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Assessing the performance of horizontally flowing subsurface wetland equipped with *Vetiveria zizanioides* for the treatment of acid mine drainage

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ABSTRACT

Contamination of different ecological spheres with acid mine drainage (AMD) has raised numerous concerns in countries with well-developed mining industries, thus calling for urgent intervention measures to redress the prevailing water pollution challenges. Due to its chemical composition and (eco)-toxicological nature, AMD can pose severe environmental damage if not properly managed. Herein, the performance of subsurface horizontally flow constructed wetland (SSHF-CW) equipped with *Vetiveria zizanioides* for the treatment of AMD was explicitly assessed. To fulfill the goals of this phytoremediation study, the experiments were administered for a period of 30 days using authentic AMD from an active gold mine; and the quality of the feed and product water was monitored daily. The results showed a slight increase in pH from 2.4 to 4.01 and a net reduction in electrical conductivity, total dissolved solids, and sulphate, registering ≥ 47.20%, ≥ 46.00%, and ≥ 33.04%, respectively. Thenceforth, there was a net removal of metal in the following order; Zn (77.75%) ≥ Fe (75.36%) ≥ Mn (67.48%) ≥ Al (55.05%) ≥ Ni (44.01%) ≥ Cu (11.36%). Interestingly, the obtained results demonstrated that *Vetiveria zizanioides* was tolerant to AMD with a tolerance index of 1.23 after 30 days, while the removed metals were partitioned amongst the substrate, plant matrices, and external factors. Chemical species accumulated by the plants were more concentrated in the roots except for Mn, which was more concentrated in the shoots. The X-ray fluorescence and X-ray diffractometers analyses revealed the presence of chemical species in the substrate, while

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Fourier transform infrared and scanning electron microscopy-energy dispersive spectroscopy analysis revealed the presence of chemical species in plants roots, confirming that substrate and plants play a huge role in pollutants removal. As such, it can be concluded that SSHF-CW equipped with *Vetiveria zizanioides* plays a major role in the removal of contaminants from AMD and could be employed in derelict mines or small operations as a passive treatment technique to phyto-remediate mine effluents.

1. Introduction

Acid and metalliferous drainage, aka acid rock drainage (ARD) or acid mine drainage (AMD), originates as a by-product of minerals mining; however, it can also occur naturally in the environment as part of the rock weathering process [1]. When sulphide ores such as pyrite, arsenopyrite, and marcasite among others are in contact with oxygen and water, they get oxidised hence releasing a leachate that is rich in dissolved chemicals. Specifically, the product water is rich in sulphuric acid, high electrical conductivity (EC), and elevated levels of dissolved chemical species. The high level of dissolved chemical species may be attributed to the acidic nature of AMD that promotes the leaching of chemical species from the surrounding geological stratas and seams [2]. According to the literature, AMD comprises of heavy metals, aluminium, iron, manganese, arsenic, sulphates, oxyanions, metalloids, rare-earth metals, and radionuclides. Due to its hazardous and toxic physico-chemical characteristics, AMD has deteriorating consequences on the receiving environment and human health on discharge and exposure [3,4]. As such, AMD is therefore seen as the most dangerous environmental hazard that is derived from mining activities, and it can remain active for an extended period of time after the mine closure [5,6]. Specifically, AMD pollutes surface and ground water resources in countries with well-developed and poorly-developed mining industries [1,7]. This has been perceived as a catastrophic threat to the public health specifically due to the embodied chemical species [8]. For instance, the study by Coetzee et al. [7] revealed the presence of radioactive substances in AMD from the Witwatersrand basin in South Africa. The presence of such substances increases the risk of cancer in humans and other living organisms according to toxicity tests [4,5]. Regulatory requirements, such as those by the World Health Organization (WHO), South African National Standard (SANS), United States Environmental Protection Agency (USEPA), Department of Environmental Affairs (DEA), and Department of Water and Sanitation (DWS) standards, require AMD to be treated before being discharged into the receiving environments. The main quest is to protect the environment for the benefit of current and future generations. Furthermore, as a trade-off, the treatment of AMD will play a pivotal role in curtailing water scarcity and aid in the reclamation of contaminated water resources [9]. Thenceforth, another added value can be associated with the potential of the recovery of precious metals and chemicals that have myriads of industrial applications [10]. Currently, there are two types of technologies that have been widely explored for the treatment of AMD; i.e., (i) active and (ii) passive treatments [11,12]. The active method entails injecting energy and chemicals to enhance the treatment process, whereas the passive treatment relies on natural energy. Moreover, the active treatment process includes attenuations of chemical species using different techniques and reagents such as lime [13], magnesite [14], sodium sulfide [11], soda ash [15], ion-exchange [16], adsorption, bio-(phyto)-remediation, filtration [17], and electrodialysis [18]. The active approach requires continuous input of resources such as chemicals, energy, and also skilled staff. Contrarily, passive methods treat the effluent by auto adjusting to a self-operating system that does not require constant monitoring and chemical and energy inputs [19]. The widely employed passive treatment processes include lime drains [20], permeable reactive barriers [21], cascaded reactors for aeration [22], biosorption [23], and phytoremediation [24]. Furthermore, passive treatment systems also use organic matter to neutralise AMD, thereby promoting the precipitation of metals and the reduction of sulphate [25]. However, passive treatment technologies cannot be considered as a long-term solution to AMD treatment because they are not efficient in treating highly concentrated AMD effluent specifically due to numerous technical problems. However, they are mainly used in abandoned mines or as a polishing step. Albeit, with finer refinements, phytoremediation has evolved as the most promising technique for the treatment of AMD, and it has been widely explored. This process is mainly governed by the affinity of plants to certain chemical species and a variety of processes such as extraction (absorption) [26], translocation [27], and phyto-stabilization [28] among others. Furthermore, different plant species have been employed in phytoremediation techniques, including *Vetiveria zizanioides* [29,30], *Phragmites australis* [31], *Eichhornia crassipes* [32], *Nauclea orientalis* [33], *Ipomea aquatica* [34], and *Pistia stratiotes* [35] and their performances also varied significantly. Due to its unique tolerance and bioaccumulation properties, *Vetiveria zizanioides* has gained attention in recent decades. Gwenzi et al. [36] assessed the performance of a sequential combination of coal ash-based adsorbents and phytoremediation using *Vetiveria zizanioides* to remove trace metals from AMD. The authors reported that this technology increased the potential of *Vetiveria zizanioides* in metal removal. Kiiskila et al. [24] designed a floating treatment wetland for remediating AMD-impacted water planted with *Vetiveria zizanioides.* The authors reported increasing pH, metal accumulation, and significant sulphate (SO_4^{2-}) removal. Furthermore, *Vetiveria zizanioides* showed tolerance with minimal change in biomass and plant growth. Kiiskila et al. [30] conducted a study to investigate the metabolic response of *Vetiveria zizanioides* to AMD; their findings revealed that *Vetiveria zizanioides* were capable of tolerating and treating AMD by increasing the pH and reducing metal and SO_4^{2-} concentrations. The Vetiver system technique has been widely explored in the remediation of AMD water, but there is limited information on the use of combining agricultural substrate and *Vetiveria zizanioides* for the treatment of AMD. Most studies used the floating wetland with the plants suspended or a hybrid of adsorption and phytoremediation but relied on nominal irrigation techniques. Furthermore, there is deficiency in terms of the effect of flowing modalities on the attenuation of chemical species from AMD using *Vetiveria zizanioides*. To the best of our knowledge, constructed wetlands with horizontal flow equipped with *Vetiveria zizanioides* have never been evaluated and employed for the treatment of AMD. This will then be the first study in design and execution to explore the use of *Vetiveria zizanioides* for AMD treatment on subsurface horizontally flowing constructed wetland (SSHF-CW). This will also highlight the fate of chemical species post the treatment process and their partitioning or translocation to different parts of the system soil (substrate) and plants matrices. The SSHF-CW is a simple, cost-effective, easy to operate, and environmentally friendly technology that can prove to be effective for the treatment of AMD [37,38]. Depending on the researcher and climatic conditions, the nature of the substrate varies, and it can either be sand, gravel, soil, root, or rhizome of aquatic plants [40,25]. Henceforth, the constructed wetland technology is widely used in wastewater treatment [40]. Like other constructed wetlands, the SSHF-CW constitutes a complex system with many different components working simultaneously to improve the performance of the system. The ultimate goal of this study is to investigate the performance of SSHF-CW in the treatment of AMD. Insights into the contribution of *Vetiveria Zizanioides*, substrate, and external factors in chemical species attenuation will also be reported.

2. Materials and methods

2.1. Acquisition of reagents, plants, and substrate

All reagents used in this study were of analytical grade (AG) and obtained from Merck, South Africa. In order to construct the SSHF-CW, shoots of *Vetiveria zizanioides* were purchased from Nandadram Ecovillage farm (Pty) Ltd in Kwa-Zulu Natal and transported to the University of South Africa, Sciences campus in Florida, Johannesburg, South Africa. The obtained soil compost which was acquired from the plant-based residue was used as wetland substrate and was purchased from Garden World, Johannesburg Proprietary Limited (Pty Ltd).

2.2 .Growth of plants

To carry out the experiments, shoots of *Vetiveria Zizanioides* were immersed under potable fresh water for approximately three weeks to allow the germination of new seeds. Following the germination of new seeds, the seedlings were transferred into small pots containing compost soil. No chemical fertilizer was added to the plants, and they were allowed to grow naturally; the plants were monitored and watered daily.

