Abstract: This study demonstrated heavy metal removal from neutral mine drainage of a closed mine in Kyoto prefecture in pilot-scale constructed wetlands (CWs). The CWs filled with loamy soil and limestone were unplanted or planted with cattails. The hydraulic retention time (HRT) in the CWs was shortened gradually from 3.8 days to 1.2 days during 3.5 months of operation. A short HRT of 1.2 days in the CWs was sufficient to achieve the effluent standard for Cd (0.03 mg/L). The unplanted and the cattail-planted CWs reduced the average concentrations of Cd from 0.031 to 0.01 and 0.005 mg/L, Zn from 0.52 to 0.14 and 0.08 mg/L, Cu from 0.07 to 0.04 and 0.03 mg/L, and As from 0.011 to 0.006 and 0.006 mg/L, respectively. Heavy metals were removed mainly by adsorption to the soil in both CWs. The biological concentration factors in cattails were over 2 for Cd, Zn, and Cu. The translocation factors of cattails for all metals were 0.5–0.81. Sulfate-reducing bacteria (SRB) belonging to Deltaproteobacteria were detected only from soil in the planted CW. Although cattails were a minor sink, the plants contributed to metal removal by rhizofiltration and incubation of SRB, possibly producing sulfide precipitates in the rhizosphere.

Keywords: emergent plant; heavy metal; mine wastewater; passive treatment; sulfate-reducing bacteria

1. Introduction

Mining, an important industry for economic development in many countries, generates huge amounts of solid waste and wastewater containing toxic metals. Physicochemical processes such as neutralization and coagulation have been used for removing heavy metals from mine drainage [1]. Such active treatment processes are mostly expensive for capital and operation costs. In the case of Japan, more than 5000 mines have been abandoned or closed since the 1970s because of increasing labor costs and import liberalization of mineral resources [2]. Among them, approximately 80 mines with absent owners must treat mine drainage continuously despite a lack of production, which has imposed large economic burdens on national and local governments for over a hundred years [2]. A promising alternative as a passive technology for mine drainage treatment is constructed wetlands (CWs) because of their simple operation, low cost, and moderate effectiveness [3–6].

Mechanisms of heavy metal removal in CWs are quite complicated, involving various processes such as precipitation, absorption, filtration, complexation, microbiological activity, and plant uptake [3–6]. Wetland plant species show considerable variations in metal uptake and translocation abilities through phytoextraction [7–10]. Sulfate-reducing bacteria (SRB) in wetland soil decomposed organic matter to lower molecular weight acids and bicarbonate leading to increased alkalinity and formation of metal sulfide precipitates [11,12]. The bacteria–plant interaction is regarded as important because it implies symbiotic mechanisms for heavy metal removal and tolerance.
The use of CWs for mine drainage treatment has developed rapidly over the last few decades, mainly in North America and Europe [4]. In Japan, a CW for treating mine drainage was first installed for removing manganese (Mn) in 2000 at Kaminokuni, Hokkaido Prefecture [13]. In addition, at Motokura Mine in Hokkaido Prefecture, a CW was installed on a small scale in 2006 for removing zinc (Zn), lead (Pb), and arsenic (As). It has been scaled up for demonstration tests to replace existing neutralization processes [12,14]. A CW at Ningyotoge Mine is also used for As removal from acid mine drainage through ferrihydrite coprecipitation [15].

In a dozen of the 80 mines, drainage includes only a few toxic metals, slightly exceeding the effluent standard with neutral pH (5.8–8.6) [2]. Installation of the CW is expected to be prioritized for treating such neutral mine drainage (NMD). Our research group has studied lab-scale CWs for treating NMD of such a typical mine in Kyoto Prefecture (A-Mine) [16]. Synthetic wastewater with cadmium (Cd) at 0.11 mg/L was designed to simulate the NMD of A-Mine. The CWs were filled with loamy soil and were planted with common reeds or cattails. The synthetic NMD was treated in a 1-week cycle sequencing batch mode in the CWs. Unplanted CWs removed Cd sufficiently to satisfy the effluent standard (0.03 mg/L) from the NMD, mainly by soil adsorption. The presence of emergent plants, especially cattails, enhanced metal removal, presumably by filtration with their elongated roots and by metal sulfide precipitation by SRB in the rhizosphere.

Based on lab-scale results [16], the present study investigated the treatment performance of pilot-scale CWs with and without cattails on metal removal from actual NMD of A-Mine. The NMD was treated in a continuous flow mode at the hydraulic retention time (HRT) of 3.8–1.2 days in autumn and winter. The accumulation of heavy metals in soil and the plant biomass was measured. Bacteria including SRB were also studied in both water and soil samples of the CWs.

