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Effective removal of iron, nutrients, micropollutants, and faecal bacteria in constructed wetlands cotreating mine water and sewage treatment plant effluent

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ABSTRACT

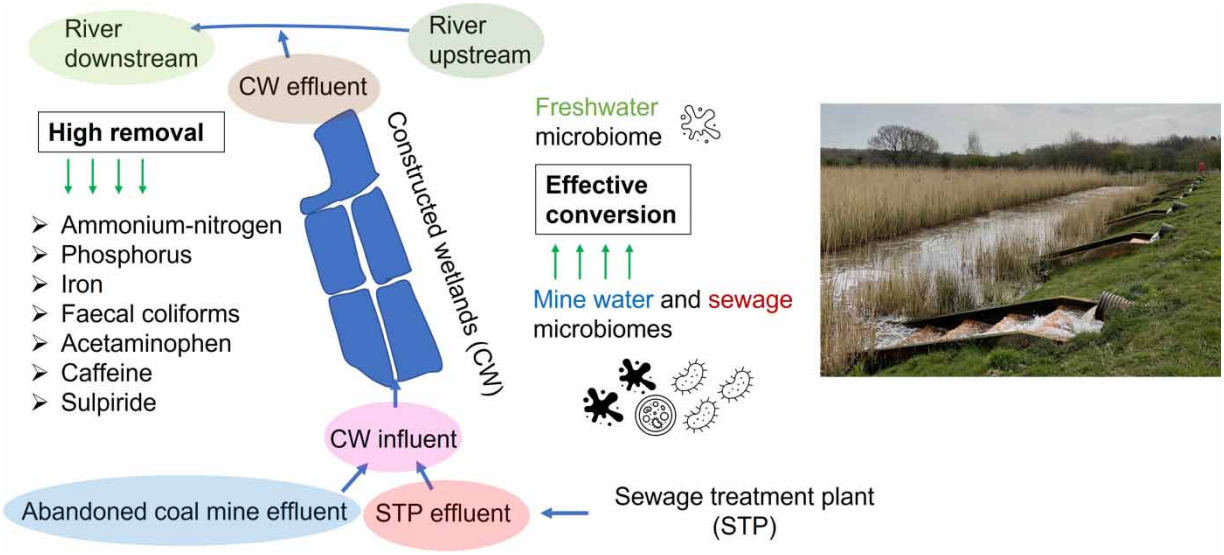
Regulators in England and Wales have set new targets under the Environment Act 2021 for freshwater quality by 2038 that include halving the length of rivers polluted by harmful metals from abandoned mines and reducing phosphorus loadings from treated wastewater by 80%. In this context, an intriguing win–win opportunity exists in the removal of iron from abandoned mines and phosphate from small sewage treatment plants by coprecipitation in constructed wetlands (CWs). We investigated such a CW located at Lamesley, Northeast England, which cotreats abandoned coal mine and secondary-treated sewage treatment plant effluents. We assessed the removal of nutrients, heavy metals, organic micropollutants, and faecal coliforms by the CW, and characterized changes in the water bacteriology comprehensively using environmental DNA. The CW effectively removed ammonium-nitrogen, phosphorus, iron, and faecal coliforms by an average of 86, 74, 98, and 75%, respectively, to levels below or insignificantly different from those in the receiving river. The CW also effectively removed micropollutants such as acetaminophen, caffeine, and sulpiride by 70–100%. Molecular microbiology methods showed successful conversion of sewage and mine water microbiomes into a freshwater microbiome. Overall, the CW significantly reduced impacts on the rural water environment with minimal operational requirements.

Key words: constructed wetlands, metals, nutrients, pathogens, river water, wastewater

HIGHLIGHTS

- The CW removed iron from mine water and phosphorus from wastewater by coprecipitation.
- The CW effectively removed nutrients, faecal bacteria, and micropollutants.
- Dissolved Cu, Zn, Mn, and total dissolved solids were not effectively removed.
- The CW converted mine water and sewage microbiomes into a freshwater microbiome.
- The CW simultaneously addressed wastewater and mine water pollution issues.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Like many other countries, England and Wales face substantial challenges in restoring their streams, rivers, and lakes to good status in line with the objectives set in The Water Environment (Water Framework Directive (WFD)) (England and Wales) Regulations 2017. Currently, only 14% of the rivers in England have good ecological status (EAC 2022). To improve this condition, the Department for Environment, Food, and Rural Affairs (Defra) has set ambitious new targets under the Environment Act 2021 for water quality by 2038 that include halving the length of rivers polluted by harmful metals from abandoned mines and reducing phosphorus loadings from treated wastewater by 80% (Defra 2022b).

