The bacteria were chosen in this study because they were used in the study by [10] to degrade organic contaminants in soil and had been shown to improve the degradation of organic contaminants by inoculating *Sphingobacterium spiritivorum* strains in the soil field. Then, *Aspergillus brasiliensis* was chosen as a bioagent in this study due to its ability to produce organic acids effective in metal bioleaching, such as tartaric, oxalic, citric, and malic [11]. In a bioremediation treatment, *Aspergillus sp.* identified from tannery waste was found to be highly effective in reducing the Chemical Oxygen Demand (COD) and color of tannery effluent [12]. In this study, the results between these two microbes were compared.

2. METHODOLOGY

2.1. Raw Materials Preparation

This study utilized fibreboard sludge and Portland cement as its primary materials. Approximately 40 kg of sludge was acquired from a fiberboard manufacturing factory prior to the sanitation stage of its wastewater treatment process. Before testing, the sludge was kept at room temperature in a sealed plastic bag to ensure its viability. Meanwhile, Portland cement was procured from a nearby hardware store as the binding agent.

2.1.1. Bacteria Preparation

The bacteria (*Sphingobacterium spiritivorum*) used in this study were isolated from sewage sludge and stored in microbeads (microbank™) at -80 °C. 200 ml of distilled water and 5.6 g of Nutrient Agar (C1225) powder were combined in a bottle (USEPA, Method FNES6). Subsequently, the bottle was shaken 3 to 4 times and autoclaved for approximately 45 minutes to achieve sterilization. Next, to ensure the agar solidified, the agar mixture was transferred into several Petri dishes and allowed to stand for 10 to 12 minutes. An inoculation loop was used to acquire a single colony of *S. spiritivorum*. The inoculation loop was streaked on a Petri dish using the quadrant method, which reduces the bacterial load at each streak to separate individual bacterial cells. A biohazard safety cabinet was used for these procedures. Since the bacterial isolates were active on days 3 or 4, the petri dishes were incubated upside-down at 37°C for 3 to 4 days, as shown in Figure 1. The parafilm closure was used. A temperature range of 30–37 °C was used as the optimal temperature for the bacteria.

A small number of frozen beads were transferred to universal bottles containing nutrient broth, and the broth was incubated at 30°C for 3 days to resuscitate the bacteria. In a bottle intended for a cube mould, 26 g of Nutrient Broth powder and 2 L of distilled water were combined to prepare bacterial broth (USEPA, Method FNES6). An autoclave machine was used to sterilize the broth for approximately 45 minutes. Four Petri dishes containing growing bacteria were combined with the sterilized broth, and the experiment was carried out in a biohazard safety cabinet. Before utilizing the broth as a bio-agent in the bioaugmentation, the bottle had to be incubated for 3 days at 30°C.



Figure 1. Process of bacterial culture in a petri dish.

2.1.2. Fungi Preparation

The fungal strain used in this study was *Aspergillus brasiliensis* ATCC® 16404TM. Initially, 200 mL of distilled water and 7.8 g of potato dextrose agar (PDA) powder were combined in a bottle using the USEPA Method FNES6. The bottle was then shaken 3 to 4 times and autoclaved for approximately 45 minutes to sterilize it. Then, to ensure the agar solidified, the PDA agar mixture in the bottle was poured into several Petri dishes and allowed to sit for 10 to 12 minutes. After that, 1 ml of the fungal spore suspension was added to the PDA agar. The biohazard safety cabinet served as the place for these procedures. As sporulation was completed after 7 days, the Petri dishes were covered with parafilm and incubated at 28°C for 7 days. The ideal temperature for the fungi was 30°C.

The USEPA standard process, Method FNES6, was used to produce the fungi broth. First, 2 L of Ultra-Pure Water (UPW) and 48 g of potato dextrose broth powder were combined in a bottle (Figure 2). An autoclave was used to sterilize the broth for approximately 45 minutes. In a biohazard safety cabinet, 4 Petri dishes containing growing fungus were combined with the sterilized broth. Before the solution was added to the cement and sludge mixture as a bio-agent, the bottle was shaken 4 to 5 times.



Figure 2. Process of mixing fungi into Ultra-Pure Water (UPW)

2.2. Measurements of Organic Contaminants and Heavy Metals Raw Materials.

Total Organic Carbon (TOC) analysis was used to quantify the carbon content of the sludge sample's organic compounds. The sludge sample was prepared as a powder prior to analysis. The TOC analysis was performed in accordance with EPA Method 9060A.

This research used the Acid Digestion method (USEPA, Method 3050B) to determine the concentration of heavy metals in sludge. Acid digestion is a common preparation method that releases heavy metals into solution by dissolving complex sample matrices, making them easier to analyze. This process guarantees that the heavy metals are present in a form that can be precisely measured with a variety of analytical methods, including Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Atomic Absorption Spectroscopy (AAS), and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

2.3. Design of S/S Method with Immediate-Inoculation and Pre-Inoculation of Bio-agent

This study focused on the hybrid method of immediate-inoculation and pre-inoculation. The immediate-inoculation and pre-inoculation were applied with bacteria (*Sphingobacterium spiritivorum*) and fungi (*Aspergillus brasiliensis*) as augmentation agents in the bioaugmentation method. For the immediate-inoculation sample, augmentation agents and cement were mixed simultaneously with Portland cement and cured for 14 days and 28 days to produce the S/S matrices. Meanwhile, for the pre-inoculation sample, augmentation agents were inoculated and stored at room temperature for 7 days before mixing with Portland cement and curing for the same period as the immediate-inoculation sample. Portland cement serves as the binder in the S/S method. For the control sample, no augmentation agents were inoculated in this sample. Only indigenous microorganisms were used for contaminant removal.

The optimum Portland cement-to-sludge ratio (70:30) employed in this study was chosen following [13]. Subsequently, 10×10^{10} CFU/ml bacterial and fungal strains were added to the hybrid method. Table 1 shows the preparations of bacteria and fungi for immediate inoculation and pre-inoculation.

Table 1. Preparation of bacteria and fungi inoculation for immediate inoculation and pre-inoculation

Sample	Portland Cement (%)	Sludge (%)	Sludge + Bacteria (%)	Sludge + Fungi (%)
14 Days				
Fungi pre-inoculation	70	-	-	30
Fungi's immediate inoculation	70	-	-	30
Bacteria pre-inoculation	70	-	30	-
Bacteria's immediate inoculation	70	-	30	-
Control sample	70	30	-	-
28 Days				
Fungi pre-inoculation	70	-	-	30
Fungi's immediate inoculation	70	-	-	30
Bacteria pre-inoculation	70	-	30	-
Bacteria's immediate inoculation	70	-	30	-
Control sample	70	30	-	-

The S/S matrices were prepared and cured for 14 days and 28 days using Portland cement as the binder. Gradually add water to the mixture, mixing until no lumps remain. The homogenized mixture was then cast into 100 mm³ molds in three layers, with each layer manually compacted using a tamping rod for at least 50 hits. In order to guarantee that air curing occurs at a regulated temperature and humidity condition, the samples were triplicated for two hydration durations, which were 14 and 28 days, following the casting process. The concrete cubes were subsequently subjected to compressive strength testing. Figure 3 shows the flowchart of the S/S matrices design.