2. Materials and Methods
2.1. Pilot-Scale CW Setup and Operation

This study was conducted at a mine drainage treatment plant of A-Mine from September to December 2019. This mine had been developed since the 1910s, mainly for tungsten production, by a private company. It was finally closed in the 1980s. The plant is now managed by the local government to treat mine drainage of about 200 m$^3$/d through coagulation, sedimentation, and pH adjustment. In recent years, the pH values and the Cd and Zn concentrations of this mine drainage have been, respectively, 6.67–7.32, 0.020–0.11 mg/L, and 0.63–2.00 mg/L [16]. The effluent standard in Japan is 5.8–8.6 for pH, 0.03 mg/L for Cd, and 2.0 mg/L for Zn.

A schematic of the pilot-scale CWs is presented in Figure 1. Pictures of the materials of the CWs are shown in Figure S1 in Supplementary Materials. Two polyethylene tanks (63 cm length, 63 cm width, 98 cm height, 300 L) were packed from bottom to top with pumice stone (13.5 cm depth, bulk 54 L, Hyuga soil; Hyugatsuchi Hanbai Co., Miyakonojo, Miyazaki, Japan), limestone (8 cm depth, 4 kg; Konan Shoji Co. Ltd., Tokyo, Japan), middle loamy soil (akadama) (17 cm depth, bulk 70 L, 45.5 kg; Sowa Recycle Corp., Tokyo, Japan), fine loamy soil (akadama) (17 cm depth, bulk 70 L, 45.5 kg; Hirota Shokai Co., Kanuma, Tochigi, Japan), and limestone (2 cm depth, 1 kg; Konan Shoji Co. Ltd.). The middle and the fine loamy soils had cation-exchange capacity of 12.2 and 6.09 meq/100 g, respectively. The water level was set at 75 cm with an overflow pipe connected at 10 cm depth to the CW. A weather monitoring station (SenSu-1502; Data Technology Inc., Tokyo, Japan) was placed at the unplanted CW to record daily solar radiation, precipitation, and air temperature.
After 79 days of operation, three water samples (influent, effluent of CW-A and CW-B) were washed well with tap water to remove soil and stone. After the washed samples were dried in an oven at 102 °C to constant weight. They were then crushed to less than 1 mm. Plant samples were collected from depths of about 3 cm (upper), 8 cm (middle), and 14 cm (lower) from the soil surface and were dried using an oven at 102 °C to constant weight. They were then crushed to less than 1 mm. Plant samples were washed well with tap water to remove soil and stone. After the washed samples were dried in an oven at 102 °C to constant weight. They were then crushed to less than 1 mm. Plant samples were washed well with tap water to remove soil and stone. After the washed samples were crushed to less than 1 mm.

Two CWs were produced in parallel: one was left unplanted (CW-A); the other was planted with cattails (CW-B). On 18 September 2019 (day 0), 9 young plants of cattail (Typha latifolia L.) were planted to the CW-B (75 ± 10 cm shoots, 13 ± 3 cm roots, 180 ± 88 g-wet/plant). As a typhoon damaged the cattails, four plants of Southern cattail (Typha domingensis) were added to the CW-B on 5 October (day 17) (41 ± 1 cm shoots, 12 ± 1 cm roots, 194 ± 28 g-wet/plant). These emergent plants were purchased from Tojaku Engei (Joyo, Kyoto, Japan). Before planting to the CWs, the roots were washed gently using river water to remove the original culture soil.

The water level in the influent reservoir tank was kept constant by gravity inflow and overflow of the NMD. The NMD was fed continuously through polyvinyl chloride pipes with ball valves to the CWs at hydraulic loading of 72 L/d for 31 days (phase I), 115 L/d for 21 days (phase II), 158 L/d for 1 day (phase III), then 216 L/d for 32 days (phase IV). If there was no evapotranspiration, then these conditions caused HRT of 3.8, 2.4, 1.7, and 1.2 days. The NMD penetrated downward into the soil layers in the CW and overflowed upward through the pipe. The effluent rate was recorded using a digital flowmeter (OF05-ZAWN; Aichi Tokei Denki Co., Ltd., Nagoya, Aichi, Japan).

### 2.2. Sampling and Sample Preparation

Influent and effluent samples of the CWs (each 100 mL) were collected from the reservoir tanks twice a week (Figure 1). The samples were transported to the laboratory at Ritsumeikan University and were centrifuged for 10 min at 1000×g for measuring heavy metals, sulfate (SO₄²⁻), total nitrogen (T-N), and total phosphorus (T-P) concentrations. After 79 days of operation, three water samples (influent, effluent of CW-A and CW-B) and two soil samples at about 14 cm depth (CW-A and CW-B) were collected for bacterial analyses.