There is a perceived shortage of sustainable treatment technologies for reliable phosphorus removal at smaller scales that demand minimal operating and maintenance expertise and are suited to northern latitudes (Bunce *et al.* 2018). However, an intriguing win–win opportunity exists in the cotreatment of mine water with secondary-treated sewage treatment plant (STP) effluent in constructed wetlands (CWs), as synergies occur if phosphate from sewage is effectively removed via precipitation with iron from mine water to form ferric phosphate in the CW sediments (Johnson & Younger 2006; Younger & Henderson 2014; Wang *et al.* 2021). Johnson & Younger (2006) found that a pilot scale CW ($25 \times 25 \times 0.45$ m, $L \times W \times H$) cotreating mine water and STP in the UK removed ammonium-nitrogen and iron levels to below the proposed discharge limits, and the rate of phosphate removal was proportional to influent iron concentrations, suggesting that the presence of iron flocs in the mine water promotes efficient phosphate removal by adsorption and precipitation. Similarly, Wang *et al.* (2021) also found that a CW microcosm ($0.89 \times 0.2 \times 0.2$ m, $L \times W \times H$) cotreating acid mine drainage and domestic wastewater in China effectively removed phosphate and iron through precipitation. The pilot trial by Johnson and Younger informed the construction of the world's first full-scale free-water surface flow CW system for cotreatment of mine water and STP effluent, which has been operating since 2005 in the Lamesley area of Northeast England, to improve the water quality of the receiving River Team (Welsh 2005; CoalAuthority 2018).

CWs are nature-based, also known as passive treatment, systems with low operational/maintenance requirements (Wang *et al.* 2021). They tackle pollution by taking advantage of natural and freely available resources such as sunlight, plants, and microbes (Wang *et al.* 2021). Younger & Henderson (2014) investigated the performance of the Lamesley CWs a decade ago in terms of the removal of biological oxygen demand, ammoniacal nitrogen, suspended solids, phosphate, and iron (Fe). We were interested in understanding the performance of this CW system more comprehensively across the wider range of relevant water quality metrics that drive impacts on receiving rivers, including emerging issues like micropollutants and microorganisms. The public and political debate about the status of the rivers in England and Wales is increasingly concerned with their suitability for recreation and wildlife as citizen and governmental initiatives seek to protect rivers for bathing and fishing (WaterUK 2021; EAC 2022). 'Rivers fit to swim in' are nowadays a stated ambition of the Environmental Audit

Committee (EAC 2022), government regulators, and authorities (DOHSC 2022). This means an increased focus on micropollutants and pathogens in rivers that may cause harm to wildlife and water users.

We, therefore, aimed to comprehensively assess how well combined treatment of abandoned coal mine water and secondary-treated wastewater in a CW protect the river environment from chemical and microbiological pollution. We hypothesized that the CW would (i) improve influent quality to meet all the treated effluent compliance limits, (ii) reduce chemical oxygen demand (COD), nutrients, heavy metals, faecal bacteria, and micropollutants beyond the mere dilution effect of blending secondary-treated wastewater with mine water, and consistently achieve the compliance limits under changeable weather conditions, (iii) create effluent that has no detrimental impact on the chemical, ecological, and bathing water status of the receiving river, and (iv) improve influent microbiome characteristics to produce treated effluent microbiomes resembling those of the receiving river.

2. MATERIALS AND METHODS

2.1. Study site and sampling schedule

The Lamesley free-water surface flow CW cells in Gateshead, Northeast England (Latitude 54°54′19.30″N, Longitude 1°35′57.8″W) cover a total area of 5.4 ha, as illustrated in Figure 1 and Figures S1–S3 in the Supplementary Information (SI). They comprise nine cells with impermeable bunds incorporating bentonite sealants. Four of the cells located to the north are arranged in two parallel series, while a five-cell design is located to the south, arranged in two parallel series of the four cells plus one cell at the end. They create two pairs of treatment streams that each converges on one of the two outfalls to the River Team. The CWs were planted with *Typha latifolia*, *Phragmites australis*, and *Iris pseudacorus*. The target water depth is around 15–50 cm. The CWs receive treated wastewater effluent from Northumbrian Water's Birtley STP where municipal wastewater equivalent to that of a population of 30,872 is treated by a combination of pre/post settling and trickling filter processes (Environment Agency 2022). The STP effluent is blended with mine water from the Kibblesworth mining site in an average ratio of about 1:4 (0.1:0.4 m^3/s wastewater:mine water) (more details are provided in Table S1 in SI).