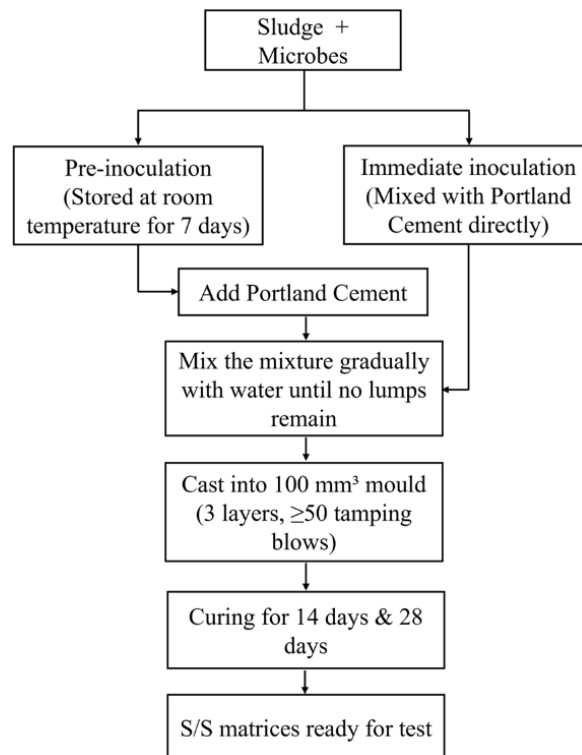


Figure 3. Flowchart of the S/S matrices design process

2.4. Compressive Strength Test

After curing, the concrete cubes were removed and thoroughly dried. Sample numbers corresponded to those listed in Table 1. Prior to testing, the dimensions and weight of each cube were recorded. Each cube was positioned vertically on the base plate of a compression testing machine, as shown in Figure 4(a), and the upper plate was lowered until it contacted the cube surface. The load was applied gradually, and the maximum compressive load at initial cracking or failure was recorded. Compressive strength was calculated by dividing this maximum load by the cube's cross-sectional area. This test is critical, as a strong S/S matrix physically immobilizes hazardous substances, whereas weak or cracked solidified materials may allow leaching of contaminants. Figure 4(b) illustrates the concrete cube after undergoing the compressive strength test, showing the point of failure.



Figure 4. (a) Compressive strength test and (b) Concrete cube of S/S matrices samples

2.5. Leachability Test

The Toxicity Characteristic Leachability Procedure (TCLP) test required that cube samples be crushed to a size of less than 9.5 mm to meet the USEPA standard (Method 1311). 5.7 ml of concentrated acetic acid, CH₃COOH, and 1000 ml of distilled water were added to a conical flask, half-filled, and the mixture was shaken to mix. After that, the 1000 ml conical flask was filled with distilled water again to the top limit line and shaken again. The pH of 400 ml of reagent water, prepared by mixing 5.7 ml of concentrated CH₃COOH with distilled water, was measured in a beaker. Subsequently, 20 g of crushed cube sample was added to the reagent water, and the mixture was placed in screw-capped polyethylene bottles. Then, the bottles were rotated at 30 rpm for 18 hours in a rotary chamber (Figure 5(a)). The collected leachate was filtered using 7 µm fiber filters, as depicted in Figure 5(b). Concentrated nitric acid (HNO₃) was used to store the leachate, and the pH was kept below 2.

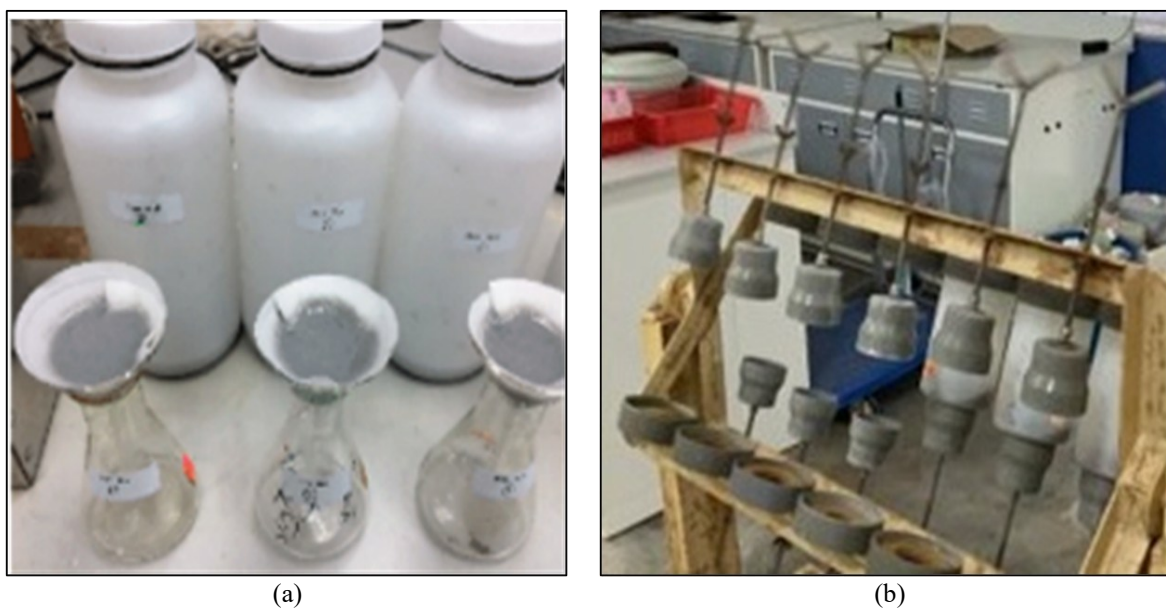


Figure 5. (a) Rotary chamber and (b) Filtration process



Figure 6. (a) HACH DR600 equipment and (b) Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

A Chemical Oxygen Demand (COD) test was used to indirectly quantify the total organic content of the collected leachate. This COD test also displayed the mass of oxygen utilized per liter of solution. The COD test also measured the amount of oxygen consumed by water pollutants during chemical oxidation. First, a 2 ml diluted, filtered sample was placed in High Range COD vials, and the leachate was kept in concentrated nitric acid (HNO₃) at pH <2. It was heated in the DRB200 reactor for 2 hours. The concentration of organic contaminants in the leachate was subsequently determined using analytical methods, with measurements conducted on the DR6000 equipment, as illustrated in Figure 6(a). The test was carried out using the USEPA Method 410.3 and the EPA-600/4-79-020 standard procedure. Heavy metal concentration in the extracted and measured samples was then examined using an ICP-MS analyzer, as depicted in Figure 6(b).

3. RESULTS

3.1. Concentration of Organic Contaminants in Raw Materials

Analysis of Total Organic Carbon (TOC) offered important information on the organic content in the fibreboard sludge. The TOC analysis measured SSM-TC (Total Carbon), SSM-IC (Inorganic Carbon), and SSM-TOC (Total Organic Carbon). Based on Table 2, a concentration of 1.000 mg/μL was determined from the SSM-TOC analysis, indicating the amount of organic carbon in the fibreboard sludge sample. The computed SSM-TOC percentage of 23.39% indicates that the fibreboard sample contained a considerable amount of organic carbon. The TOC result of 23.39% was categorized as a high organic content in the sludge [14]. Depending on the source and method of treatment, sludge was classified as high range when it ranged within 15% to 25% [14]. TOC values in the 20% to 28% range, which were higher, indicated that the sludge was better suited for organic fertilizer applications or composting [15].

Table 2. Summaries of results for TOC analysis

Type	Analysis	Manual Dilution	Density	Result
Unknown	SSM-TOC	1.000	1.000 mg/μL	SSM-TOC: 23.39% SSM-TC: 23.40% SSM-IC: 0.00959%

Table 3. Summary of SSM-TC analysis

Area	CNV	Abs C	Concentration	SD Concentration	Weight	Volume
1400	1400	7020 μg	23.40%	0.000	30.00 mg	30 μL

Table 4. Summary of SSM-IC analysis

Area	CNV	Abs C	Concentration	SD Concentration	Weight	Volume
0.4755	0.4755	2.897 μg	0.00959%	0.000	30.20 mg	30 μL

However, the total carbon content of the fibreboard sample, as determined by the SSM-TC analysis, was 23.40% (Table 3). The consistency of the carbon content across measurements is suggested by this finding and the lack of a large standard variation in the concentration.

On the other hand, Table 4 shows that the SSM-IC analysis revealed the presence of inorganic carbon molecules at a low concentration of 0.00959%. The small standard deviation adds further evidence of the validity of this finding.