2.3. Sampling and characterization of AMD water

The field AMD water used in this study was collected from the Sibanye Gold mine, an active mine in Krugersdorp, Gauteng, South Africa (26.0963°S and 27.8077°E). During the AMD sampling, some parameters were determined *in situ* using a multi-parameter probe (HANNA instruments, Johannesburg, RSA). These included pH, total dissolved solids (TDS), and electrical conductivity (EC). The initial concentration of the metals (Al, Cu, Fe, Mn, Ni and Zn) in the collected AMD was analysed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), whereas the initial sulphate (SO_4^{2-}) concentration was analysed using Ion chromatography (IC). The parameters were analysed following the "Standard Methods of the Examination of Water and Wastewater" [41].

2.3.1. Quality assurance and quality control (QA/QC)

In this study project, QA/QC was applied to ensure reliable results. The experiment was conducted in triplicates to determine the reproducibility of the results. Thenceforth, the analyses were also done in triplicates, and the data reported as the mean value. The results were considered acceptable when the difference among the triplicate was less than 10%. The values that were below the detection limit (BDL) was determined by referring to USEPA guidelines for data analyses [42], while the accuracy of the analysis was monitored following the guidelines of the National Institute of Standards and Technology (NIST) for water quality standards.

2.4. Experimental setup

2.4.1. Horizontally flowing wetland design and description

Two experimental pilot plants consisting of two reservoirs tank of 1000 liters capacity each containing AMD and fresh water for control were set up. Two small round plastic basins with a 100 liters capacity were used as the wetland cells (treatment and control). The diameter of each basin measured d = 62cm, and the height was h = 45 cm (Figure 1). Young *Vetiveria zizanioides* (section 2.2) were transferred from the growing pots into the two basins containing the organic matter (substrate). The two basins containing 20 plants (0.00662 plant per square centimeter or 66.2 plants per square meter on a large scale) were connected to the reservoir tank via a pipe to create two wetlands (one for treatment and one for control – Figure 1). A plastic drip was incorporated at the bottom of each tank to regulate the flow rate of water (AMD water for the treatment wetland and fresh water for the control wetland) from the tank to the wetland cell. The water (AMD water and fresh water for control) flowed in continuous drops from the tanks to the wetland cell for the duration of the experiment. The water entered the plant wetland system via an inlet zone and flowed horizontally under the surface of the substrate until it reached the outlet zone, where leachates were collected. The water column inside the wetland was maintained by blocking the outlet at the beginning of the experiment, thereby allowing the water to reach the desired height and followed by the regulation of the outflow rate of the water to the same flow rate as the inflow.

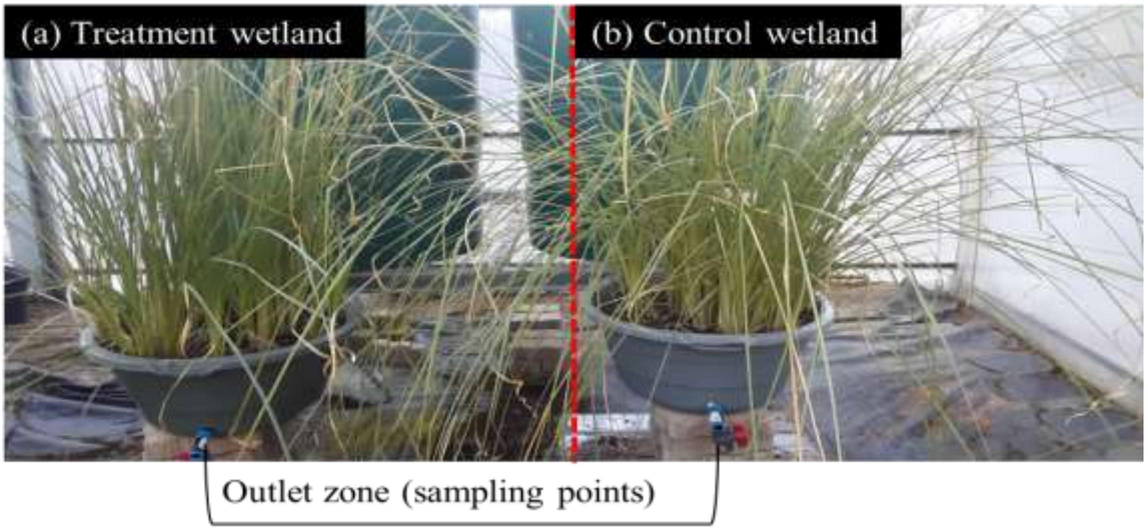

Fig. 1. Experimental set up of the SSHF-CW system, treatment (a) and control (b).

2.4.2. Pre-treatment of AMD water and substrate characterization

Prior to the experimental phase, the AMD water was filtered to remove the total suspended solid (TSS) susceptible to clogging the pipe. The elemental composition and mineralogical composition of substrate were determined. The elemental composition was done before and after the experiment using X-ray fluorescence (XRF). The analyses were performed using a Thermo Fisher ARL-9400 XP+ Sequential XRF with winXRF Software. The mineralogical composition was done using X-ray diffraction (XRD), and analyses were performed using a Philip PW 1710 diffractometer equipped with graphite secondary monochromatic.

2.4.3. Hydrology of the system

The system was calibrated using a plastic drip, which allowed maintaining a very low flow rate and allowed the wastewater to flow into the system in continuous drops for 30 days. The average inflow and outflow (Qav) were determined according to Equation 1.

$$\mathrm{Qav} = \frac{\mathrm{Qin} + \mathrm{Qout}}{2} \quad (1)$$

Where, Q_{in} is the average inflow of wastewater into the system, Q_{out} is the average outflow of wastewater from the system, and Q_{av} is the average flow rate of wastewater through the system (m^3/d). The calculated Q_{av} was 0.0012 m^3 or 1.2 liters. The hydraulic retention time (HRT) was determined using Darcy's law as given in Equation 2 [43].

$$\mathrm{HRT} = \frac{\mathrm{As} \times \mathrm{d} \times \mathrm{n}}{\mathrm{Qav}} \quad (2)$$

Where, As = 3.14 × $(0.31)^2$ is the surface area of the wetland (m^2), d = 0.2 is the water depth in the wetland (m), n = 60 is the porosity of the wetland substrate (%), Q_{av} = 0.0012 is the average flow through the wetland (m^3/d), and HRT is the hydraulic retention time (days).

$$\mathrm{HRT} = \frac{3.14 \times (\mathrm{r})^2\ \mathrm{d} \times \mathrm{n}}{\mathrm{Qav}}$$

$$\mathrm{HRT} = 30.17 \text{ days}$$

The determined HRT was 30.17 days. During that period, the hydraulic system of each pilot plant was monitored daily to ensure a steady flow of water within the system. At the end of the experiment (just after the 30th day), the plants were harvested from both wetlands (treatment cell and control) for metal analysis.

2.4.4. Product water and soil sampling

Water samples (experiment and control) and substrate (soil samples) were collected daily from each wetland from Day 1 to the last day of the experiment (Day 30). The substrate samples were collected from the bottom of each wetland (Figure 1). Water samples were collected into amber glass bottles of 500 mL. After collection, the samples were divided into two sub-samples of 400 mL and 100 mL. The sub-samples of 400 mL were used for the in-situ analysis of pH, TDS, and EC, while the 100 mL subsamples were used for the analysis of exsitu parameters, which included metals (Al, Cu, Fe, Mn, Ni, Zn) and SO_4^{2-}.

2.4.5. Analytical methods

The product water from both wetlands (control and treatment cell) was analysed following the standard methods for examining water and wastewater [41]. The EC, pH, and TDS were determined *in situ*, as mentioned in section 2.3, and the manufacturer's manual was followed with reference to the standard methods for examining water and wastewater [41]. Prior to analysis for metal concentrations, the water samples were filtered using a 0.22 μm pore syringe filter membrane to remove particles, followed by preservation with two drops of HNO_3 concentrated to prevent aging and immediate precipitation of metals. The filtrates were refrigerated at 4 °C prior to analysis by ICP-OES using an Agilent 5100 ICP-OES System (Agilent Technologies Inc., Santa Clara, CA, USA). The sulphate (SO_4^{2-}) was analysed using ion chromatography (IC). The soil samples were air dried for five days, sieved, and one gram (1g) was digested in a mixture of 5 mL of 55% nitric acid (HNO_3) and 2 mL of 30% hydrogen peroxide (H_2O_2) using microwave digestion (Shanghai Sineo Microwave Chemistry Technology Ltd, China) [44]. Once the digested samples were cooled to room temperature, the solutions were filtered through a 0.22 μm pore syringe filter membrane and analysed for metal concentrations by Agilent 5100 (ICP-OES) with a detection limit of 0.0001 mg/L and a margin error of 10%.

2.5. Treatment efficiency of subsurface horizontal flow-constructed wetland (SSHF-CW)

2.5.1. Efficiency of SSHF-CW on pH increment

The pH was measured daily, and the increment was calculated after every five-day period. The pH increment (I) was determined as illustrated in Equation 3 [37].

$$\mathrm{I} = \mathrm{pHf} - \mathrm{pHi} \tag{3}$$

Where, *pHf* is the final pH value after 30 days retention time, *pHi* is the pH value of the feed AMD water, and *I* is the increment of pH after 30 days retention time.