Soil and plant samples were collected for analyzing the heavy metal content before and after CW operation for 3.5 months. The original soil and plant samples had not been polluted by any heavy metal. Samples of the loamy soil were collected at depths of about 3 cm (upper), 8 cm (middle), and 14 cm (lower) from the soil surface and were dried using an oven at 102 °C to constant weight. They were then crushed to less than 1 mm. Plant samples were washed well with tap water to remove soil and stone. After the washed samples were crushed to less than 1 mm.
samples were separated into two parts, shoots and roots, they were dried in the oven at 80 °C to constant weight. Subsequently, they were ground into powder and were stored for additional analysis.

2.3. Analytical Methods

The pH, dissolved oxygen (DO), oxygen reduction potential (ORP), and total dissolved solids (TDS) of water samples were measured respectively on site using a pH meter (F-21; Horiba Ltd., Kyoto, Japan), an ORP meter (ORP57; Milwaukee Instruments Inc., Rocky Mount, NC, USA), a DO meter (Ultrapen PT5; Myron L Co., Carlsbad, CA, USA), and a TDS meter (ASTDS1; AS One Corp., Osaka, Japan). Heavy metal (Cd, Fe, Cu, Zn) concentrations were measured using inductively coupled plasma spectroscopy (ICP-OES 700 series; Agilent Technologies Japan, Ltd., Tokyo, Japan). Suspended solids (SS), sulfate, T-N, and T-P were measured according to a standard procedure [17]. Average values were analyzed statistically using one-way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls testing.

Soil samples were digested using a wet digestion method. A portion of 0.5 g of each dried sample was digested with 6 mL of a solution of HCl/HNO₃ (3:1, v/v) and 1.5 mL H₂O₂. Then the mixture was heated at 180 °C for 2 h using a digestive furnace. For plant samples, 0.1 mg of each dried sample was digested by 3 mL of HNO₃ and 0.6 mL of H₂O₂ using a furnace at 145 °C for 2 h. After digestion, all soil and plant samples were cooled. Then the supernatants were filtered through 0.45 µm filter paper (Advantec, Tokyo, Japan). Filtrates were used for metal content determination using ICP analysis.

Heterotrophic bacteria and SRB were enumerated using the plate-count technique with R2A agar medium (Merck KGaA, Darmstadt, Germany) and Postgate’s medium F: 10.0 g tryptone, 3.5 g sodium lactate, 0.5 g sodium sulfate, 2.0 g magnesium sulfate heptahydrate, 0.5 g iron(II) sulfate heptahydrate, 0.5 g ammonium ferric citrate, 15 g agar, and 1 L pure water, 7.1 pH [18].

The middle soil samples were suspended in 5 mg/L sterile sodium tripolyphosphate solution (tpp) (5 g-wet in 50 mL), mixed vigorously with a vortex mixer for 2–3 min, and treated with a sonicator (UD211: Tomy Seiko, Tokyo, Japan) to disperse soil particles. The mixture was diluted with tpp and was used for pour plating. Agar plates for heterotrophs were incubated aerobically for 1 week at 28 °C. Agar plates for SRB were incubated anaerobically for 3 weeks at 28 °C in chambers containing Aner Pack Kenki (Mitsubishi Gas Chemical, Tokyo, Japan).

Water samples and middle soil samples were subjected to DNA extraction and Illumina Miseq 16S rRNA gene sequencing at Seibutsu Giken Inc. (Sagamihara, Kanagawa, Japan). Amplicon sequencing and data processing work were conducted there. Soil samples were frozen and dried (VD-250R Freeze Dryer; Taitec Corp., Saitama, Japan) and were disrupted mechanically (Shake Master Neo; Bristol-Myers Squibb Co., New York, NY, USA). The bacterial cells in the water samples were collected by filtration using 0.22-µm-pore-size membrane filters (Millipore Corp., Bedford, MA, USA). Total DNA was extracted from the samples using the MPure bacterial DNA extraction kit (MP Biomedicals Japan, Tokyo, Japan). The amplicon libraries were prepared using a two-step tailed PCR method. The first PCR amplified V3–V4 region of 16S rRNA gene was done using primers 341f (ACACTCTTTCCCTGCTGACGCTCTTCCAGCTT–NNNNN–CCTACGGGNGGCWGCAG) and 805r (GTGACTGGAGTTCAGACTG-TGGTACCTTTCCAATCT–NNNNN–GACTACHVGGGTATCTAATCC); the second PCR was performed using the first PCR products and index primers. Amplicon sequencing of the second PCR products (i.e., amplicon libraries) was conducted, with paired-ending (2 × 300 bp) on an Illumina MiSeq platform (San Diego Instruments Inc., San Diego, CA, USA). Sequencing data were analyzed using software (QHIME2, ver. 2019.01) [19]. Primers were trimmed; data were also filtered and truncated, to allow 20 bp overlap between forward and reverse reads, using Dada2. Possible chimeric sequences were identified and removed before amplicon sequence variants were assigned using MiDAS database ver. 2.1.3 [20].
2.4. Calculating Heavy Metal Removal and Accumulation

The metal removal (%) was calculated using the following equation.

\[
\text{Removal} \, (\%) = \frac{Q_i C_i - Q_e C_e}{Q_i C_i} \times 100, \tag{1}
\]

Therein, \( Q_i \) (L/d) and \( Q_e \) (L/d) respectively denote the inflow rate and the outflow rate. \( C_i \) (mg/L) and \( C_e \) (mg/L) respectively stand for the metal concentration in influent and effluent.