From the TOC results, the higher range of the organic carbon content in the fibreboard sludge needed to be remediated to ensure it could reduce the potential environmental risks and be safe to be disposed. The presence of organic content in Portland cement is generally negligible, as the material is primarily composed of inorganic constituents, including iron oxide, limestone, silica, and alumina [16]. Any organic contaminants were burnt out during the high-temperature kiln process used in Portland cement production [16]. Furthermore, research indicated that the hydration and strength development of cemented soil can be inhibited by the presence of organic matter [17]. Other studies have also shown that an increase in organic matter weakens the mechanical properties of treated soils by interfering with cementitious binding, resulting in reduced strength and greater susceptibility to cracking [18].

3.2. Concentration of Heavy Metals in Raw Materials

The concentrations of heavy metals in the raw materials, including Portland cement and fibreboard sludge, are shown in Table 5. The concentration of heavy metals in each raw material was determined by averaging triplicate samples. The information presented allows for comparison of the concentrations of heavy metals (Cr, Cu, Zn, As, and Pb) in Portland cement and fibreboard sludge with USEPA standards. Waste exceeding the regulatory limit is classified as hazardous and must be properly managed.

Table 5. Concentration of heavy metals in raw materials

Heavy Metals	Formula	Concentration (mg/L)		Standard USEPA limit (mg/L) [19]
		Portland Cement	Fibreboard Sludge	
Chromium	Cr	36	4.06	≤ 0.1
Copper	Cu	8	3.79	≤ 1.3
Zinc	Zn	81	87.60	≤ 5.0
Arsenic	As	19	0.79	≤ 0.01
Lead	Pb	15	2.52	≤ 0.02

The results showed that all heavy metal elements in the Portland cement and fibreboard sludge exceeded the USEPA standard. Zn showed the highest concentration in the Portland cement and fibreboard sludge, with 81 mg/L and 87.60 mg/L, respectively. Therefore, remediation was necessary to reduce the toxicity of these harmful metal components in the environment. Mixing Portland cement as a binder with fibreboard sludge in the S/S method treatment may reduce heavy metal leaching. S/S method is an immobilization technique that uses binders to change the mobile metal (loid)s in the sludge into less mobile forms [20]. The study discovered that in polluted soil, Portland cement, fly ash, quicklime, and blast furnace slag can be used as composite materials to significantly stabilize heavy metals such as Cu, Cr, Zn, and Pb [21]. Fixation of heavy metals can be improved by adding more cement [3].

This research implemented the combination of S/S and bioremediation (bioaugmentation) to immobilize both heavy metals and organic contaminants. The combination treatment method was used because contaminant substances remain in the matrices and are not destroyed, posing a serious threat to the safety of solidified samples when the binder does not absorb or react with them to form a stable component. The discussion on the Chemical Oxygen Demand (COD) concentration for the combination method is discussed in the next section.

3.3. Comparison of Chemical Oxygen Demand (COD) Concentration for Immediate Inoculation and Pre-Inoculation

Due to the high organic content of the sludge, the COD concentration was selected to indirectly quantify organic contaminants in the leachate. The test will show the mass of oxygen consumed per Liter of solution. COD measures the amount of oxygen required to oxidize contaminants. The efficiency of the combination method (S/S) and bioaugmentation in reducing COD concentration was demonstrated in Figure 7. Based on data from 14 days, immediate-inoculation and pre-inoculation for both fungi and bacteria yielded better results than the control sample. In the control sample, only indigenous microorganisms are reducing the COD concentration in the leachate. Based on this result, the indigenous microorganisms are unable to reduce the COD concentration. The potential of fungal and bacterial inoculation can help reduce COD concentration.

By comparing fungi and bacteria, fungi's immediate inoculation shows the lowest COD concentration, which means fungi can work immediately during the mixing process with cement. In contrast, bacteria require an adaptation period to utilize COD for their vital physiological and biochemical processes. Unbalanced nutrient levels and unfavorable conditions, such as high pollutant concentrations, temperature, moisture content, and pH, may affect the success of bacterial pollutant degradation [22]. Fungi are filamentous organisms that can break down complex organic pollutants, such as phenolic compounds, as soon as they encounter them and can react quickly to the presence of contaminants [23,24]. Fungi have demonstrated their immediate usefulness in bioremediation applications by degrading pharmaceutically active chemicals (PhACs) through complex enzymatic systems without major lag [25].

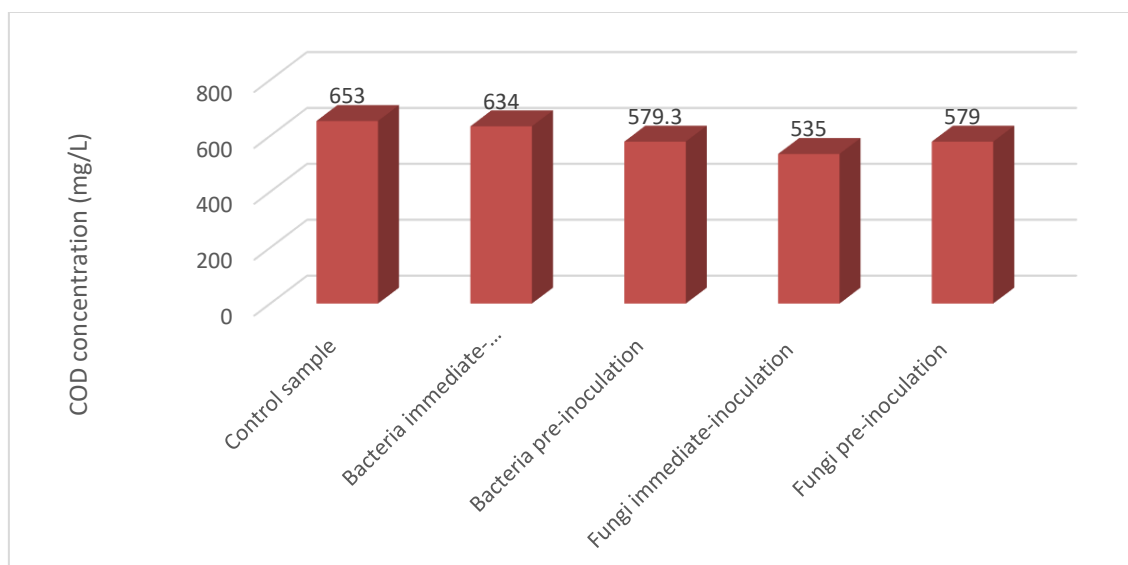


Figure 7. COD concentration (mg/L) for immediate-inoculation and pre-inoculation on 14 days of the curing period.

The experiment was extended to a 28-day curing period, and the focus was on immediate inoculation with fungi and bacteria, showing an increase in COD concentrations from 14 to 28 days. The control sample also shows the same trend, with COD concentrations increasing from 14 days to 28 days. Only samples with pre-inoculation show a reduction of COD concentrations over a longer duration. Microorganisms that fail to survive in S/S matrices under non-indigenous conditions may necessitate immediate inoculation. The organic content of the mixture increases due to the presence of dead microorganisms. This statement was supported

by [26], which found that the remains of dead microorganisms contribute to soil organic matter, indicating that these residues are essential for increasing soil organic carbon content.

However, the results of the pre-inoculation method showed effectiveness: after 28 days, pre-inoculation with bacteria and fungi resulted in lower COD concentrations than those observed with immediate inoculation. It was shown that the adaptability of these bio-agents under the early 7-day inoculation condition (pre-inoculation) enhanced their ability to degrade organic contaminants, thereby reducing the COD concentration, as shown in Figure 8.