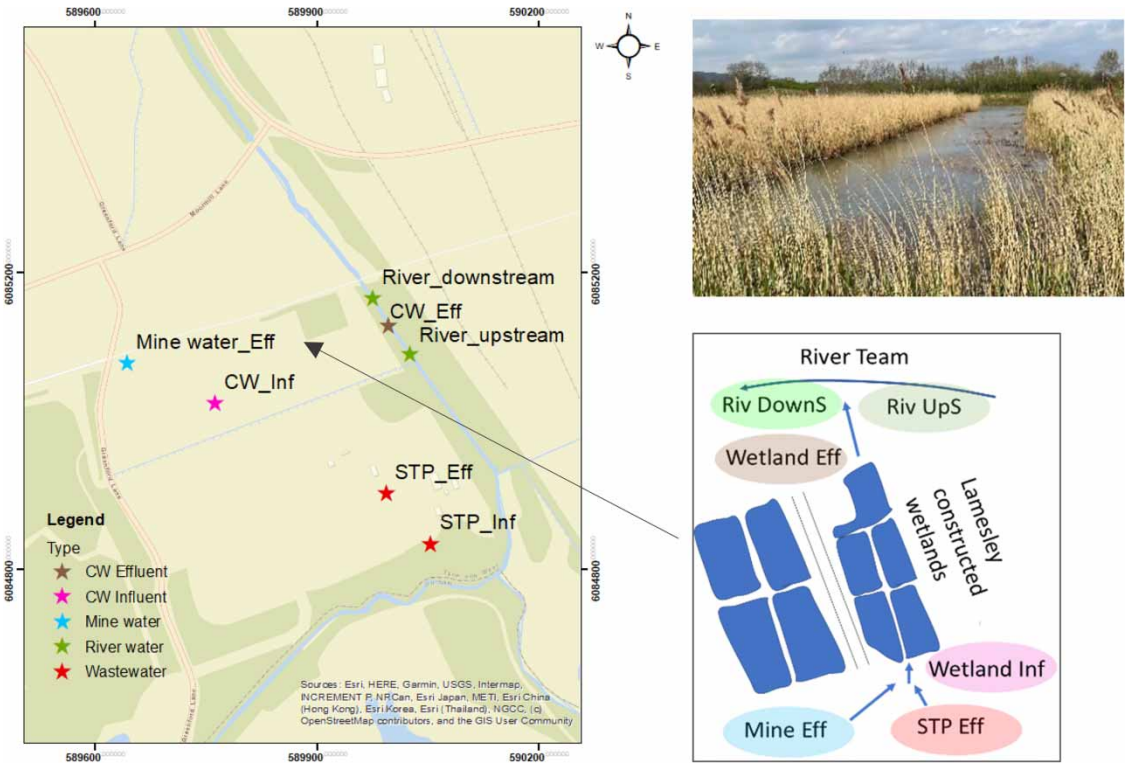


Figure 1 | Sampling locations: STP influent, STP effluent, mine water effluent, CW influent, CW effluent, river upstream, and river downstream.

Hence, the mine water dilutes the STP effluent and contributes 80% of the total flow to the CW. The hydraulic retention time (HRT) is about 10 h, as estimated from the CW area (54,000 m^2) multiplied by the average depth of the wetland (0.325 m) and divided by the flow rate of (0.5 m^3/s or 1,800 m^3/h). The two waters are mixed in underground header tanks, then routed into the CW via aeration cascades. More detailed information is provided by Welsh (2005) and Younger & Henderson (2014).

We collected grab samples from seven locations around the CW area (Figure 1) comprising STP influent, STP effluent, mine water effluent, CW influent, CW effluent, and river upstream and downstream of the CW discharge. We investigated the performance of the five-cell wetland, because its outfall is located upstream of that of the four-cell wetland. We conducted the sampling in March, May, July, and August 2021 covering the spring period (March and May) and summer period (July and August) for every sample except the STP influent that was only obtained in May, July, and August. The STP influent characteristics have already been reported in a previous publication (Zan *et al.* 2023). Weather (rainfall) conditions for the sampling events are summarized in Table S2, in the SI. A scoping study with a reduced sampling schedule had been conducted in winter, January 2020 (before the outbreak of the COVID-19 pandemic and related laboratory lockdowns).