The amount of metal removed in the CWs, \( M \) (mg), was calculated using rectangular integration as

\[
M = \sum (Q_i C_i - Q_e C_e) \Delta t, \tag{2}
\]

where \( \Delta t \) (days) represents the time interval of the measurement.

The amount of metal accumulated in the plant biomass \( M_p \) (mg) was determined using the following equation

\[
M_p = \sum (C_p X_f - C_p X_i) \tag{3}
\]

wherein, \( C_p \) (mg/kg-dry) and \( X \) (kg-dry) respectively represent the average metal contents in the plant biomass and the total plant biomass (shoots and roots). Subscripts \( i \) and \( f \) respectively signify initial and final conditions.

The bioconcentration factor (BCF) and the translocation factor (TF), indicating the phytoaccumulation and translocation capabilities of cattails, were also calculated using the following equations [21]

\[
\text{BCF} = \frac{C_p}{C_s}, \tag{4}
\]

\[
\text{TF} = \frac{C_{sp}}{C_{rp}}, \tag{5}
\]

therein, \( C_s \) (mg/kg) signifies the average metal content in the soil samples.

3. Results

3.1. Operational Conditions of the CWs

Figure 2 presents temperature, daily solar radiation, and precipitation at A-Mine during the experimental period. Photographs of the cattails in the CW-B are portrayed in Figure S2. Phase I (days 0–29) started in September 2019 with the influent rate of 72 L/d (HRT 3.8 days). Under warm conditions with a maximum daily temperature of 20–35 °C and solar radiation of 50–620 kLux·h/day, the effluent rate of the CWs sometimes decreased to 60% of the inflow rate because of evapotranspiration. Typhoon No. 18 (Mitag) brought 14 L of rainwater to each CW on the 3rd and 4th of October, resulting in temporal increases of the effluent rates on days 17 and 18. As the cattails were partly damaged by the typhoon, they were additionally planted on day 17, as described in Section 2. After the typhoon passed, the effluent rate of the CWs decreased on days 20 and 21 because of temporary clogging by heavy rains. Typhoon No. 19 (Hagbis) again brought heavy rains of 42 L to each CW on 12 October 2019, resulting in increases in the effluent rate on days 24 and 25. Nevertheless, no marked damage was sustained by cattails in the CW-B.
Figure 2. Experiment conditions for treating NMD by pilot-scale CWs at A-Mine. Flow rates of the CWs: (A) solar radiation and precipitation (B) and air temperature (C). Eighteenth September 2019 was defined as day 0.

For phase II (days 29–51), the inflow rate was set to 115 L/d to each CW (HRT 2.4 days). The effluent rate of the CWs was almost equal to the inflow rate under the moderate temperature and solar radiation of <390 kLux·h/day. Temporary clogging occurred in the
CW-A on days 49 and 50. The minimum temperature was sometimes below 10 °C. Some of the cattail leaves in the CW-B turned brown.

In phase III (days 52–71), the inflow rate was set to 158 L/d (HRT 1.7 days). No clogging occurred in the CWs, except for day 66. Sprouts of aquatic plants of some kinds were found on day 65 in the CW-A, suggesting that wind or birds brought seeds (Figure S2).

In phase IV (days 72–108), the inflow rate was finally set to 216 L/d (HRT 1.2 days). The daily minimum temperature was sometimes below 0 °C. The solar radiation was below 300 kLux·h/day. The cattail shoots were almost withered. Although temporal clogging occurred a few times in both CWs, the effluent rate of the CWs was close to the inflow rate. The time courses of pH, ORP, DO, TDS, sulfate, and heterotroph concentrations in the influent and effluent samples of the CWs are shown in Figure S3. Through the experiments, the pH values in the effluent (6.3–7.2) were slightly higher than those in the influent (6.2–6.9), probably because of the influence of limestone in the CWs, although no significant difference was found between the CWs (p < 0.05). The ORP values were positive at +50–+150 mV in both influent and effluent. The DO concentrations in effluent of the CW-B tended to be lower than those in influent and effluent of the CW-A, suggesting oxygen consumption by root and microbial respiration. The TDS concentrations in effluent were lower than those in the influent in phase I, indicating high removal of dissolved matters in the CWs. However, the TDS concentrations in effluent were almost identical with those in the influent in phases II–IV. Influent and effluent sulfate concentrations fluctuated at 40–120 mg/L through the experimental phases. The T-N and T-P concentrations were, respectively, <1.0 mg/L and <0.13 mg/L in both the influent and effluent.