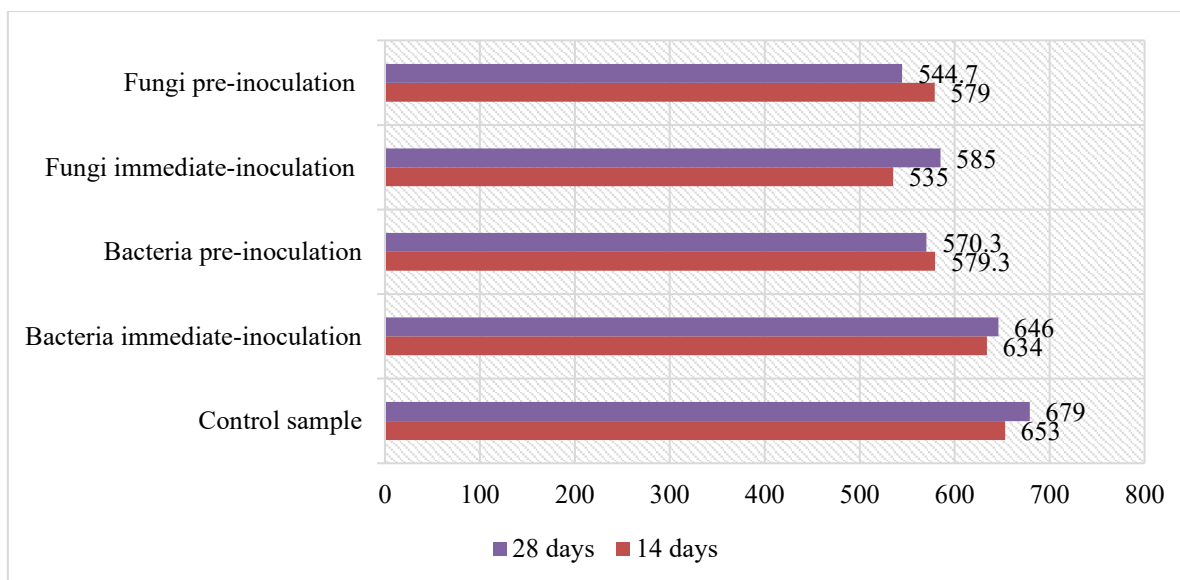


Figure 8. Comparison of COD concentration (mg/L) for immediate-inoculation and pre-inoculation between 14-day and 28-day curing periods.

In addition, pre-inoculation with fungi shows better results than with bacteria. It was implied that the ability of *Sphingobacterium spiritivorum* to break down the organic contaminants in the fibreboard sludge might be limited. These conditions demonstrated that the adaptability of the indigenous microorganism was lower than that of *Sphingobacterium spiritivorum* and *Aspergillus brasiliensis* during pre-inoculation.

The selection of the bio-agents greatly impacted the rates of the COD concentration reduction. The research conducted by [27] showed that the microbiological species involved were essential for the replication and enhancement of the bioaugmentation process. The microbial population's variety and functionality contributed to the repair of contaminated environments [28, 29].

In conclusion, comparing the reduction in the COD concentration between the immediate-inoculation and pre-inoculation procedures emphasized the importance of introducing the bio-agents in a timely manner when using the combination method of S/S and bioaugmentation. Based on this study, pre-inoculation appeared as the recommended strategy for lowering COD concentration.

3.4. Comparison of Heavy Metals Concentration for Immediate Inoculation and Pre-Inoculation

Because heavy metals in Portland cement and fibreboard sludge exceeded the USEPA standard, this parameter was also measured in the leaching test. In this study, the concentrations of heavy metals, including chromium (Cr), copper (Cu), zinc (Zn), arsenic (As), and lead (Pb) were measured and compared using the combination method, namely S/S and bioaugmentation

method, with two inoculation methods, which were immediate-inoculation and pre-inoculation after 14 days of curing period, as shown in Figure 9.

The results in Figure 9 demonstrated that the pre-inoculation sample showed a greater reduction in heavy metals than the immediate-inoculation samples. All heavy metal concentrations were increased for the immediate-inoculation sample compared to the control sample, except for Cr (bacteria immediate-inoculation). Based on this finding, the combination method is not recommended for immediate inoculation. The bioaugmentation process in the S/S matrices may be inhibited by the presence of cement. Microbial life can be adversely affected by cement, which can change the chemical and physical environment [30,31]. Furthermore, a crucial part of the bioaugmentation process, microbial growth and activity can be inhibited by high pH levels. The process of mixing sludge with cement increases the pH level in the S/S matrices.