2.5.2. Efficiency of SSHF-CW in metals and sulphate removal

The treatment efficiency of SSHF-CW in metals and SO_4^{2-} removal was gradually determined during the duration of the experiment. The metals and SO_4^{2-} concentration were analysed daily, and the removal efficiency (RE) of each of the parameters of concern was calculated after every five-day period using Equation 4 [45].

$$RE = \frac{\mathrm{Ci} - \mathrm{Cf}}{\mathrm{Ci}} \times 100 \tag{4}$$

Where, *Ci* is the initial concentration of each parameter in the AMD water, *Cf* is the final concentration of each parameter after 30 days retention time, and *RE* is the removal efficiency of each parameter after 30 days retention time (in percentage).

2.6. Plant harvesting

Plants from each wetland (treatment cell and control cell) were harvested without damaging the roots and rinsed separately with distilled water to remove dust, soil, and mineral particles. The plants were air dried at 25°C for one week and weighed to determine the tolerance index using Equation 5. The tolerance index (TI) is a phytoremediation factor that allows for evaluating plant sensitivity to pollutants [46]. TI is the ratio between a variable measured in the treated plant and that in the control plant.

$$\text{Tolerance index (TI)} = \frac{\text{biomass of plant growing in AMD cell}}{\text{Biomass of plants growing in potable fresh water cell}} \tag{5}$$

2.6.1. Digestion of plants and metal content analysis

The plants were separated into roots and shoots, and each portion of the plants was ground using a mortar and pestle. One gram of each portion was added to a mixture solution of 10 mL of 65% HNO_3 and 4 mL of 35% H_2O_2, followed by digestion using a microwave digester [44]. The digestate samples were then filtered using a 0.22 μm pore syringe filter membrane, followed by metal analysis using ICP-OES. The metals content in the plants allowed the researchers to determine the bio-concentration factors (BCF) and the contribution of the substrate, plants, and external factors in metal removal. The BCF of each metal was calculated as illustrated in Equation 6 to determine the quantity of the metals that moved from the wetland to the plant [47].

$$\mathrm{BCF} = \frac{\text{Metal concentration in plant tissue}}{\text{Final concentration of metals in sediment}} \quad (6)$$

2.6.2. *Functional group and morphological properties of the Vetiveria zizanioides roots*

The *Vetiveria zizanioides* roots were ground, and the functional groups were determined using a Perkin-Elmer Spectrum 100 Fourier Transform Infrared Spectrometer (FTIR) equipped with a Perkin-Elmer Precisely Universal Attenuated Total Reflectance (ATR) sampling accessory with a diamond crystal. The morphological properties and spot analysis were determined using SEM-EDS. The grounded root samples were mounted on carbon tape that was attached to an Al ends. The samples were viewed in a JEOL 5400 LV SEM with an attached KEVEX electron detector (JEOL, Tokyo, Japan). They were viewed in low vacuum mode, and the metals were detected with a Sigma EDS spectrometer.

2.6.3. *Partitioning of chemical species between substrate, plant and external factors*

The metal content in the substrate was determined before and after the experiment. The results together with the results of the metals content in the plant and product water allowed the researchers to estimate the content of metal retained in the wetland as given in Equation 7. The substrate's contribution in overall metal removal is given in Equation 8; the contribution of external factors in metal removal is shown in Equation 9.

$$\mathrm{Mw} = \mathrm{X} - \mathrm{Y} \quad (7)$$

Where, X (mg/L) is the initial concentration of metal in AMD water,

Y (mg/L) is the final metal content in AMD water, and Mw (mg/L) is the metal content retained in the wetland.

$$\mathrm{Ms} = \mathrm{Mz} - \mathrm{Mt} \quad (8)$$

Where, Mt (mg/L) is the initial content of metal in substrate, and

Mz (mg/L) is the final metal content in substrate, and Ms (mg/L) is the substrate contribution in overall metals removal.

$$\mathrm{ExF = Mw\text{-}\ (Ms+Mp)} \quad (9)$$

Where, ExF (mg/L) is the external factors contribution in overall metals removal.

3. Results and discussion

The results of this study will be discussed in the categories of aqueous samples and solid sample studies.

3.1. *The effect of SSHF-CW in 30 days retention time on final pH*

The effect of SSHF-CW on pH in 30 days of retention time was evaluated, and the results are shown in Figure 2.

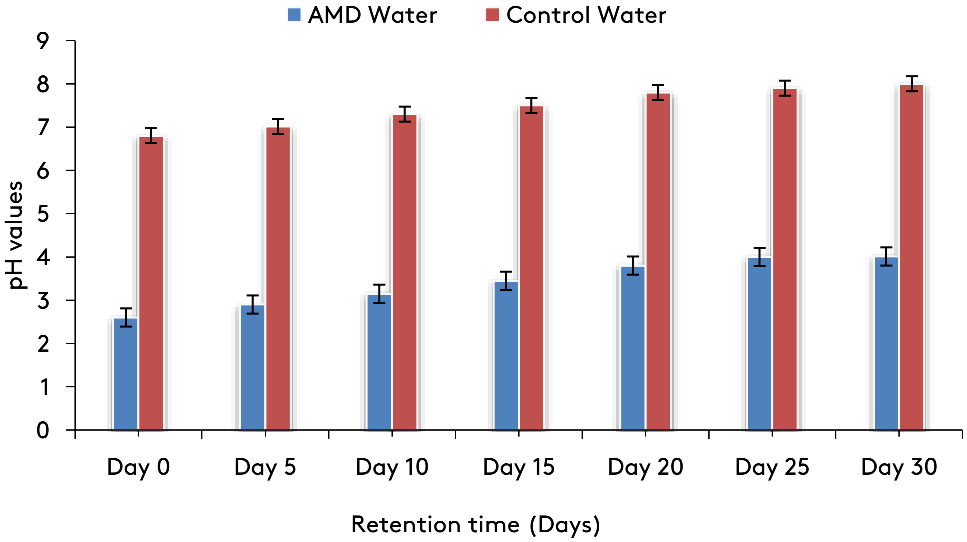

Fig. 2. The effect of SSHF-CW on treated AMD pH in 30 days under an everage flow rate of 1.2 liters/day.

As shown in Figure 2, the pH of the solution increased from 2.6 to 4.01 and 6.8 to 8.01 for the experiment and control, respectively. An increase in pH is explained by the biochemical processes occurring in the substrate. For their growth, plants release acid to mineralize organic compounds and enhance nutrient absorption leading to increased pH [48]. An increase in pH accelerates the precipitation of metal and reduction of hydrogen ions, thereby reducing the acidity of AMD water. In addition, some cations released from the substrate, used as growing media, also contribute to the reduction of ion hydrogen (H^+) activity [49]. Moreover, this may perhaps contribute to the increase of pH of product water after 30 days of retention time. Furthermore, a slight increase was observed for both the experimental and control cells but at different incremental values. An increase in pH may also be attributed to the SO_4^{2-} reduction in the treated AMD water since the contact between SO_4^{2-} and wetland substrate can promote the growth of sulphate reducing bacteria (SRB), which leads to an increase in the alkalinity of the medium thereby raising the pH of the water [49]. The findings are consistent with previous studies conducted by Lizama Allende et al. [39] with different media and plants. In the wetland environment, plants play an important role in the fluctuation of pH. In fact, during photosynthesis, aquatic plants absorb carbon dioxide (CO_2) from water, and this can raise the pH of the water due to the attenuation of carbonates that forms carbonic acid [50].

3.2. The effect of SSHF-CW on electrical conductivity (EC)

The effect of SSHF-CW on EC in 30 days of the retention time is illustrated in Figure 3.

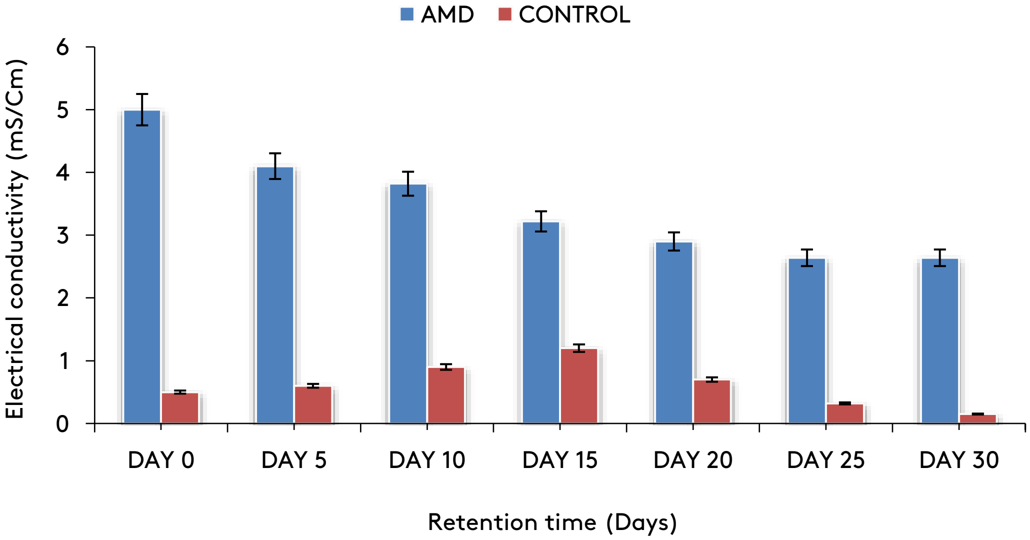

Fig. 3. The effect of SSHF-CW on electrical conductivity in 30 days under an average flow rate of 1.2 liters/day.