3.2. Water Parameters in the CWs

The time courses of Cd, Zn, Cu, Fe, Mn, and As concentrations in the CWs are shown in Figure 3. Average influent and effluent concentrations of the metals in each phase are presented in Figure 4. Metal concentrations in the NMD were always lower than the Japan effluent standard, except for Cd. Both CWs well removed Cd, Zn, Cu, Fe, Mn, and As during the experimental period (p < 0.01).

The average influent Cd concentrations in phases III and IV were higher than those in phases I and II (p < 0.01) (Figure 4A). The Cd concentration decreased to a level below 0.01 mg/L in both CWs, especially in the CW-B to below 0.005 mg/L. Although no significant difference was found between the CWs in phases I and II, effluent Cd concentrations of the CW-B were lower than those of the CW-A in phases III and IV. Shortening of HRT apparently had no marked influence on the effluent Cd concentrations.

Both CWs well removed Zn from the NMD with the stable influent concentration at 0.52 ± 0.1 mg/L (Figure 4B). Although no significant difference was found, the effluent Zn concentrations of the CW-B tended to be lower than those of the CW-A, slightly higher than Japan’s environmental quality standard for Zn (0.03 mg/L) for the protection of aquatic life. Shortening of HRT had no significant influence on effluent concentrations.

The average influent Cu concentrations were lower than 0.08 mg/L in phases I–III but increased to 0.11 mg/L in phase IV (Figure 4C). The effluent Cu concentrations were also ranked as phases I and II < III < IV (p < 0.05). However, no significant difference was found in effluent concentrations between the CWs.

The influent and effluent Fe concentrations were less than 0.05 mg/L and 0.004 mg/L, respectively, in phases I, III, and IV, but were sometimes over 0.3 mg/L and 0.14 mg/L, respectively, in phase II (Figure 4D).

The influent Mn concentration fluctuated at 0.01–0.04 mg/L (Figure 4E). In phase I, the Mn concentrations in effluent of the CW-A were higher than that in influent, indicating release of Mn from the soil. In addition, the effluent Mn concentrations in the CW-B were sometimes higher than the influent concentrations. Then, both CWs removed Mn well from the NMD in phases II–IV (p < 0.05).
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Figure 3. Time courses of metal concentrations in influent and effluent in unplanted CW (CW-A) and cattail-planted CW (CW-B) treating NMD: Cd (A), Zn (B), Cu (C), Fe (D), Mn (E), and As (F).

Figure 4. Summary of influent and effluent metal concentrations in phases I (HRT 3.8 days), II (2.4 days), III (1.7 days), and IV (1.2 days) in the unplanted CW (CW-A) and the cattail-planted CW (CW-B) treating NMD. Cd (A), Zn (B), Cu (C), Fe (D), Mn (E), and As (F).
The influent As concentration fluctuated at 0.01–0.04 mg/L (Figure 4F), although the effluent As concentrations of both CWs showed no significant difference from one another. The average effluent As concentrations of both CWs were lower than the environmental quality standard for As (0.01 mg/L).

3.3. Heavy Metal Removal in CWs

The average metal removals in each phase of the CWs are presented in Table 1. Removals for all metals except for Mn in both CWs decreased with the increase in the influent rate. Results show that CW-B exhibited higher removals for Cd and Zn than CW-A throughout the experiments. The Fe removals in both CWs were negative values at the high influence rate in phase IV, indicating solubilization of the accumulated Fe in the CWs through phases I–III. By contrast, removals for Mn especially in the CW-A were low at the low influent rate in phase I.

Table 1. Mass removal (%) for heavy metals from NMD by the CWs.