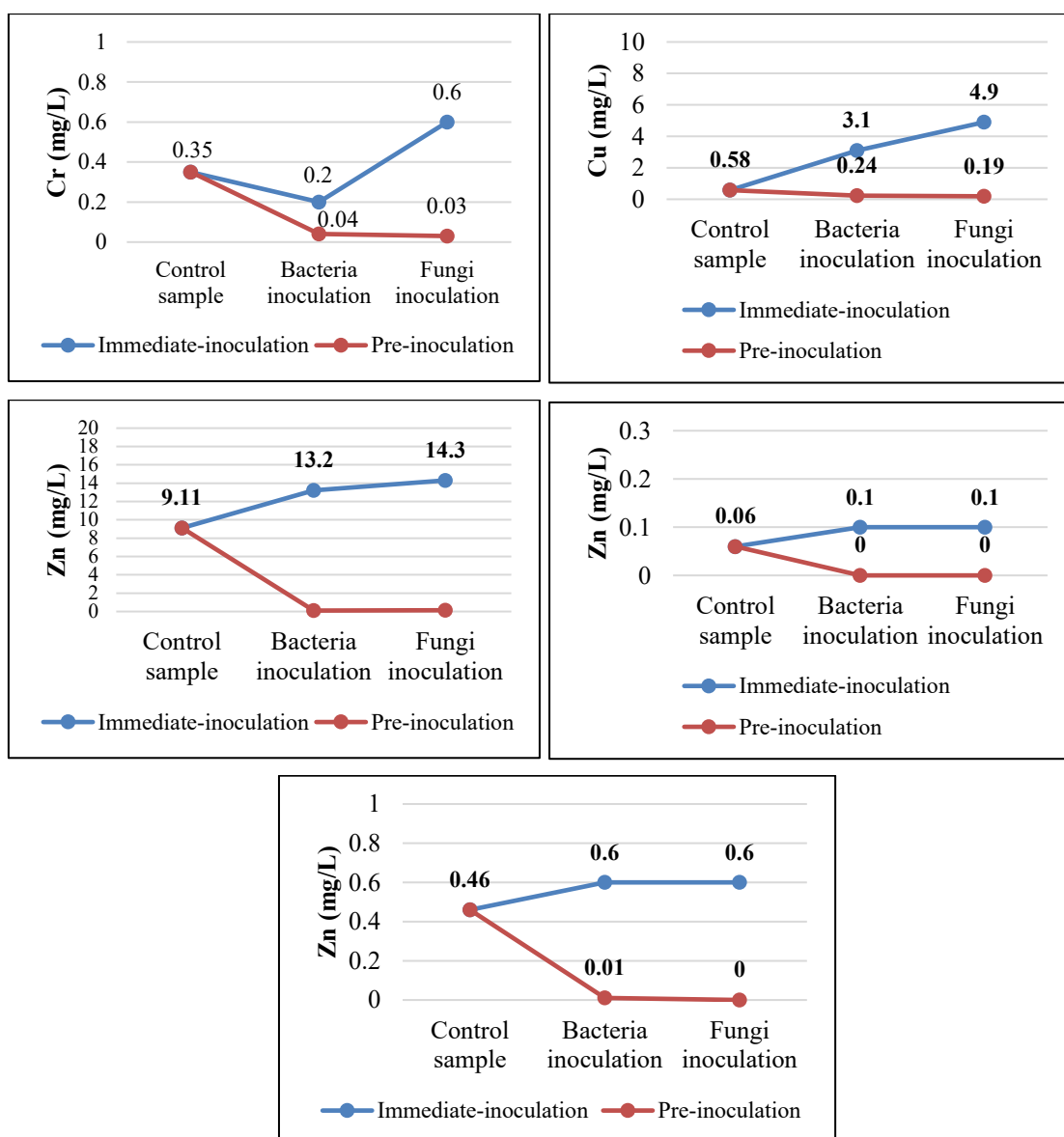


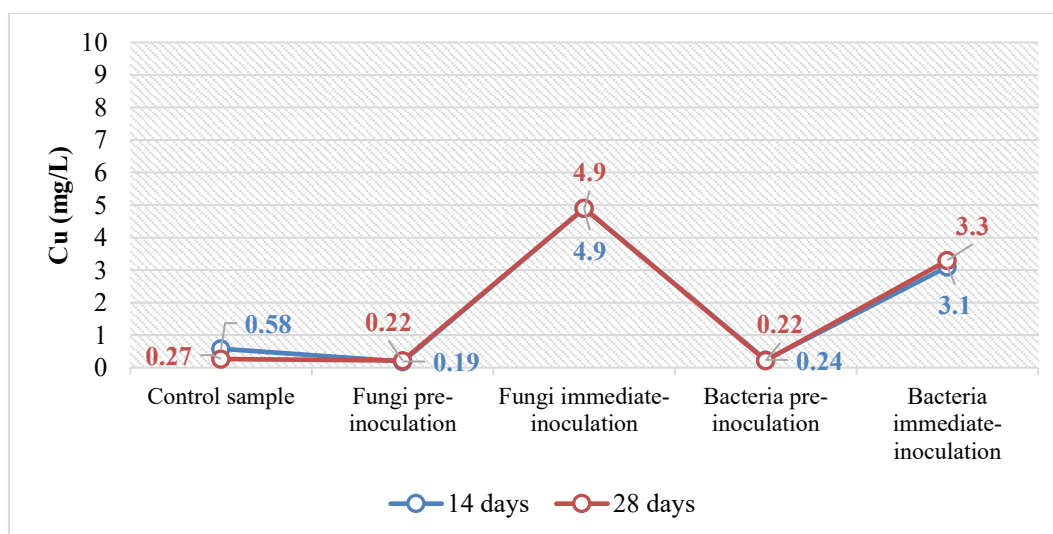
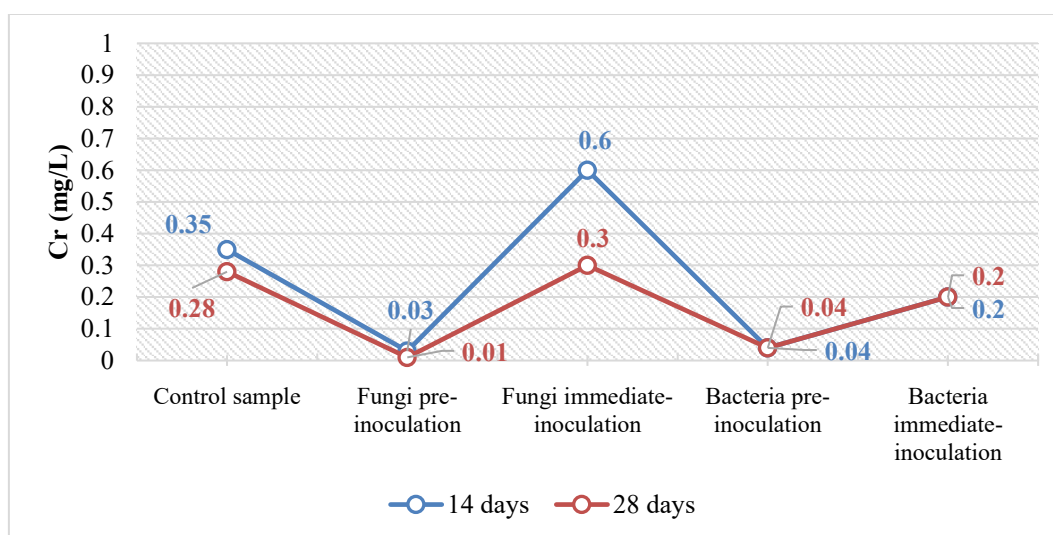
Figure 9. Heavy metals concentration (mg/L) for immediate-inoculation and pre-inoculation on 14 days of the curing period.

By comparison, pre-inoculation with fungi appeared to be the most effective method for reducing heavy metal concentrations, except for Zn. For Zn, pre-inoculation with bacteria

shows a greater reduction in Zn concentration. Fungi are very useful as bioagents because they contain specialized enzymatic systems that can degrade various organic and inorganic contaminants [32]. The main components of these enzymatic systems are extracellular and intracellular enzymes, each with a specific function in the degradation process [23]. Laccase and peroxides were the key extracellular enzymes that catalyzed the breakdown of complex contaminants. Moreover, fungal bioremediation capacities are greatly enhanced by intracellular enzymes such as cytochromes [23].

Based on the results in Figure 10, the concentration of heavy metals decreased after a 28-day curing period, except for Cu and As, for both bacteria and fungi, following immediate inoculation. However, better results were obtained for pre-inoculation samples, in which As was not detected after 28 days of the curing period. Whereas for Cu, a slight reduction was observed with bacterial pre-inoculation and a slight increase with fungal pre-inoculation. For Cr, Zn, and Pb, the pre-inoculation showed a greater decrease in the concentration of heavy metals after 28 days compared to immediate-inoculation.

The concentration of heavy metals in fibreboard sludge treated with bacteria and fungi decreases clearly during this time, indicating that the early formation of microbial consortium speeds up the bioaugmentation process and leads to more noticeable decreases in the heavy metal's concentration over time.