2.2. Conventional water quality analysis

We analysed the water samples for temperature, pH, electrical conductivity, total dissolved solids (TDS), salinity, and dissolved oxygen (DO) in-situ using a precalibrated, handheld probe (Extech Instruments, Nashua, NH, USA) and a HQ40D Digital two-channel multimeter (HACH, Manchester, UK). We measured alkalinity using a digital titrator (HACH, Manchester, UK). COD, ammonium-nitrogen (NH_4^+-N), nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N), total nitrogen (TN), phosphate-phosphorus (PO_4^{3-}-P), total phosphorus (TP), and fluoride (F^-) were determined using HACH cuvette test kits LCI400, LCK339, LCK341, LCK238, LCK349, LCK349, and LCK323, respectively. Cuvette tests were performed following the manufacturer's instructions and evaluated in a HACH DR6000 Ultraviolet and Visible Spectrum Spectrophotometer. For quality assurance, we verified the cuvette tests with a blank solution (deionized (DI) water) and known concentration standards prepared from the respective nutrient salts to ensure that the result from the cuvette tests agreed with the standard concentration by $\pm 5\%$. We also filtered the water samples through a cellulose acetate syringe filter (0.45 μm, 25 mm; VWR International, UK) to measure for anions using a Dionex High Pressure Ion Chromatography instrument (ThermoFisher, UK) and for dissolved organic carbon using a carbon analyser (Vario TOC cube, Elementar Analysen Systeme GmbH, Germany). Additionally, filtered water samples were acidified with 1% v/v concentrated nitric acid and analysed for metals using a Varian Vista-MPX Inductively Coupled Plasma-Optical Emission Spectrometer or Agilent Inductively Coupled Plasma Mass Spectrometry 7700 Series instrument, as appropriate for the metal concentration (Mayes *et al.* 2021). Certified 1,000 ppm standards (accuracy of $\leq \pm 1.0\%$; VWR Chemicals, VWR International, UK) were diluted using 1% nitric acid solution for preparing calibration standards. Blanks and standards were run every seven samples to check analytical accuracy and precision.

2.3. Micropollutant analysis

We performed micropollutant analysis according to EPA method 1694 via Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS/MS) (Waters, Elstree, UK). The instrument consists of a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, USA) coupled to a Xevo TQ-S™ triple quadrupole mass spectrometer (Waters Micromass, Manchester, UK), equipped with electrospray ionization (ESI) interface (Waters, Watford, UK). The mass spectrometer was operated in both ESI modes using multiple reaction monitoring. Triplicate CW influent and effluent samples were analysed for each sampling event. We first submitted subsamples to the University of Bath for non-target screening via their liquid chromatography–high resolution mass spectrometry (LC-HRMS) system (Agilent, CA, USA). The LC-HRMS analyses were performed using an Agilent QTOF 6545 with Jetstream ESI spray source coupled to an Agilent 1260 Infinity II Quat pump HPLC with a 1260 autosampler, column oven compartment, and variable wavelength detector. We selected the most notable compounds for subsequent, quantitative UPLC-MS/MS analysis at Newcastle University. We quantified eight compounds comprising acetaminophen, diethyltoluamide (DEET), caffeine, carbamazepine, sulfapyridine, venlafaxine, sulpiride, and cetirizine. The detailed methodology including column details and instrument operating condition for both LC-HRMS and UPLC-MS/MS is provided in SI, Section 1.2 with Tables S3–S4.

2.4. Microbial analysis and data processing

We analysed faecal coliforms by membrane filtration according to Method 8074 (HACH, Manchester, UK). We also performed molecular microbiology methods using a combination of MinION nanopore sequencing of 16S rRNA gene

amplicons and quantitative polymerase chain reaction (qPCR) methods, as previously described (Acharya *et al.* 2020a; Zan *et al.* 2022). We processed sequencing data using Matlab© (Version R2019b, Mathworks, Portola Valley, CA, USA) for multivariate data analysis (cluster, principal component analysis (PCA), and analysis of similarities (ANOSIM)). We downloaded the taxonomic classification and quality of barcoded reads from the EPI2ME dashboard as a CSV file that contained information on run and read IDs and read accuracy, barcodes, and NCBI taxa IDs for classified reads. Then, we processed the CSV file with Matlab© scripts published elsewhere (Zan *et al.* 2023). The detailed methodology including instrument details is provided in SI, Section 1.3 with Tables S5–S7 and Figure S4.

2.5. Data curation and statistical analysis

Data screening found suspected contamination of the mine water sampling tap by ingress of STP effluent from the blending tank in May and August, as explained in more detail in SI with Figures S5–S6. We, therefore, excluded these two outlier samples (mine water samples in May and August) from the final analysis. This exclusion did not affect the CW performance evaluation, as CW influent and effluent samples were available for all sampling events.