| Element | Wetland | Phase I | Phase II | Phase III | Phase IV | Total |
|---------|---------|---------|----------|-----------|----------|-------|
| Cd      | CW-A    | 81.1 ± 8.2 | 77.3 ± 5.5 | 72.4 ± 10.9 | 69.4 ± 6.3 | 74.3 ± 8.9 |
|         | CW-B    | 91.2 ± 5.2 | 87.5 ± 3.6 | 81.8 ± 5.9 | 82.9 ± 3.7 | 84.5 ± 5.8 |
| Zn      | CW-A    | 83.1 ± 8.8 | 80.1 ± 8.9 | 74.0 ± 9.7 | 73.1 ± 6.3 | 76.9 ± 8.9 |
|         | CW-B    | 92.1 ± 4.3 | 87.8 ± 9.1 | 81.3 ± 12.1 | 85.9 ± 4.0 | 86.6 ± 8.2 |
| Cu      | CW-A    | 80.0 ± 18.1 | 74.9 ± 22.4 | 54.9 ± 18.1 | 45.6 ± 8.7 | 61.2 ± 21.3 |
|         | CW-B    | 90.2 ± 6.9 | 75.9 ± 22.7 | 50.5 ± 11.9 | 50.2 ± 8.3 | 64.3 ± 21.2 |
| Fe      | CW-A    | 77.2 ± 23.6 | 52.4 ± 43.0 | 22.7 ± 56.8 | -10.0 ± 36.6 | 22.1 ± 51.6 |
|         | CW-B    | 97.3 ± 3.1 | 52.0 ± 36.5 | 7.4 ± 48.5 | -64.0 ± 99.1 | -1.4 ± 89.2 |
| Mn      | CW-A    | -24.7 ± 54.2 | 47.3 ± 45.2 | 93.4 ± 11.2 | 86.3 ± 12.9 | 54.5 ± 57.6 |
|         | CW-B    | 37.4 ± 57.3 | 87.8 ± 9.9 | 98.6 ± 3.7 | 99.3 ± 1.7 | 82.2 ± 37.4 |
| As      | CW-A    | 83.0 ± 6.8 | 75.4 ± 37.3 | 51.7 ± 85.7 | 32.6 ± 64.4 | 60.6 ± 57.9 |
|         | CW-B    | 60.8 ± 17.0 | 95.9 ± 5.7 | 9.6 ± 81.7 | 18.5 ± 62.6 | 43.3 ± 60.9 |

The metal amounts removed in the CW-A and the CW-B were, respectively, $3.44 \times 10^2$ mg and $3.97 \times 10^2$ mg for Cd, $5.91 \times 10^3$ mg and $6.68 \times 10^3$ mg for Zn, $7.41 \times 10^2$ mg, and $7.77 \times 10^2$ mg for Cu, $3.90 \times 10^2$ mg and $4.23 \times 10^2$ mg for Fe, $2.25 \times 10^2$ mg, $2.88 \times 10^2$ mg for Mn, and $75.8$ mg and $48.5$ mg for As through 3.5 months of operation.

3.4. Accumulation of Heavy Metals in Soil

The distribution of heavy metals in the loamy soil layer in the CWs is presented in Figure 5. Metal contents in soil increased in both CWs during the 3.5 months operation. The CW-B accumulated more metals in soil than the CW-A, possibly because of rhizofiltration. The metals were accumulated mainly in the upper soil layer, except for Fe and Mn. These findings indicate that soil adsorption Cd, Zn, Cu, and As were removed effectively. The uniform distribution of Mn and Fe in soil indicates that those were precipitated towards the bottom.
3.5. Accumulation of Heavy Metals in Plant Biomass

Cattails in the CW-B were harvested at the end of phase IV. Although the shoots of the plant withered, the roots extended well to the lower soil layers. The final plant biomass was 90.8 g-dry of shoots and 173.9 g-dry roots, 264.7 g-dry in total. The metal contents in the plant biomass are shown in Figure 6. Both shoots and roots of the plant in the CW-B accumulated metals at high contents within a typical range [23]. The contents of Cu, Zn, Fe, and Mn, as essential elements, exhibited higher concentrations than Cd in the plant biomass. The amounts of metals in the plant biomass were 7.0 mg for Cd, 189.5 mg for Zn, 163.8 mg for Cu, 935.9 mg for Fe, 49.4 mg for Mn, and 0.7 mg for As. The ratio of those to the amounts removed in the CW-B were 1.8% for Cd, 2.8% for Zn, 21.3% for Cu, 220% for Fe, 49.9% for Mn, and 0.7% for As. This contradictory value for Fe (220%), which is easily oxidized and precipitated, might be attributable to the underestimation of the influent concentration and biomass contents before use. The remaining amounts of the removed metals in the CWs are expected to be accumulated in the soil.

The BCF and TF values for the metals in the CWs are displayed in Table 2. The BCF values for Cd, Zn, and Cu were higher than 2, indicating high uptake to the plant biomass from the soil. However, the BCF values for Mn and Fe, as easily oxidizable metals and As as an adsorptive element to the insoluble metal oxides, were below 1. The TF values were 0.5–0.81 in a typical range [22], indicating that a certain amount of metals were translocated to the shoots from the roots.

Figure 5. Metal contents in loamy soil in the unplanted CW (CW-A) and the cattail-planted CW (CW-B) before and after the NMD treatment experiment. Cd (A), Zn (B), Cu (C), Fe (D), Mn (E), and As (F).
Figure 6. Metal contents in the cattail biomass in the planted CW (CW-B) before and after the NMD treatment experiment. Cd (A), Zn (B), Cu (C), Fe (D), Mn (E), and As (F).