As shown in Figure 3, the EC decreases from 5 mS/cm to 2.64 mS/cm within a period of 30 days; whereas in the control wetland, EC remains constant (0.5 mS/cm) for the first five days and slowly increases up to 1.5 mS/cm on the 10th day and decreases from 1.5 mS/cm to 0.15 mS/cm on day 30. This reduction of EC in treatment wetland may be the consequence of chemical species reduction from AMD, leading to reduced TDS and EC due to less metal (dissolved) [51]. An increase in EC of the control wetland may be attributed to the presence of pollutants containing ions coming from the mixture of potable fresh water with the substrate. However, the effects of those pollutants were insignificant in the treatment wetland. The decrease from day 15 can be attributed to the sedimentation of pollutants in the substrate and accumulation by the plant.

3.3. The effect of SSHF-CW on total dissolved solid (TDS)

The effect of SSHF-CW on total dissolved solid (TDS) reduction is illustrated in Figure 4.

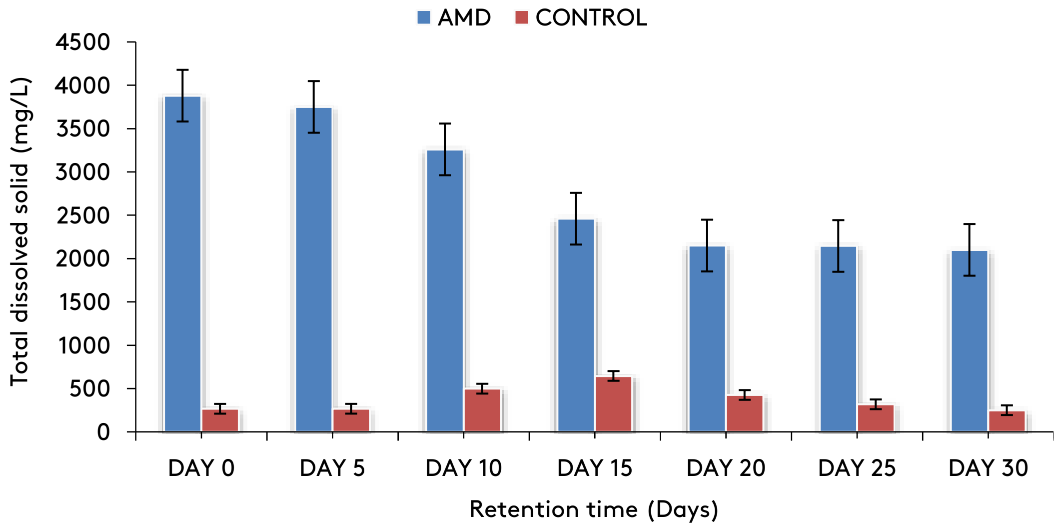

Fig. 4. Variation in total dissolved solids with variation in the retention time (days) under an average flow rate of 1.2 liters/day.

As clearly portrayed in Figure 4, after 30 days of retention time, the TDS of AMD is reduced from 3880 mg/L on day 0 to 2160 mg/L on day 20 and remained almost constant. In the meantime, the TDS of the freshwater (control cell) increased slowly from 266.5 mg/L to 845.08 mg/L on day 10 and decreased to 250.02 mg/L on day 30 (Figure 4). TDS is the concentration of all the combined inorganic substances present in a liquid in the dissolved form [52]. TDS is comprised of an inorganic salt such as calcium, magnesium, potassium, sodium, and SO_4^{2-}. The reduction of TDS in AMD treatment cell may be the consequence of pollutant reduction in AMD water. The reduction of TDS may also be attributed to the process of sedimentation/precipitation occurring within the wetland's substrate. In fact, there is always sedimentation within the wetland substrate, and this process allows some particles to settle down, thereby improving the efficiency of the wetland. In some cases, pollutants adhere to the particles of organic matter in suspension, thereby improving the efficiency of wetland [53]. The slight increase of TDS in the control wetland may be attributed to pollutants arising from the substrate but with insignificant effects on the treatment wetland. In fact, the water flows horizontally from the inlet to the outlet through the porous medium under the substrate, which initially contained a low concentration of pollutants. The decrease of TDS from day 15 may be attributed to the process of sedimentation occurring within the wetland substrate and pollutant uptake by the plants.

3.4. Effect of SSHF-CW on sulphate concentration

The effect of SSHF-CW on the removal of SO_4^{2-} from AMD is illustrated in Figure 5.

The findings revealed that SSHF-CW with *Vetiveria zizanioides* grass had the potential to reduce SO_4^{2-} concentration from 3137 mg/L to 2100.5 mg/L within 30 days HRT (Figure 5). The removal of SO_4^{2-} may be attributed to the following biochemical process. SO_4^{2-} is reduced biologically in an acidic medium, as illustrated in Equation 10.

$$\mathrm{SO_4^{2-}} + \mathrm{CH_3COOH} + 2\mathrm{H^+} \rightarrow \mathrm{HS^-} + 2\mathrm{HCO_3^-} + 3\mathrm{H^+} \tag{10}$$

This equation clearly shows that electrons are transferred from the acetic acid (energy source) to the electron acceptor (SO_4^{2-}) to form bisulphide (HS^-). Depending on the environmental conditions, hydrogen sulphide can be released as a gas and can be ionized to HS^- and S^{2-} or precipitates as a polysulfide, elemental sulphur, or metal sulphide [54]. This process is a key to metal removal in the subsurface wetland. For instance, the study of Chen et al. [55] revealed that Dissimilatory Sulfate Reducing Bacteria (DSRB) are only active in anaerobic conditions and are responsible for SO_4^{2-} reduction in subsurface constructed wetlands (SSCW) since SSCW provides anoxic zones where DSRB can be effective. Or in this study, SO_4^{2-}

removal is correlated with the removal of metals such as zinc, which may explain why SO_4^{2-} concentration decreases simultaneously with metal concentrations.

3.5. Effect of retention time on the removal of metals from AMD

Variation in the contents of metals removed as a function of the retention time is illustrated in Figure 6 (a, b).

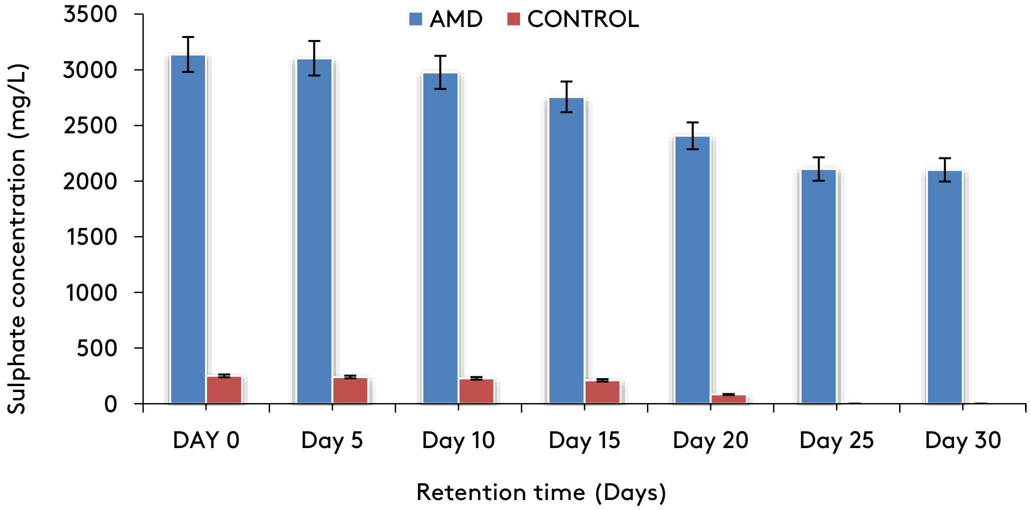

Fig. 5. Variation in the concentration of sulphate with varying retention times (days) under an average flow rate of 1.2 liters/day.

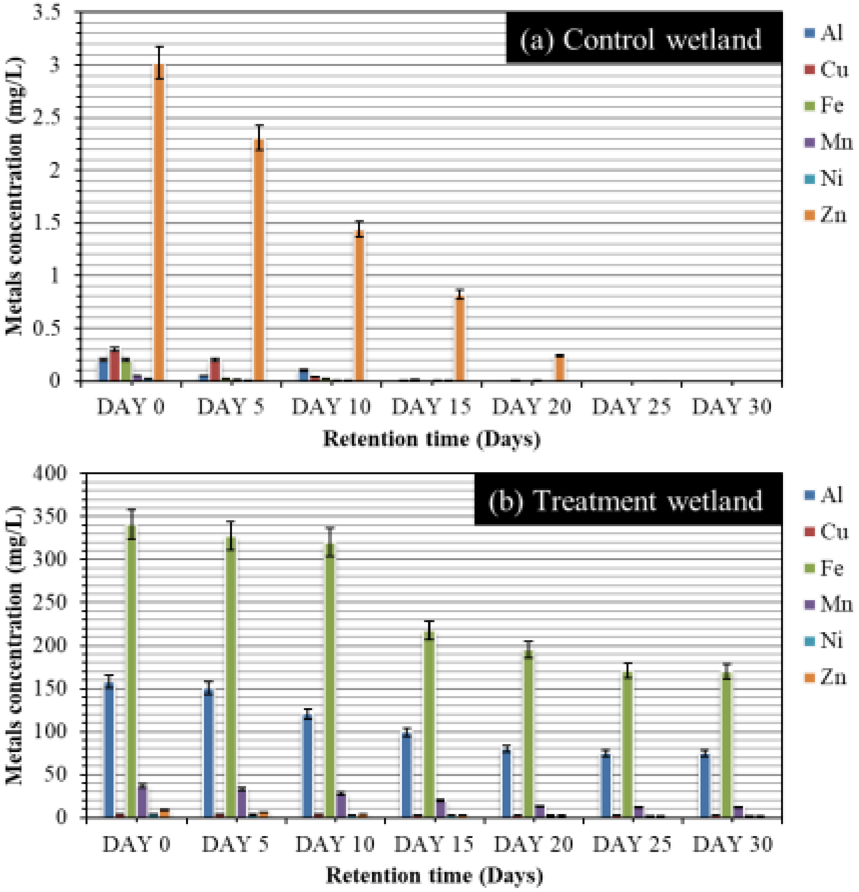

Fig. 6. (a and b). Variation of metals concentration in control wetland (a) and treatment wetland (b) in 30 days hydraulic retention time under an average flow rate of 1.2 liters/day.