We used MS Excel© and two-tailed *t*-tests to evaluate the null hypothesis that there is no difference between the mean values of two sample groupings of interest, or one-tailed *z*-tests to investigate if the mean value of a parameter meets the desired standard. We used Primer7 software (primer-e, Auckland, New Zealand) to investigate the linkage between environmental parameters and microbial communities using the BEST (Bio-Env) procedure as described by Clarke *et al.* (2014).

3. RESULTS AND DISCUSSION

3.1. Characteristics of STP influent and effluent and mine water

Table 1 summarizes the conventional water quality parameters measured at different treatment stages as mean values and standard deviations of all the sampling events, while corresponding data for each event are provided in SI with Tables S8–S11.

The untreated sewage (STP influent) had a pH of 8 with high NH_4^+-N, COD, and faecal coliform levels. After primary/secondary settling and trickling filter treatment, the secondary-treated sewage was of neutral pH, and the COD and NH_4^+-N concentrations were below the compliance limit for the STP effluent of 125 and 40 mg/L, respectively (*z*-test, *p*-values $<$ 0.01, Table 1). The mine water was not acidic (pH of 7) and had substantial alkalinity levels, presumably because of a contact with sandstone in overlaying geological strata, which helps establish a stable pH level, in line with previous studies reporting a pH of 7.1 with 755 mg/L $CaCO_3$ alkalinity for discharge from the Kibblesworth mine (Banks *et al.* 1997; Younger & Henderson 2014). Heavy metal levels in the mine water were higher for Fe and Mn, but lower for Zn, Cu, and As, as compared with the STP influent and effluent. We measured metals as dissolved concentration to avoid damaging analytical instruments, while the permissible limit for Fe was set for the total Fe concentration. But even the dissolved Fe in the mine water exceeded the permissible limit for total Fe substantially (*z*-test, *p*-value $>$ 0.05, Table 1). Similarly, the Fe level previously reported for this mine water was also high (6,000 μg/L, for total Fe) (Younger & Henderson 2014). Further treatment is thus required to reduce the Fe level in the mine water.

3.2. Blending effects and CW influent characteristics

The CW influent showed similar alkalinity, salinity, conductivity, and TDS characteristics to the mine water, and the pH remained at 7. High levels of Fe and Mn were also maintained. Since mine water had higher Fe, Mn, conductivity, TDS, salinity, and alkalinity than the STP effluent and contributed four of five parts of the blended water, only slight changes to these parameters were expected. Meanwhile, the STP effluent had much higher nutrient and faecal coliform, Cu, and Zn levels than the mine water. After the blending, an 80% reduction of these metrics will result simply from the dilution of the STP effluent with the mine water. All of this can be seen in Table 1 by comparing STP effluent, mine water, and CW influent characteristics.

3.3. CW treatment effects and compliance with discharge standards

Dilution by blending is no solution for pollution and the actual treatment achieved by the CW is revealed by comparing the effluent and influent characteristics. From Table 1, the COD level in the CW effluent was 26% lower than in the CW influent and complied with the compliance limit (125 mg/L). The NH_4^+-N concentration was significantly reduced by 86.1% from 0.91 ± 0.50 mg/L in the influent to 0.13 ± 0.16 mg/L in the effluent (*t*-test, *p*-value $<$0.05), which is well below the

Table 1 | Conventional water quality parameters of the STP influent and effluent, mine water, and CW influent and effluent relative to the UK wastewater treatment work's compliance limits