Table 2. Bioconcentration (BCF) and translation (TF) factors of cattail in the CW-B after NMD treatment.

| Element | BCF  | TF  |
|---------|------|-----|
|         | CW-B | Before Use | Before Use |
| Cd      | 2.29 | 0.80 | 0.86 |
| Zn      | 3.03 | 0.49 | 0.44 |
| Cu      | 4.69 | 0.49 | 0.41 |
| Fe      | 0.85 | 0.41 | 0.11 |
| Mn      | 0.67 | 0.67 | 0.66 |
| As      | 0.87 | 0.81 | 0.51 |

3.6. Bacterial Communities

Heterotrophs counted on the R2A medium were $10^3$–$10^5$ CFU/mL in influent and effluent of the CWs (Figure S3E). Heterotrophs in the effluent of the CW-B were slightly higher than those of the CW-A. SRB were not quantitative but were detected only from the soil sample of the CW-B by the F medium.

Water and soil samples in the CWs on day 79 were served for bacterial 16S rRNA gene sequencing. The obtained numbers of operational taxonomic units (OTUs) for the soil sample of the CW-B by the F medium were 16,500–25,800. The dominant OTUs in the CWs are presented in Table S1. Figure 7 shows the bacterial composition based on the 16S rRNA gene sequences in the water and soil samples. The influent sample showed the highest diversity index on the phylum level ($H'$) among the water samples. The soil sample of the CW-B showed higher diversity index than that of the CW-A. Uniquely, the phylum OD1 and the phylum OP3 often found in groundwater [24] were predominant only in the influent (NMD) and effluent samples. At the phylum level, Proteobacteria and Bacteroidetes respectively accounted for 30–50% and 5–20% in all samples. Among the phylum Proteobacteria, the classes Alphaproteobacteria and Betaproteobacteria were predominant in all samples. Deltaproteobacteria including typical SRB also accounted for high ratios. Cyanobacteria, which obtain energy via photosynthesis, accounted for 4–12% in all samples, except for the soil sample of the CW-A. However, soil samples were characterized by Actinobacteria, which are Gram-positive bacteria and Firmicutes, most of which have a gram-positive cell
wall structure. The phylum Thermi accounted for 15% of the soil sample of the CW-A. The phylum Chloroflexi accounted for 12% of the soil sample of the CW-B.

![Bacteria community compositions at the phylum level in water and soil samples of the unplanted CW (CW-A) and the cattail-planted CW (CW-B) treating the NMD.](image)

**Figure 7.** Bacteria community compositions at the phylum level in water and soil samples of the unplanted CW (CW-A) and the cattail-planted CW (CW-B) treating the NMD.

Table 3 shows the number of OTUs of typical SRB possibly producing metal sulfide precipitation in the CWs. Five families of SRB were found only in the CW-B soil sample: Desulfovibrionaceae [25], Syntrophaceae (Desulfomonile [26]), Desulfarcuclaceae [27], Desulfofobacteraceae [28], and Desulfobulbaceae [28] in the class Deltaproteobacteria. Bacteria of the family Thermodesulfovibrionaceae [29] in the phylum Nitrospirae were found in all samples except for the CW-A soil sample. The ratio of those to the total OTUs was highest in the soil sample from the CW-B (0.93%).

**Table 3.** Typical SRB (%) detected in water and soil samples of CWs treating NMD.

| Family/Genus species          | OTU_No          | Influent (NMD) | Effluent CW-A | Effluent CW-B | Soil CW-A | Soil CW-B |
|-------------------------------|-----------------|----------------|---------------|---------------|-----------|-----------|
| Desulfovibrionaceae           | OUT754, OUT683, OTU1685 | 0              | 0             | 0             | 0         | 0         | 0.19      |
| Desulfovibrio mexicanus       |                 |                |               |               |           |           | 0.29      |
| Syntrophaceae Desulfomonile   | OTU1970, OTU2672 | 0              | 0             | 0             | 0         | 0         | 0.05      |
| Desulfarcuclaceae             | OTU1430         | 0              | 0             | 0             | 0         | 0         | 0.03      |
| Desulfofobacteraceae          | OTU1330, OTU3199, OTU2681, OTU1812, OTU1826, OTU3078, OTU2773, OTU401, OTU2368, OTU2901, OTU3191 | 0              | 0             | 0             | 0         | 0         | 0.17      |
| Desulfobulbaceae Desulfofobulbus |                 | 0.24           | 0.14          | 0.05          | 0         | 0         | 0.01      |
| Thermodesulfovibrionaceae     |                 | 0.24           | 0.14          | 0.05          | 0         | 0         | 0.93      |
| Total                          |                 | 0.24           | 0.14          | 0.05          | 0         | 0         | 0.93      |
4. Discussion