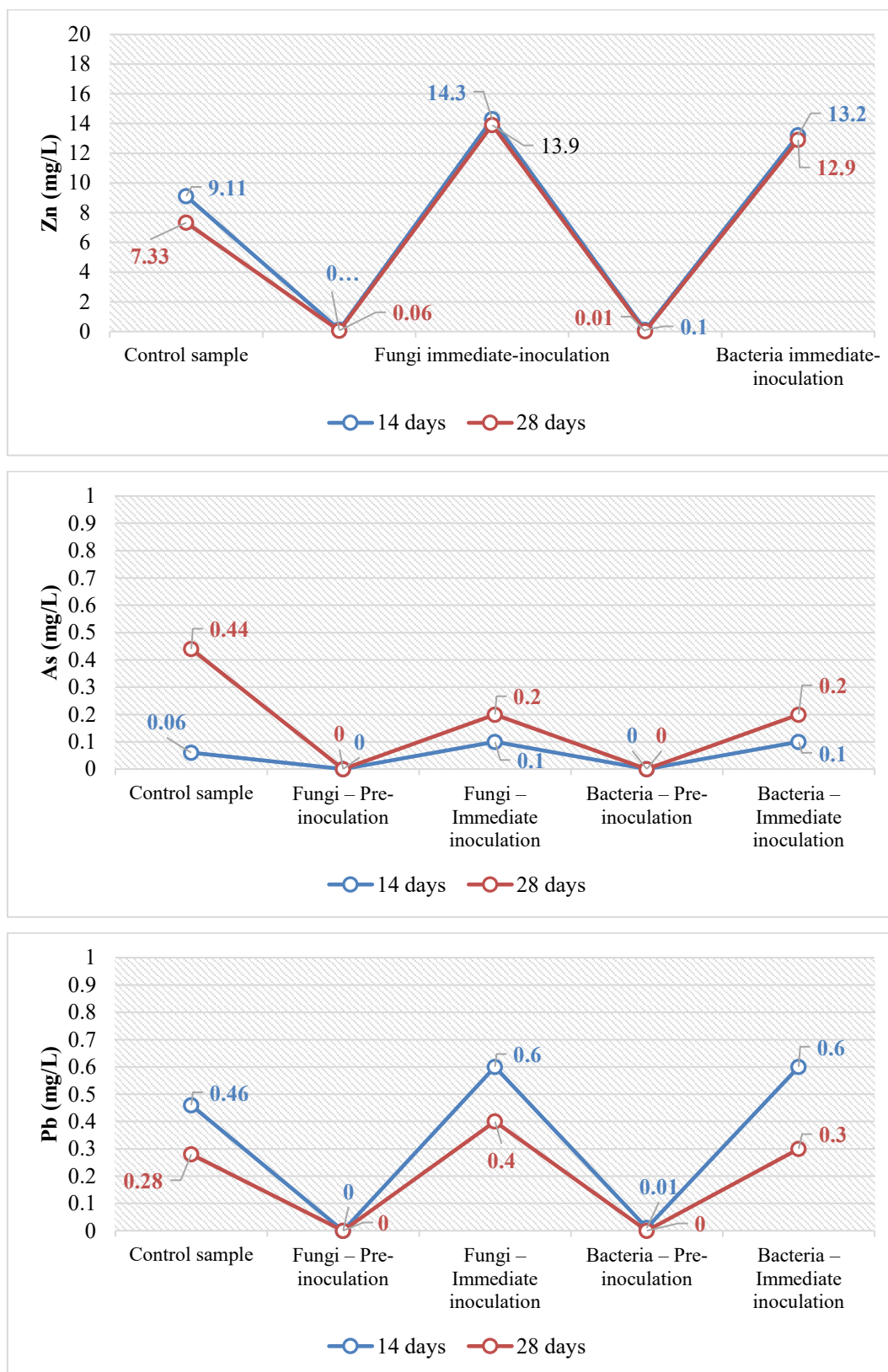


Figure 10. Comparison of heavy metals (mg/L) concentration between 14 and 28 days of curing

3.5. Statistical Comparison of Immediate Inoculation and Pre-Inoculation

The bar chart utilizes a logarithmic scale to effectively illustrate the substantial differences in values among the various parameters that have been developed. This approach allows for a

clearer comparison, particularly when the values vary widely in magnitude. As depicted in Figure 11, the logarithmic scale enables the visualization of these differences in a more manageable way, making it easier to interpret the data.

Independent t-tests were conducted to analyze the effects of immediate inoculation relative to pre-inoculation across a range of parameters, as presented in Table 6. The results showed statistically significant differences in three key parameters: COD ($p = 0.013$), Cu ($p = 0.001$), and Zn ($p = 0.0001$). Moreover, no significant differences were observed for Cr ($p = 0.08$), As ($p = 0.095$), and Pb ($p = 0.097$), as the p-values are greater than 0.05.

These findings indicate that the immediate-inoculation treatment generally resulted in higher concentrations of all measured parameters than the pre-inoculation treatment. Notably, the most pronounced differences were observed for COD, Cu, and Zn, suggesting that pre-inoculation may have a substantial positive effect on these specific parameters. This suggests the potential benefits of pre-inoculation in reducing the concentrations of these critical substances.

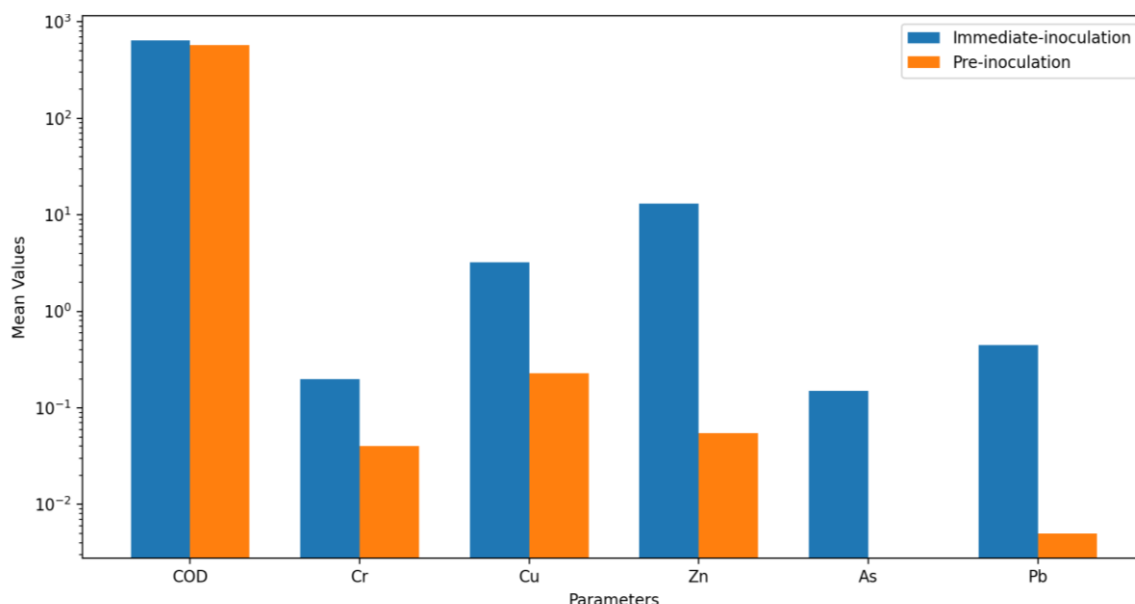


Figure 11. Comparison of parameter concentrations on a logarithmic scale

Table 6. Result of t-test analysis across all parameters

Parameter	Mean		t-statistic	p-value	Significance
	Immediate Inoculation	Pre-Inoculation			
COD	640.0	574.8	8.6933333333	0.0129750861	*
Cr	0.2	0.04	0.0001	0.08	***
Cu	3.2	0.23	29.5526045492	0.0011430451	*
Zn	13.05	0.055	82.979683843	0.0001451984	*
As	0.15	0.0	3.0	0.0954659663	***
Pb	0.45	0.005	2.9650198907	0.0974113588	***

* $p \leq 0.01$: Highly Significant, ** $p \leq 0.05$: Significant, *** $p > 0.05$: Not Significant

COD, Cu, and Zn, selected as key parameters, were thoroughly analyzed to evaluate and compare the roles and effects of bacteria versus fungi in the inoculation method. The Shapiro-Wilk test results indicate that the datasets for Zn and Cu do not follow a normal distribution, as p-values fall below the 0.05 threshold, as shown in Table 7. This finding suggests that

parametric tests, which assume normality, would not be appropriate for analyzing these data. As a result, the Mann-Whitney U test was applied as a nonparametric statistical method that does not rely on the assumption of normality to compare the presence of bacteria and fungi across three specific parameters.

Upon conducting the Mann-Whitney U test, the ranks of the data for both bacteria and fungi were analyzed. As shown in Table 8, p-values from this analysis indicate no statistically significant differences between the two groups (bacteria and fungi) in Zn and Cu measurements. This finding suggests that the presence of either bacteria or fungi does not significantly influence the levels of these parameters. Therefore, any observed variations in COD, Zn, and Cu levels between the two groups may be attributed to random variability rather than a systematic difference.

Table 7. Statistical comparison between bacteria and fungi using the Shapiro-Wilk test

	Shapiro-Wilk test		
	COD	Zn	Cu
Bacteria	0.2470*	0.0323**	0.0479**
Fungi	0.3046*	0.0331**	0.0255**

* $p > 0.05$: *t*-test acceptable, ** $p < 0.05$: Use Mann-Whitney U

Table 8. Mann-Whitney U Test results for Cu and Zn concentrations (bacterial treatment)

	Mann-Whitney U Test	
	Zn	Cu
Bacteria		
Fungi	0.4857**	1.0000**

* $p \leq 0.05$: significant difference, ** $p > 0.05$: not significant

The differences between the two inoculation methods, namely immediate inoculation and pre-inoculation, underscore the critical importance of carefully selecting the optimal timing for inoculation before applying the binder in the stabilization/solidification (S/S) method. Immediate inoculation involves adding microbes directly into the sludge at the time of mixing, while pre-inoculation requires preparing the microbes in advance and allowing them to establish themselves before binder application. This timing is essential, as it can significantly affect the effectiveness of microbial interactions with the sludge. However, this study found no significant differences in effectiveness between the bacterial and fungal species used in these inoculation methods. This lack of significance suggests that both types of microorganisms can be used interchangeably without affecting the overall outcomes of the S/S process. Based on the findings of this study, the inoculation method for enhancing contaminant removal in cement matrices contributes new knowledge to the sludge remediation process.