	STP influent	STP effluent	Mine water	CW influent	CW effluent	Compliance limit
NH_4^+-N (mg/L)	47.78 ± 27.06	3.07 ± 1.90	0.21 ± 0.26	0.91 ± 0.50	0.13 ± 0.16 (86.1%)	<40 and 3.5[a,b]
NO_2^--N (mg/L)	0.19 ± 0.15	0.98 ± 1.43	0.004 ± 0.00	0.17 ± 0.18	0.06 ± 0.06 (62.5%)	N/A
NO_3^--N (mg/L)	1.95 ± 1.38	24.97 ± 9.07	0.29 ± 0.24	6.81 ± 2.28	5.14 ± 2.30 (24.6%)	N/A
TN (mg/L)	80.73 ± 19.11	35.75 ± 9.26	1.40 ± 0.40	9.42 ± 4.1	6.43 ± 2.73 (31.6%)	N/A
PO_4^{3-}-P (mg/L)	5.87 ± 2.71	2.70 ± 0.93	0.25 ± 0.35	0.86 ± 0.14	0.23 ± 0.07 (73.1%)	N/A
TP (mg/L)	6.89 ± 2.96	2.86 ± 0.96	0.23 ± 0.32	1.00 ± 0.20	0.26 ± 0.08 (74.2%)	N/A
Fluoride (mg/L)	1.22 ± 0.47	0.64 ± 0.18	0.60 ± 0.10	0.59 ± 0.15	0.59 ± 0.15	N/A
Alkalinity (mg/L $CaCO_3$)	273.00 ± 47.84	51.00 ± 19.24	545.00 ± 7.07	408.25 ± 71.51	436.50 ± 84.08	N/A
Salinity (mg/L)	530.00 ± 29.82	396.75 ± 61.67	1,525 ± 35.36	1,205.00 ± 283.37	1,320.00 ± 154.92	N/A
pH	8.41 ± 0.13	7.20 ± 0.31	7.25 ± 0.23	7.12 ± 0.14	7.54 ± 0.19	N/A
Conductivity (μS/cm)	1,072.67 ± 64.27	804.00 ± 105.87	2,985 ± 49.5	2,335.00 ± 523.10	2,595.00 ± 238.12	N/A
TDS (mg/L)	749.00 ± 44.19	578.75 ± 84.55	2,100.00 ± 28.28	1,656.25 ± 374.37	1,815.00 ± 154.16	N/A
DO (% saturation)	22.10 ± 11.21	52.75 ± 15.81	62.79 ± 11.03	61.31 ± 8.30	62.17 ± 9.91	N/A
COD (mg/L)	636.83 ± 362.25	65.89 ± 23.69	11.88 ± 0.38	22.41 ± 4.71	16.38 ± 4.33 (26.0%)	<125[c]
DOC (mg/L)	22.77 ± 2.98	15.61 ± 2.01	3.44 ± 2.51	7.27 ± 3.12	6.72 ± 2.00	N/A
Temperature °C	17.13 ± 2.81	14.03 ± 5.95	11.60 ± 9.33	13.40 ± 5.99	13.73 ± 5.23	N/A
Faecal coliform (log_{10}CFU/100 mL)	6.49 ± 0.35	4.98 ± 0.37	0 CFU/100 mL	3.87 ± 0.46	3.28 ± 0.27 (74.8%)	N/A
Heavy metals (μg/L)[d]						
Fe	40.00 ± 10.00	45.00 ± 17.32	2,057.5 ± 689.43	880.00 ± 635.94	18.75 ± 8.54 (97.9%)	<2,000[a,e,f]
Mn	26.67 ± 15.28	43.75 ± 11.09	1,137.50 ± 38.89	756.25 ± 95.95	516.25 ± 296.18 (31.7%)	N/A
Pb	0.43 ± 0.15	0.00 ± 0.00	0.05 ± 0.07	0.07 ± 0.08	0.04 ± 0.04 (45.4%)	N/A
Zn	35.00 ± 8.66	57.50 ± 9.57	5.00 ± 7.07	27.50 ± 9.57	27.50 ± 28.72 (0.0%)	N/A
Cu	18.80 ± 12.65	10.00 ± 11.55	0.10 ± 0.15	1.66 ± 1.29	3.71 ± 1.59 (–)	N/A
As	0.88 ± 0.19	0.75 ± 0.29	0.21 ± 0.29	0.32 ± 0.23	0.17 ± 0.11 (48.4%)	N/A

Notes: Results were reported to two decimal places as Mean ± *SD*. STP effluent and CW influent and effluent were sampled in March, May, July, and August, the STP influent, in May, July, and August, and the mine water, in March and July. The numbers in parentheses represent the average percent removal of nutrients, COD, heavy metals, and faecal coliforms in the CWs.

[a]The Water Resources Act (1991): Consent to Discharge from the Environment Agency (consent number 235/1891): site-specific consent for Birtley sewage treatment work and Lamesley CWs.

[b]The limit for STP effluent from (i) and CW effluent from (ii), respectively.

[c]The Environment Agency's compliance limits for treated wastewater discharge.

[d]Dissolved metal concentration (μg/L).

[e]The limit for CW effluent.

[f]For total iron (Fe) (μg/L).

compliance limit specified for Lamesley CW discharge (3.5 mg/L) (*z*-test, p-value < 0.01). Nitrification of ammonium coupled with denitrification can remove TN in CWs (Vymazal 2007; Younger & Henderson 2014; Wang *et al.* 2021). Accordingly, mean values of all measured forms of nitrogen (NH_4^+-N, NO_2^--N, NO_3^--N, and TN) were reduced in the CW effluent, relative

to the CW influent. There was also a significantly lower TP concentration in the CW effluent than the influent (*t*-test, *p*-value < 0.01).