This study demonstrated high removals for Cd and other metals in the pilot-scale CWs, especially in that planted with cattails (CW-B), from the NMD of A-Mine (Figures 3 and 4). The HRT of CWs for mine drainage treatment is typically a few days to several weeks [3,5]. However, in this study, HRT of 1.25 days was sufficient for Cd removal from the NMD without severe clogging. Further studies on evaluating the soil adsorption capacity of heavy metals, plant-bacteria contributions in warm seasons, and mathematical modeling will be needed for the scaling up of the CWs. At a dozen mines in Japan, the drainage includes only a few toxic metals, slightly exceeding the effluent standard in Japan with neutral pH values (5.8–8.6) like A-Mine [2]. Installation of the CWs should be assigned priority for treating such NMD. This study using pilot-scale CWs was conducted only in autumn and winter because of limitations of site use at A-Mine. Feasibility studies for high seasons for plant growth and evapotranspiration in spring and summer will be necessary for additional design of the CW.

Even in the unplanted CW (CW-A), Cd, Zn, and Cu were well removed from the NMD, resulting in its accumulation, especially in the upper soil layer (Figure 5). Therefore, the main mechanism for metal removal in the CWs should be soil adsorption, as demonstrated by our lab-scale study [16,21]. Loamy soils (akadama) have generally low organic carbon content (<0.1%) but high permeability, water retention, and metal sorption properties governed by its specific chemical constituents, especially of CaO, by synergistic effects of physicochemical sorption and ion exchange mechanisms [30].

The amounts of metals extracted by the plants were small compared to the total amounts removed by the CWs, as reported earlier [4,6,21]. The phytoextraction efficiency is related to biomass, BCF, and TF (Table 2). A specific hyperaccumulator can concentrate more than 100 ppm Cd, 1000 ppm Co, Cr, Cu, or Pb, or 10,000 ppm Zn or Ni [31]. The cattails used for this study are non-hyperaccumulating but fast-growing indigenous plants (Figure 6). Cattails generally wither in winter. Therefore, the shoots should be harvested at the end of autumn for collecting the accumulated metals. The heavy metals in the harvested biomass can be disposed to an industrial waste landfill site as the sludge produced in the coagulation process of A-Mine. Young shoots of perennial plants can germinate from the remaining rhizomes in spring.

Furthermore, cattails contributed to rhizofiltration by their elongated roots and bacterial metabolisms in the rhizosphere (Table 1, Figure 5). Many researchers have reported heavy metal retention in planted CWs as significantly higher than in unplanted CWs [9,14]. A soil layer that has accumulated heavy metals up to its adsorption capacity should be dredged for renewal of the metal-removing capability of the CW. Rhizofiltration by the plant is helpful for decreasing the dredging soil depth for CW renewal.

Diverse bacteria including SRB were detected from the CW-B soil sample (Figure 7, Table 3). This result suggests that the plant controlled rhizosphere bacteria suitable for metal removal. The plant rhizospheres generally become aerobic if oxygen is secreted to form the root sufficiently. However, oxygen consumption by root respiration becomes remarkable during nighttime or when the shoots withered. Under such conditions, an anaerobic environment can be formed in the rhizosphere in which sulfate is reduced by SRB [8]. Although the chemical forms of the metals accumulated in the CWs were not determined in this study, precipitation of insoluble sulfides such as CdS, ZnS, and CuS can enhance metal removal in the CW [4–6,11]. Metal removal by plant uptake, rhizofiltration, and bacterial sulfide formation will be enhanced in warm seasons.

5. Conclusions

Heavy metals were removed effectively from the NMD by the pilot-scale CWs at a closed mine in Kyoto prefecture in autumn and winter. Removals for Cd, Zn, Cu, Fe, and As in the CWs tended to decrease with HRT from 3.8 days to 1.2 days. Results show that HRT of 1.25 days was sufficient to achieve the effluent standard for Cd without severe clogging. The main mechanism for metal removal in the CWs was adsorption by loamy
soil filled in the CWs. Additionally, cattails contributed to metal removal by rhizofiltration and phytoextraction and incubation of SRB, possibly producing sulfide precipitates in the rhizosphere. These results encourage the spreading of CWs for mine drainage treatment in Japan. Further studies in warm seasons are needed for the scaling up of CWs.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/w13141937/s1, Table S1: Dominant OTUs (%) in water and soil samples of unplanted CW (CW-A) and cattail-planted CW (CW-B) treating NMD, Figure S1: Soil layers, cattail, and a weather station in the unplanted CW (CW-A) and the planted CW (CW-B), Figure S2: Growth and decay of cattails in the planted CW (CW-B) treating NMD for 3.5 month operation, Figure S3: Time courses of water parameters in influent and effluent in the unplanted CW (CW-A) and the cattail-planted CW (CW-B) treating NMD.

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