4. DISCUSSION

The results of this study indicate that pre-inoculation with both fungi and bacteria markedly improves the stabilization of COD and heavy metals compared with immediate inoculation. This enhanced performance is clearly illustrated in the comparative analyses presented in Figures 12 and 13, which highlight the superior effectiveness of pre-inoculated treatment.

As shown in Figure 12, pre-inoculation resulted in greater reductions of COD and heavy metals than immediate inoculation after 14 days, as reflected by the percentage contributions of each treatment. For COD, pre-inoculation accounted for approximately 55–60% of the total reduction, exceeding the contribution of immediate inoculation and indicating enhanced organic matter degradation following microbial acclimatization. A similar trend was observed for heavy metals, where pre-inoculation consistently contributed the largest percentage of removal across all metals studied. Bacteria pre-inoculation accounted for more than 60% of chromium (Cr) and copper (Cu) reduction, while fungal pre-inoculation contributed nearly 70% of zinc (Zn) removal. For arsenic (As) and lead (Pb), pre-inoculated systems contributed over 65% of the total reduction, whereas immediate inoculation contributed comparatively little. Overall, the dominance of pre-inoculation in percentage reduction for both COD and heavy metals indicates that microbial adaptation prior to exposure significantly improves treatment efficiency. These findings highlight pre-inoculation as a more effective strategy for achieving rapid contaminant reduction within short treatment durations.

At 28 days, the percentage contribution analysis, as depicted in Figure 13, shows that pre-inoculation resulted in greater reductions in COD and heavy metals than immediate inoculation. For COD, pre-inoculated treatments accounted for approximately 55–60% of the total reduction, indicating improved organic matter stabilization following microbial adaptation. For heavy metals, pre-inoculation consistently dominated the percentage contribution across all metals studied. Reductions in Cr and Cu were largely driven by bacterial and fungal pre-inoculation, accounting for more than 40–50% of total removal, whereas Zn reduction exceeded 60% under pre-inoculated conditions. Similarly, As and Pb removal was predominantly associated with pre-inoculation, accounting for over 65% of the total reduction, whereas immediate inoculation contributed comparatively little. These results confirm that microbial pre-conditioning enhances long-term contaminant stabilization, making pre-inoculation a more effective treatment strategy over extended durations.

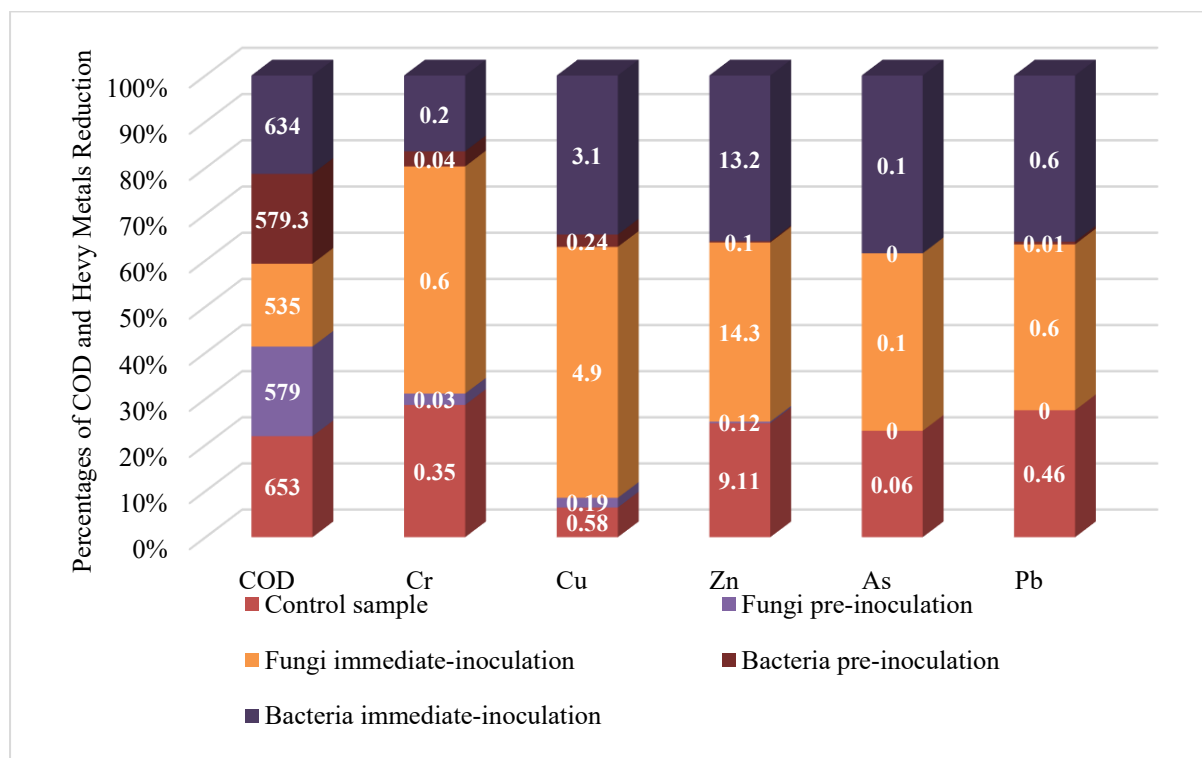


Figure 12. Comparison of the percentage reduction for COD and heavy metals in 14 days

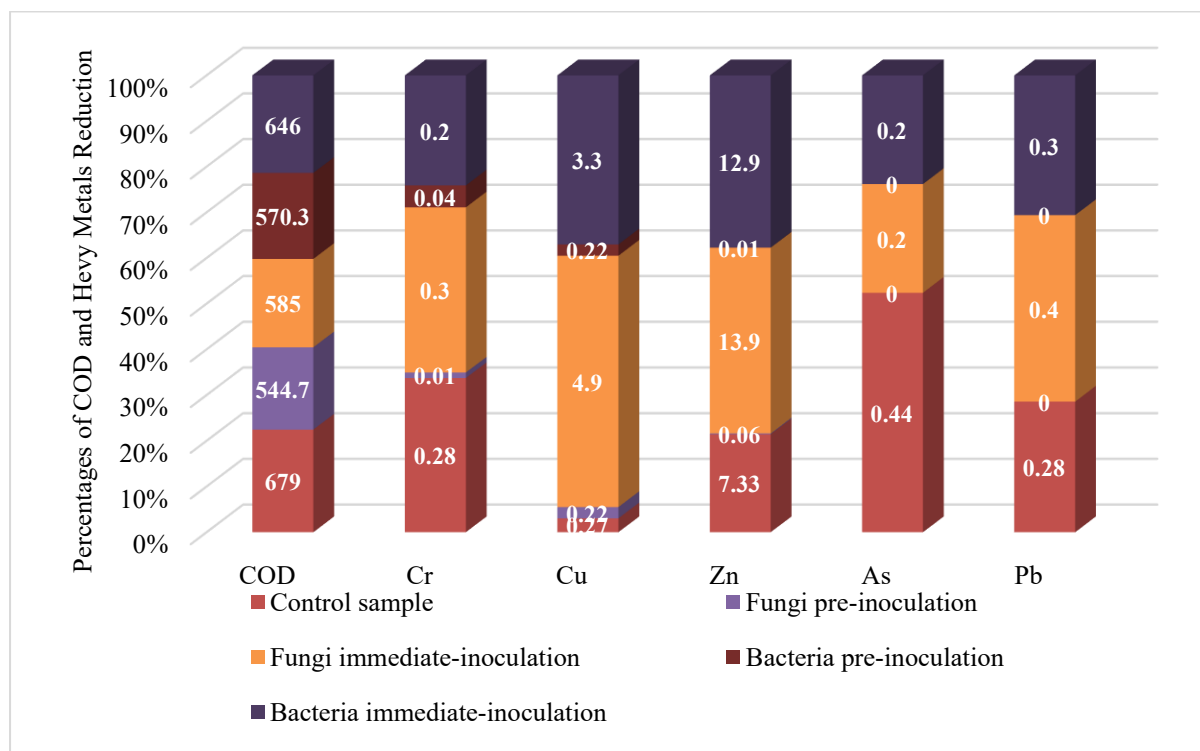


Figure 13. Comparison of Percentage Reduction for COD and Heavy Metals in 28 Days