The concentration of metals in the control (a) and experiment (b) wetlands are shown in Figure 6 (a,b). For the experiment wetlands (Figure 6b), the concentration of the metals decreased with an increase in retention time. Specifically, from the beginning to the end of the experiment, Fe decreased slightly from day 0 to day 10 (06.10% removal), significantly from day 10 to day 25 (46.63% removal), and remained almost constant until day 30, while Al decreased slowly from day 0 to day 25 and remained constant until day 30 whereas other metals decreased slightly from day 0 to day 20 and remained constant until day 30 (Figure 6b). In the control wetland (Figure 6a), the concentration of metals decreased slowly, and in the end, all metals were below the detection limit. The decreasing of metal concentrations in both wetlands was likely due to sedimentation, chemical transformation, filtration, external factors, and accumulation by the plants. In the control wetland, the decrease of the metals concentration until below the detection limit of 0.0001 mg/L can be explained by the low concentration of metals in the freshwater used as control liquid, continuous sedimentation, chemical transformation, plant uptake, and external factors. The metal removal in the SSHF-CW is influenced by various biological processes such as sedimentation, filtration, and plant uptake. In addition, metals are immobilized by the media and rhizomes through complexation or chelation, followed by accumulation by the plants [40,56]. Metals play a huge role in plant metabolism and homeostasis. For instance, Fe contributes to the synthesis of chlorophyll, and it is essential for the maintenance of the chloroplast structure [57]. Zn is one of the eight essential micronutrients needed by plants in small amounts but crucial to plant development; it plays an important role in a wide range of processes such as growth hormone and defense mechanisms in plants [58]. Ni deficiency affects plant growth, leading to the plant's senescence, reduces nitrogen metabolism and Fe accumulation, and plays a huge role in disease resistance [59]. Cu is an essential element of many proteins and enzymes involved in photosynthesis and the respiration process of the plant [60].

3.6. Variation of metals in the substrate

The metal concentration in the substrate was evaluated in mg/kg, and the results are illustrated in Figure 7 (a and b).

As shown in Figure 7a, the concentration of metal increases with the HRT, from Day 0 to day 10, followed by a decreasing plateau. The increasing phase can be attributed to the continuous load of metal in the wetland since it was a continuous flow experiment. The major part of metals concentration was retained in the substrate, thereby confirming the results reported in the finding of Chen et al. [61] and Le et al. [62]. They revealed that the substrate in SSHF-CW played an important role in metal removal by retaining them through the sedimentation process, thereby enhancing their accumulation by plants. The period of increase in metal concentration was followed by the decreasing phase, and this can be attributed to continuous sedimentation, chemical transformation, filtration, biological assimilation, accumulation by the plants, and external factors (evaporation, volatilization) [63] (Figure 7a). In the meantime, metal concentration in the substrate from the control wetland constantly decreased from the beginning to below the detection limit of 0.0001 mg/kg at the end of the retention time (Figure 7b); this may be attributed to the low initial concentration of metals in the potable fresh water, continuous sedimentation, chemical transformation, biological assimilation, accumulation by plants, and external factors contributing to the overall metal removal. Comparing the results from both wetlands (treatment and control), it follows that the substrate played a huge role in metal removal in the constructed wetland. In addition, to provide the growth medium for plants and microorganisms, the substrate offered hydraulic conditions for water flow and also removed pollutants directly by interception, sedimentation, adsorption, and precipitation [64].

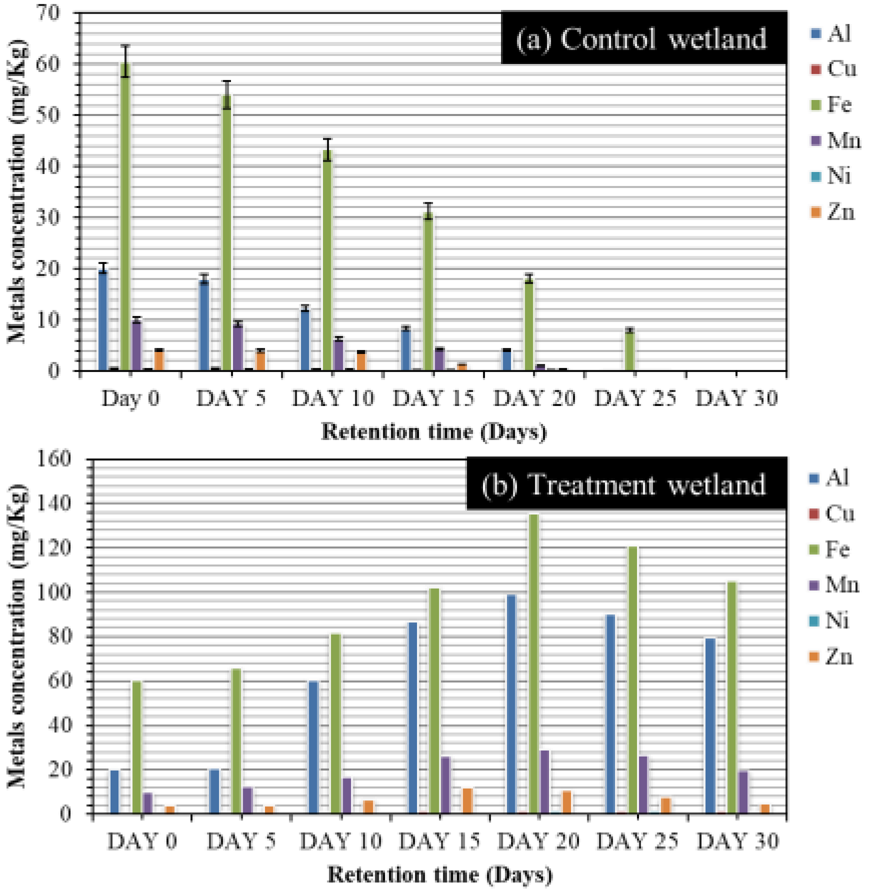

Fig. 7. (a and b). Variation of metals concentration in substrate: (c) experiment and (d) control in 30 days hydraulic retention time and under an average flow rate of 1.2 litres/day.

3.7. Efficiency of subsurface horizontal flow constructed wetland (SSHF-CW)

3.7.1. The efficiency of SSHF-CW in pH increment

The efficiency of SSHF-CW in increasing the pH of the AMD water was determined, and the result is illustrated in Table 1. From Table 1, it follows that after 30 days of HRT and under an average flow rate of 1.2 liters/day, the SSHF-CW increased the pH by as much as 1.4 units. The pH in the control cell was raised by as much as 1.2 units. However, the final pH value of the AMD water was out of the range of the water quality guidelines standard for effluent discharge as set by the DEA and DWS for effluent discharge.

Table 1. Performance of the SSHF-CW in raising the pH of AMD

Wetland	**pH**		
	Initial pH	Final pH	Increment
Treatment wetland	2.6	4.00	1.4
Control wetland	6.8	8.01	1.2

3.7.2. Efficiency of SSHF-CW on the removal of metals and sulphate

The removal efficiency of metals and sulphate by SSHF-CW is illustrated in Figure 8.

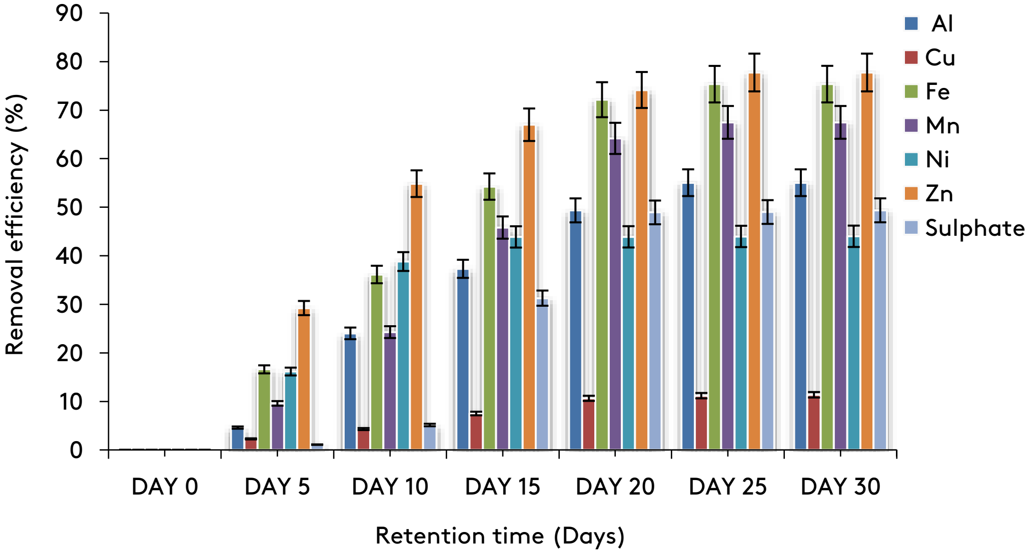

Fig. 8. Removal efficiency of metals and sulphate by SSHF-CW in 30 days hydraulic retention time and under an average flow rate of 1.2 liters/day.