For the heavy metals, the dissolved Fe concentration was reduced by 97.9% from 880.00 $\pm$ 635.94 μg/L in the CW influent to 18.75 $\pm$ 8.54 μg/L in the effluent, which is more than a factor of 100 below the limit for the total Fe at 2,000 μg/L (*z*-test, *p*-value < 0.01). The simultaneous TP and Fe removal is the result of the sorption of phosphate by ferric hydroxide and precipitation as ferric phosphate (Dobbie *et al.* 2009; Younger & Henderson 2014). This CW is of neutral pH, and with the influent aeration in cascades, the Fe removal through oxidation and precipitation is enhanced (Wang *et al.* 2021). Ferrous iron (Fe^{2+}) is rapidly converted to ferric iron (Fe^{3+}) in oxidizing conditions and ferric iron can form ferric hydroxide at a pH of more than 3.5 (Jarvis *et al.* 2012). Moreover, with the mixture of wastewater and mine water, suspended solids in the wastewater provide nuclei and counterions (phosphate) for the generation of iron flocs, hence accelerating the precipitation of ferric hydroxide (ochre) and ferric phosphate (Johnson & Younger 2006). Certain microbes can also contribute towards the formation of such phosphate minerals by transforming organic phosphorus into phosphate (Gadd 2010). In this surface flow CW, the resulting sediment deposits did not cause any blockages, but after 15 years, the accumulated sediment needed to be removed to create free space for further treatment (NorthumbrianWater 2022).

In contrast to Fe, there was no significant difference in the Mn levels of the CW effluent and influent (*t*-test, *p*-value > 0.05). The persistent level of Mn is likely because the hydroxide solubility product of Mn is higher than for Fe, and very high pH ($\approx$10) is required to immobilize Mn as hydroxide to get adsorbed onto the soil/sediment or get removed as Mn hydroxide precipitates (Jarvis *et al.* 2012). Furthermore, Mn removal is inhibited by high Fe concentrations in water (Neculita & Rosa 2019). Pb, Zn, Cu, and As were also inefficiently removed in the CWs, which could again be ascribed to complex geochemical factors. For example, Zn requires a pH of 8.2 for effective removal as its hydroxide (Jarvis *et al.* 2012). Cu concentrations were highly variable in both CW influents and CW effluents presumably due to the re-dissolution of the Cu precipitates and because they were also being released back by plants, but without a statistically significant difference in the mean values. These observations are important amendments to previous work, which only monitored Fe to assess heavy metal removal (Younger & Henderson 2014).

3.4. Variation in nutrient and heavy metals removal between sampling events

Tables S13–S14 in SI show how the concentrations and removal efficiencies (%) of the key pollutants by the CW varied between the four sampling events in March, May, July, and August. From the rainfall data in the 24 h before the sampling, May stood out as the sampling event with the wettest conditions and the highest STP effluent loading (Table S2 in SI). This wet weather seemed to affect nutrient removal. There was only 37% removal of NH_4^+-N in May, versus >90% removal in the other three sampling events. A slightly negative removal of NO_3^--N also occurred in May, versus the positive removal in the other three sampling events. The performance of biological nitrogen removal will be influenced by rainfall and implications for HRT, as well as temperature (García *et al.* 2003; Shingare *et al.* 2019). Regarding temperature, winter sampling in our scoping study showed efficient removal of nutrients, faecal coliforms, and Fe in January (Table S15 in SI). A surprising lack of seasonal variation in biological nutrient removal by this CW was previously noted by Younger & Henderson (2014), who attributed it to the steady temperature of mine water all year round. The buffering of temperature variation is thus another benefit of cotreating mine water with sewage in CWs.

3.5. Removal of organic micropollutants in the CW

Figure 2 shows that the CW removed the eight monitored micropollutants with variable efficiencies. There were high removals (70–100%) of acetaminophen, caffeine, and sulpiride, and low to moderate removals of DEET, carbamazepine, sulfapyridine, venlafaxine, and cetirizine in March, July, and August when the weather was dry. Similarly, Ilyas & van Hullebusch (2020) reported on the high removal of acetaminophen and caffeine in free-water surface flow CW, which most likely results from aerobic biodegradation. The aeration cascades of the studied CW can hence enhance the removal of these compounds by improved aerobic conditions. In line with our findings, poor to moderate removals were previously reported for carbamazepine, venlafaxine, and DEET, which is likely a consequence of the poorer biodegradability of these compounds (Ilyas & van Hullebusch 2020). Also, DEET has low light sensitivity and poor photodegradability (Li *et al.* 2017). Decreased removals of nearly all compounds were observed when the weather was at the wettest. The seemingly negative removal of caffeine, sulfapyridine, and cetirizine in the CW in May could be attributed to rainfall effects. The rainfall in the 24 h before the sampling likely reduced the concentrations of these compounds in the influent, while the levels at the