The stabilization performance of pre-inoculation and immediate inoculation S/S treatments is presented in Table 9, where COD, Cu, and Zn were identified as the most influential contaminants based on statistical significance analysis. Focusing on these key parameters provides a clearer understanding of treatment effectiveness. Consistent with the percentage-based reduction analysis, pre-inoculation contributed a larger share of the reductions in COD and heavy metals than immediate inoculation, particularly for COD, Cu, and Zn. The higher percentage reductions observed in pre-inoculated systems confirm that microbial pre-conditioning enhances the efficiency of contaminant stabilization, supporting its effectiveness as a superior S/S treatment strategy.

Table 9. COD, Cu, and Zn levels for immediate inoculation and pre-inoculation (14 and 28 days)

Treatment	14 days			28 days		
	COD (mg/L)	Cu (mg/L)	Zn (mg/L)	COD (mg/L)	Cu (mg/L)	Zn (mg/L)
Fungi pre-inoculation	579	0.19	0.12	544.7	0.22	0.06
Fungi's immediate inoculation	535	4.90	14.30	585	4.90	13.90
Bacteria pre-inoculation	579.3	0.24	0.10	570.3	0.22	0.01
Bacteria's immediate inoculation	634	3.10	13.20	646	3.30	12.90
Control samples	653	0.58	9.11	679	0.27	7.33

COD reduction at 28 days was more pronounced in pre-inoculated samples (fungi: 544.7 mg/L; bacteria: 570.3 mg/L) than in immediate-inoculated samples (fungi: 579.0 mg/L; bacteria: 579.3 mg/L), suggesting that allowing microbial populations to establish prior to treatment accelerates organic matter degradation. Heavy metal immobilization, particularly for Cu and Zn, was markedly improved under pre-inoculation at 28 days, with Cu concentrations

as low as 0.22 mg/L for fungi and bacteria, Zn concentrations were reduced to 0.06 mg/L in fungi and 0.01 mg/L in bacteria under pre-inoculation, whereas under immediate inoculation, Cu and Zn levels remained substantially higher (Cu: fungi 4.90 mg/L, bacteria 3.30 mg/L; Zn: fungi 13.90 mg/L, bacteria 12.90 mg/L). Control samples consistently exhibited the highest COD and intermediate metal levels, highlighting the contribution of microbial inoculation to both organic matter breakdown and metal stabilization. These results highlight the practical advantage of pre-inoculation for sludge remediation, as both organic and inorganic contaminants can be effectively stabilized prior to disposal or reuse.

Pre-inoculation treatment in the S/S method developed in this study demonstrates significant improvements over prior fungal bioaugmentation studies. As shown in Table 10, previous studies have investigated fungal strategies for soil remediation and heavy metal removal, and filamentous fungal consortia applied to landfill and contaminated soils exploit synergistic interactions to enhance pollutant degradation and metal immobilization [34, 35]. Indigenous soil fungi offer a sustainable remediation approach with minimal ecological impact [36]. This non-living fungal biomass and liquid-phase treatments have also been used to remove heavy metals via extracellular binding.

These studies predominantly relied on simultaneous inoculation or non-S/S matrices, which can delay microbial adaptation and reduce stabilization efficiency. In contrast, the pre-inoculation approach in this study allowed microbial communities, including *Aspergillus brasiliensis* and *Sphingobacterium spiritivorum*, to establish metabolic activity before exposure to the sludge matrices. This led to accelerated COD reduction and markedly lower residual concentrations of Cu and Zn. To our knowledge, this is the first study to integrate pre-inoculated microbes into S/S matrices for simultaneous organic and heavy-metal stabilization, emphasizing both the novelty and industrial scalability of the approach, as shown in Table 10.

Table 10. Comparison of previous fungal bioaugmentation studies and the present pre-inoculation S/S approach.

Study	Fungi	Method Approach	Key Findings	Novelty
Hassan <i>et al.</i> , 2020 [34]	Filamentous fungal consortia	Simultaneous inoculation	Metal removal (Cu ~52%) over long-term incubation	Did not integrate fungi into S/S; slower adaptation
Hassan <i>et al.</i> , 2020 [35]	Fungal consortia	Simultaneous inoculation	Enhanced metal removal vs. control	Soil-based; no pre-inoculation; not S/S
Dusengemungu <i>et al.</i> , 2022 [36]	Indigenous fungi (<i>Aspergillus</i> , <i>Cladosporium</i> , <i>Geotrichum</i>)	Soil bioaugmentation	High Zn removal; metal tolerance	Soil system; not cemented; no pre-inoculation
Current study (2025)	<i>Aspergillus brasiliensis</i> + <i>Sphingobacterium spiritivorum</i>	Pre-inoculation	High reductions of COD and heavy metals	First to integrate pre-inoculated microbes into S/S matrices

From a practical perspective, these findings have clear implications for industrial waste treatment. Pre-inoculation could be integrated into S/S processes at water treatment plants, industrial sludge management facilities, or contaminated site remediation projects. By establishing active microbial populations beforehand, treatment efficiency can be improved, potentially reducing curing times and lowering the residual toxicity of treated sludge.

Furthermore, the methodology can be scaled to larger systems by pre-culturing microbial inocula in controlled bioreactors before mixing with bulk sludge, offering a feasible route for industrial-scale application.

Overall, this study not only demonstrates the technical benefits of pre-inoculation over immediate inoculation but also provides a framework for applying microbial S/S strategies in real-world sludge remediation scenarios. Future work should explore optimization of inoculum size, curing conditions, and microbial consortia combinations to maximize treatment efficiency across diverse sludge types and heavy metal compositions.

5. CONCLUSIONS

According to the TOC analysis, the sludge contained 23.39% organic carbon, indicating a high organic content in the fibreboard sludge. The heavy metals present in both the sludge and Portland cement included Cr, Cu, Zn, As, and Pb, all of which exceeded USEPA standards. After 28 days, the control sample and the pre-inoculated bacteria and fungi showed lower COD concentrations than the immediate-inoculated samples. When comparing the COD concentrations of bacteria and fungi, the fungi with pre-inoculation exhibited the lowest COD concentration at 544.7 mg/L after 28 days, while bacteria with pre-inoculation had a concentration of 570.3 mg/L after the same period. Additionally, based on the heavy metal analysis, the pre-inoculation samples showed a greater reduction in heavy metal concentrations than the immediate-inoculation samples. Specifically, the pre-inoculation with fungi had the most substantial impact on lowering heavy metal concentrations, achieving levels of 0.01 mg/L for Cr, 0.22 mg/L for Cu, 0.06 mg/L for Zn, and 0 mg/L for both As and Pb after 28 days. Statistically proven that pre-inoculation is better than immediate-inoculation with a significant p-value of 0.0001 for COD, Cu, and Zn. The results from the pre-inoculation method were particularly noteworthy. However, there is no significant difference between the two microbes used in this study. Pre-inoculation with a co-culture of bacteria and fungi is recommended to further enhance the solid-state treatment method, providing a promising approach for industrial sludge management. Future research is required to evaluate scalability for practical and sustainable waste treatment applications.

ACKNOWLEDGEMENT

This study was supported by Universiti Tun Hussein Onn Malaysia (UTHM) through GPPS & Publisher's Office (vot Q776 and E15216).

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