As shown in Figure 8, the removal of the chemical species was in the following order: Zn (77.75%), > Fe (75.36%), > Mn (67.48%), > Al (55.05%),> $SO4^{2-}$ (49.36%), > Ni (44.01%), > Cu (11.36%). The higher removal efficiency of Zn, Fe, Mn, and Al was expected. Other studies by Vymazal [65] and Bakhsoodeh et al. [66] have shown that the removal efficiency of Zn is usually high in SSHF-CW. The high percentage of Zn (77.75%) removed may be attributed to the sequestration of Zn as zinc sulphide that was formed by combining with the sulphide generated by sulphate reducing bacteria in the wetland's substrate [67]. The removal efficiency of Fe (75.36%) in SSHF-CW is low compared to FWS-CW, where it is usually very high (above 90%). The removal of Fe can be attributed to two biochemical processes: (i) precipitation of Fe that occurs at a pH ≥ 3.5 and (ii) the activities of DSRB that reduces the SO_4^{2-} concentration and enhances the sequestration of Fe in the form of iron sulphide [68]. The 75.36% removal efficiency of Fe may also be attributed to its importance in plant metabolism since Fe deficiency in plants leads to common nutritional disorders. It is an essential micronutrient for almost all living organisms and plays a crucial role in the metabolic process such as DNA synthesis, respiration and photosynthesis [69]. Like Fe, the removal efficiency of Mn is low in SSHF-CW compared to FWS-CW; this may be attributed to the anaerobic status of SSHF-CW that retains Mn in a less soluble form (Mn^{4+}), thereby reducing its mobility in the system. The low Ni removal efficiency (44.01%) may be the result of Ni co-precipitation with Mn and Fe-oxyhydroxides since the co-precipitation of Ni with other metals delays the oxidation of Ni, thereby reducing its mobility in the solution [71]. Many processes such as sedimentation, complexation, and plant uptake participate in the metal removal in the wetland. The sedimentation process allows the metal to settle down; however, their accumulation by plants is selective since plants take up metal according to their metabolic needs and this justifies the different patterns observed in metal removal efficiency. SO_4^{2-} removal may be attributed to the plant needs in metabolism and the reduction of metal since metal reduction leads to reduction of sulphate salts such as $FeSO_4$, $ZnSO_4$, and $CuSO_4$ [72].

3.8. Tolerance index and bio-concentration factor

3.8.1. Tolerance index

The tolerance index was determined as described in section 2.5, and a TI of 1.23 was obtained for *Vetiveria zizanioides.* For this experiment, the TI value is 1.23 (> 1), which according to Kumar et al. [46], translates into a net increase in biomass and reveals that *Vetiveria zizanioides* has developed a tolerance; this may be justified by the fact that *Vetiveria zizanioides* grown in the AMD remained green and healthy throughout the experiment. The resistivity and tolerance of *Vetiveria zizanioides* to harsh conditions may be attributed to its genetic characteristics. In fact, *Vetiveria zizanioides* have a straight and stiff stem that allows it to withstand the high hydraulic loading rate of water [73. In addition, *Vetiveria zizanioide* grass, up to 2m high, can survive in a relatively deep-water flow. As a result, it can increase the retention time in a wetland, thereby improving the performance of the wetland [74].

3.8.2. Bio-concentration factor

In wetland, the BCF is the ratio between concentrations of a chemical compound in a plant to the chemical compound in the sediment [75]. A bio-concentration factor greater than 1 indicates the potential of the plant to uptake pollutants. On the contrary, a BCF lower than 1 (BCF < 1) indicates the inability of plants to accumulate pollutants. The BCF was calculated for all metals in the treatment wetland, and the results are reported in Figure 9. However, the BCF of metal could not be calculated in the control wetland since the final concentration of metal in the sediment was below the detection limit of 0.0001 mg/kg.

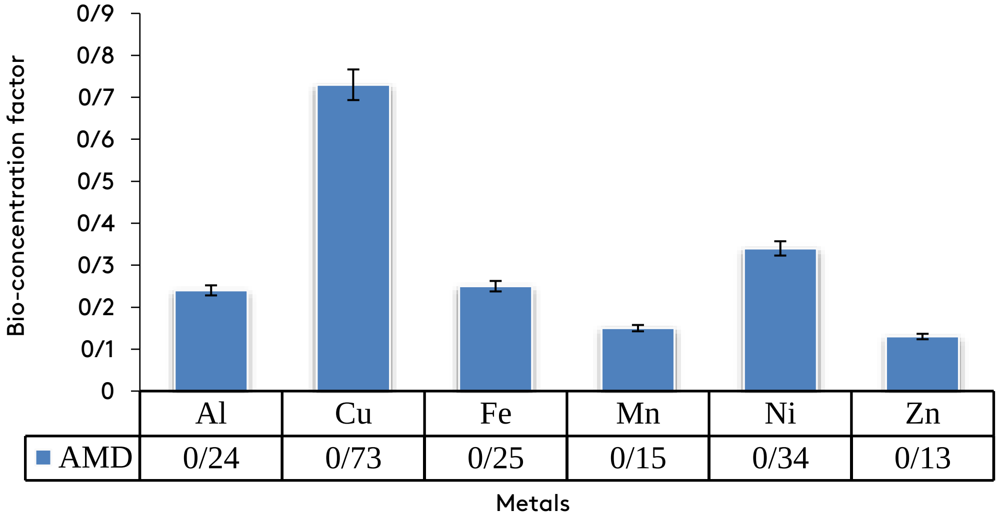

Fig. 9. Bio-concentration factor of metals.

As shown in Figure 9, the BCF for different elements (metals) varies significantly. Figure 9 depicts that in the treatment wetland, the BCF of all metals were lower than 1 (BCF < 1) and range in the following order: Cu (0.73) >Ni (0.34) >Fe (0.25) > Al (0.24) > Mn(0.15) > Zn (0.13). In this experiment, none of the metal was sufficiently accumulated by *Vetiveria zizanioides* during the 30 days HRT. This merely means that after 30 days, the metals were more retained by the wetland substrate, and it may require more time for the *Vetiveria zizanioides* to accumulate a sufficient quantity of metal to be qualified as a hyper-accumulator (BCF > 1). The BCF results justify the findings obtained in section 3.4, which revealed that the substrate played a huge role in overall metal removal in the constructed wetland. However, in the control wetland, the BCF of all metals (Al, Cu, Fe, Mn, Ni and Zn) could not be determined since their final concentration in the substrate was below the detection limit of 0.0001 mg/kg. The variation in BCF values of metals could be explained by the importance of the given metal in the plant metabolism. For instance, metals such as Cu, Fe, Zn, and Mn act as important co-factors for many enzymes and are all-important for both mitochondrial and chloroplast functions [73,74]. The BCF values may also be influenced by the initial concentration of metal in the AMD water. The determination of the metal content in the plant and substrate allowed the researchers to calculate the quantity of metal retained by the wetland substrate.

3.9. Contribution of substrate, plant, and other factors in overall removal of the metal

The contribution of substrate, Vetiver, and other factors in metal removal was determined, and the results are presented in the Table 2.

Table 2. Heavy metals removal by wetland based on substrate, plant, and external factors contributions to overall metals removal.

Parameters	**Al**	**Cu**	**Fe**	**Mn**	**Ni**	**Zn**
Components	(%) Percentage removal					
Substrate	93.20	70.70	60.00	73.31	53.08	69.77
Plant	4.10	20.46	35.17	18.22	29.85	20.75
External factor	2.70	8.77	5.02	8.45	17.06	9.48

Table 2 shows that each component of the wetland contributed to metal removal. The wetland substrate played a huge role in metal removal contributing to 93.2%, 70.7%, 60%, 73.31%, 53.08 and 69.77% for Al, Cu, Fe, Mn, Ni, and Zn, respectively of the overall removal. The contribution of *Vetiveria zizanioides* in the metal (Al, Cu Fe, Mn, Ni and Zn) removal is a small fraction of the overall removal, contributing to 4.10%, 20.46%, 35.17%, 18.22%, 29.85% and 20.75% removal of Al, Cu, Fe, Mn, Ni, and Zn, respectively while external factor contributed to minor fraction of the overall removal with contribution ranged in the order: Zn Ni (17.06%) > Zn (9.48%) > Cu (8.77%) > Mn (8.45) > Fe (5.02%) > Al (2.70%). External factor include: Evaporation, volatilization, biological assimilation may also contribute significantly to metal removal in constructed wetland. In the wetland system, substrate plays a vital role such as providing support to plant growth, serve as energy source for biogeochemical reaction among others [78]. However, in the biochemical processes occurring in the wetland system, sulfides and others minerals in the substrate may also contribute to the removal of metal in the constructed wetland [66]. Despite the smaller contribution of the plant (*Vetiveria zizanioides*) to the overall metal removal, it plays a crucial role in the treatment of wastewater using a constructed wetland. For instance, it promotes the settling of suspended solids [79]. Like other plants used in the constructed wetland, the Vetiver's rhizosphere provides the substrate and supporting media suitable environment for the growth of microorganisms, which play an important role in overall metal removal in the constructed wetland [77-79]. In addition, oxygen transported by *Vetiveria zizanioides* from the aerial parts to the roots creates a suitable condition in the rhizosphere for the proliferation of bacteria and for the promotion of various chemical and biochemical reactions, which accelerate metal sedimentation in the substrate and further accumulation by plants [68,71].

3.10. Characterization of the solid samples

3.10.1. X-ray fluorescence analysis

The elemental composition of raw and reacted soil samples is shown in Table 3.

Table 3. Elemental composition of raw and reacted soil samples.

Sample	**Initial soil (wt. %)**	**Control soil (wt. %)**	**AMD reacted soil (wt. %)**
Fe	83.6917	82.9824	89.0974
Rb	5.3379	5.0927	5.0196
Sr	3.3187	4.2368	3.0746
Ti	4.3548	3.6349	3.4322
Mn	1.9175	2.6888	2.6185
Zn	0.9745	0.9276	0.6774
Zr	-----------	0.2212	1.1057
Cl	-----------	0.2156	---------
I	-----------	----------	2.3647
Cr	0.3846	----------	0.3182
Ca	-----------	----------	0.4676

As shown in Table 3, after the reaction of the substrate with AMD, the elements Zr, I, Cr and Ca were found to be present. Thenceforth, the levels of Fe and Mn increase, indicating the formation of a new mineral phase, whereas Ti, Zn, Rb, and Cr decreased, indicating possible dissolution into the aqueous solution.

3.10.2. X-ray diffraction analysis

X-ray diffraction analysis was done to determine the mineralogical composition of the soil from the experimental and control wetland; the results are reported in Figure 10.Figure 10 depicts that the X-ray diffraction patterns of the substrate from the experimental wetland (1) and control wetland (2) show various peaks spread over 2θ between 12° and 68° but at different intensities. The peak at 2θ = 21°, 27.5°, and 51° correspond to quartz (SiO_2), which is the major mineral composition of compost soil [83]. The peak at 2θ = 21°, 26°, 31°, 37°, 55°, and 60° correspond to calcite, which supplies calcium for plant nutrition [84]. The peak at 2θ = 40° corresponds to cellulose, which originates from the plant cell wall [83]. The peak at 2θ = 46° may correspond to the silica present in the compost soil. Silica affects the binding of nutrient elements to soil particles [85]. The peak at 2θ = 68° may correspond to dolomite, which is one of the fertilizers found in compost soil [86]. The different peaks of calcite (2θ = 21°, 26°, 31°, 37°, 55° and 60°), the peak of silica (2θ = 46°), and the peak of dolomite (2θ = 68°) indicate that the conditions are suitable for the precipitation of calcium, magnesium, and iron bearing species. The calcium ions (Ca^{2+}) reacted with carbonic acid from AMD to form calcium carbonate, as illustrated in Equation 11.

$$\mathrm{Ca^{2+}} + 2\mathrm{HCO_3^-} \rightarrow \mathrm{CaCO_3} + \mathrm{H_2O} + \mathrm{CO_2} \qquad (11)$$

Simultaneously to Equation 11, silica reacted with the acid in the AMD water through ion exchange, leading to a slight increase in pH.

3.10.3. Fourier transforms infrared spectroscopy analysis

The metal functional groups of the raw and reacted roots are presented in Figure 11 and Table 4 lists the identified metal functional groups and their respective wavelengths.

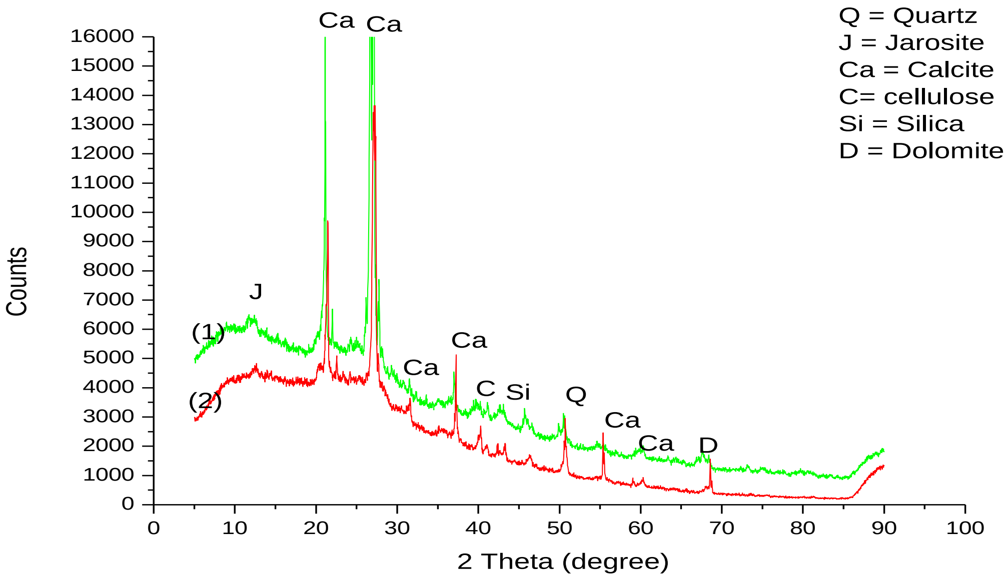

Fig. 10. (1-2). XRD patterns of substrate: experiment (1) and control (2).

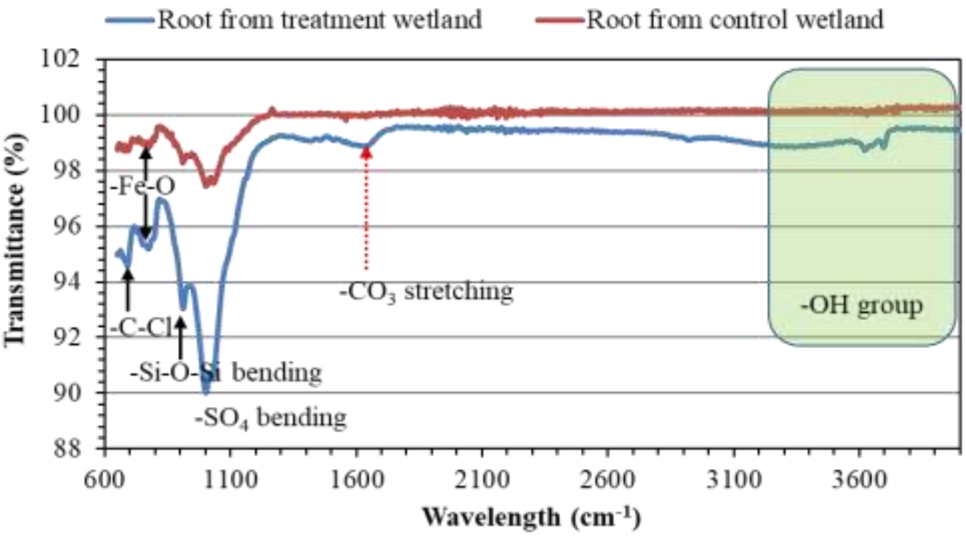

Fig. 11. The metal functional groups of raw and reacted roots.

Table 4. Metals functional groups and their references

Wavelength	Functional group	References
805.5	C-Cl	[87]
883.5	Fe-O	[88]
966.5	Si-O	[89]
1034.5	SO_4	[90]
1711	C=O	[91]
3707	O-H	[89]

As shown in Figure 11, the spectrum of both roots begins by a series of stretching vibration with a peak at 805.5 cm^{-1} for both roots, followed by a strong vibrations between 920 and 958.5 cm^{-1} for the control root as well as between 966.5 and 1034.5 cm^{-1} for the root from the treatment. After the vibration, the spectrum of root from the control wetland shows a straight band from 1264.5 to 3974 cm^{-1}, while the spectrum of the root from the treatment wetland shows a stretching vibration with a band at 1648 cm^{-1} and a doublet with a band at 3617.5 and 3707 cm^{-1}. The vibration observed from both roots may be attributed to the stress factor, while the stretching vibration observed in the root from the treatment wetland may result from pollutants accumulation.

3.10.4. Scanning electron microscope-electron dispersion spectrometry analysis

To understand the mode of accumulation of metal by *Vetiveria zizanioides* and the formation of the mineral phases, scanning electron microscope (SEM) and energy dispersive X-ray spectroscopy (EDS) were performed for the roots from both wetlands (treatment and control). The results are shown in Figures 12 and 13.

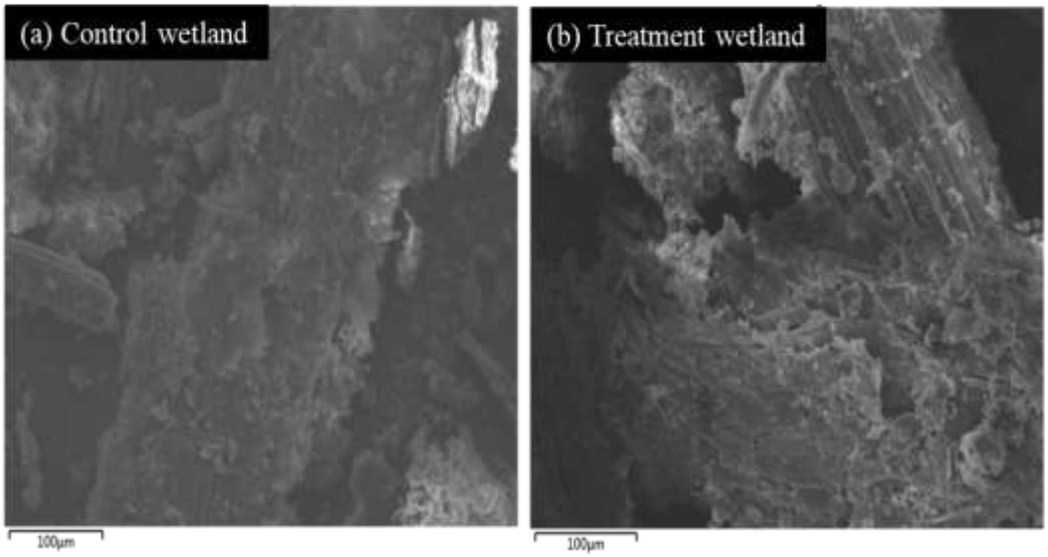

Fig. 12. (a and b). SEM image of root from control wetland (a) and from treatment wetland (b).

As shown in Figure 12, the morphological properties of the roots from the control and treatment set-up are evaluated using SEM analysis. Specifically, this is done to acquire a better understanding of the mode of interaction between AMD and *Vetiveria zizanioides.* SEM was performed to examine the structural changes that may have happened in the roots of *Vetiveria zizanioides* as a result of stress factors caused by the acidic media, elevated concentration of salt (SO_4^{2-}), and high level of metal in the AMD. The SEM images of the roots from the treatment wetland (Figure 12b) and those from the control wetland (Figure 12a) revealed noticeable differences. The image with the root from the treatment wetland showed an aggregate of pallettes, while the SEM image of the root from the control wetland showed a sort of large cubic mass. The aggregate of palettes observed in the SEM image of the root from the treatment wetland could be attributed to metal and other pollutants accumulated by the plant. Therefore, leading to elevated percentages of pollutants such as Al, Fe, K, and Si, as shown in EDS analysis of the roots from the treatment wetland (Figure 13a). The presence of Ca, C, Mg, and O in both EDS may be attributed to metals such as manganese, iron, and carbonate, whereas the presence of silicon in the roots from both wetlands (Figure 13 a and b) may be attributed to silicon in the biological plant material. This confirms the findings of Nylese et al. [92]. They revealed a high percentage of silicon in the plant material after EDS analysis. The result concurs with the findings obtained by Kiiskila et al. [29], which revealed a noticeable change in the morphology of the *Vetiveria zizanioides* roots grown in AMD water. In fact, plants use their cell wall as a defense compartment to respond to toxic conditions. According to Rich et al. [93], toxic metals are accumulated by plants using the cell wall. The plant cell wall is rich in proteins, amino acids, and phenolics that are able to bind metal in order to render them less or non-toxic [94]. When plants accumulate metal using the cell wall, it leads to the formation of crystal-like deposits that saturate the cell wall and thickens it. And this demonstrates that *Vetiveria zizanioides* accumulated and sequestrated toxic metal away from its sensitive parts. This perhaps is a biological strategy of *Vetiveria zizanioides* to respond and adapt to a very toxic environment such as AMD water.

4. Chemical species of untreated and AMD treated with SSHF-CW

The results of the chemical species of treated AMD were used to compare the Department of Water and Sanitation (DWS) and Department of Environmental Affairs (DEA) guideline standards for effluent discharge, and the results are presented in Table 5.

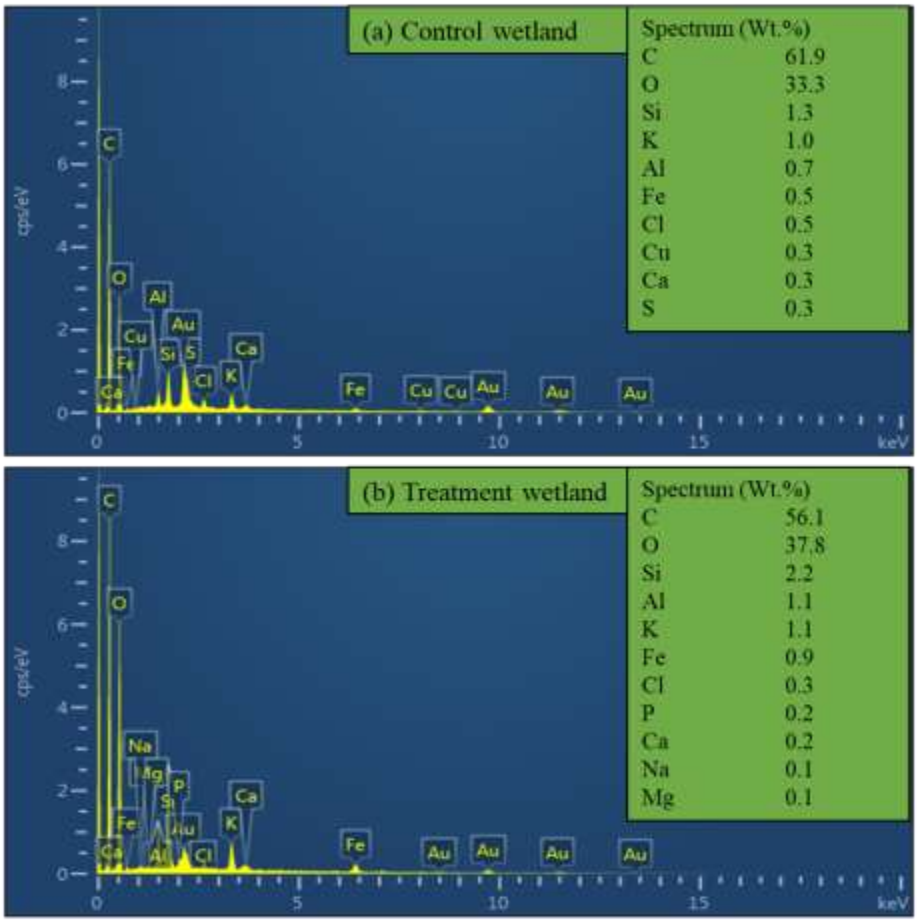

Fig. 13. Elemental composition of roots from the experimental wetland (a) and control wetland (b).

Table 5. Concentrations of chemical species for untreated and AMD treated with SSHF-CW (all units in mg/L except pH and EC.

Parameters	Feed AMD	Limit	Product water	% Removal
pH	2.6	6-10	4.01	1.41
TDS (mg/l)	3380	2400	2100	37.86
EC (μS/cm)	5000	1500	2640	47.2
Al (mg/L)	158	20	71.02	55.05
Fe (mg/L)	341	50	84.02	75.36
Mn (mg/L)	37	20	12.03	67.48
Cu (mg/L)	4.2	20	3.72	11.36
Zn (mg/L)	8.55	20	1.90	77.75
Ni (mg/L)	3.92	10	2.19	44.01
Sulphate (mg/L)	3137	2400	1588.57	49.36

As shown in Table 5, the pH is very acidic (2.6) with notable levels of TDS and EC. This may be the results of high levels of broken down chemical species. After the treatment of AMD, the pH increases slightly from 2.6 to 4.01, whereas EC and TDS notably decrease. The concentration of metals (Al, Cu, Fe, Mn, Ni and Zn) and SO_4^{2-} are observed to have decreased significantly after the interaction with SSHF-CW. And this is attributed to the settling of metals or deposited as sediment followed by accumulation by *Vetiveria zizanioides*. However, the DWS water quality guidelines [95] have not been met except for Ni; therefore, there is a need to associate or integrate subsurface horizontal flow constructed wetland with another type of water treatment technology to enhance the performance of the system.

5. Conclusions

This eco-friendly and passive study successfully demonstrated the feasibility of SSHF-CW to improve the quality of AMD water treated with *Vetiveria zizanioides.* The plants denoted the high toleratiion rate for acidic conditions through its resistance to de-generating and this has been confirmed by the tolerance index of ≥1. The results showed a slight increase in pH from 2.4 to 3.8 and a net reduction in electrical conductivity (EC), total dissolved solids (TDS), and sulphate with ≥47.20%, ≥46%, and ≥33.04%, respectively. Thenceforth, there was a net removal of metals, with Zn registering the highest removal percentage of 77.75%, followed by ≥Fe (75.36%), ≥Mn (67.48%), ≥Al (55.05%), ≥Ni (44.01%), and ≥Cu (11.36%). The selected pollutants were significantly removed from the AMD after 30 days of retention time; however, they failed to meet the DEA and DWS limits for effluent discharge hence requiring a polishing technology to enhance the efficacy of the system. Furthermore, the metal removal was partitioned between the substrate, plant accumulation, and external factors, with the substrate contributing more for the metal removal except with Cu and Ni, where the contribution percentage of the substrate was 10.05 and 30.59, respectively. Metal accumulation by plants accounted only for a small percentage of the overall metal removal. In light of the highlighted findings, this study would need to be integrated with another effective technology to enhance its performance and ensure that the product water complies with the stipulated specifications, guidelines, and standards. This will play an enormous role in curtailing the impact of AMD on the environment and other ecological compartments.

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