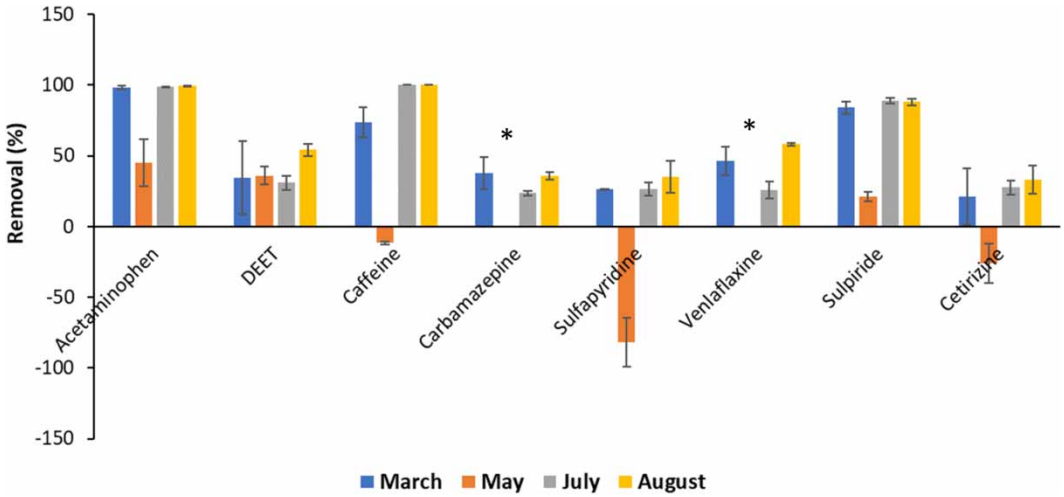


Figure 2 | Removal efficiency (%) of acetaminophen, DEET, caffeine, carbamazepine, sulfapyridine, venlaflaxine, sulpiride, and cetirizine by the CW in March, May, July, and August. Error bars were calculated as a standard deviation of triplicate samples. *Data were excluded if the removal was not significantly different from zero.

outlet could still be high from the prerain higher influent concentrations (Sossalla *et al.* 2020). Overall, a significant treatment benefit was observed in terms of micropollutant removal in the CW, which adds to the previously reported benefits of effective nutrient and Fe removal (Younger & Henderson 2014). The micropollutant concentrations are provided in Table S17 in SI.

3.6. Removal of faecal bacteria and putative pathogens in the CW

Faecal coliforms were reduced by 74.8% in the CW (Table 1), although without statistical significance owing to the high variability of concentrations. Additionally, in July we analysed for the abundance of *Escherichia coli*-producing extended-spectrum *β*-lactamases, which gives them resistance to commonly used antibiotics, including penicillins and cephalosporins (Rawat & Nair 2010) (Table S16 in SI). We found a substantially lower abundance of them in the CW effluent as compared with the influent (*t*-test, p-value < 0.01), indicating that the CW could remove the antibiotic-resistant *E. coli*.

With molecular methods more detailed insight can be obtained with regard to the microbial water quality, although there are currently no related standards. This may change in the future as molecular methods are increasingly being used to monitor wastewater treatment and attribute faecal pollution to its sources (Ahmed *et al.* 2019; Bunce *et al.* 2020). As seen in Figure 3, there was an overall higher absolute abundance of sequenced 16S rRNA genes attributed to bacterial genera-containing putative human pathogens (i.e., *Aliarcobacter*), or faecal indicator bacteria, as well as specific genetic markers quantified by qPCRs for *E. coli* (*rodA*) and human host-associated *Bacteroides* (HF183), in the CW influent compared with the effluent in all sampling months. For *Vibrio cholerae*, a qPCR assay was also carried out, but the genetic marker (*ompW*) was not detected in any of the samples, contrary to our findings in Ethiopia (Hiruy *et al.* 2022), where the cholera is endemic (Park *et al.* 2022).

There was >82% removal of the absolute abundance of genes from both *Aliarcobacter* and the faecal indicator bacteria by the CW in all sampling months. For *Aliarcobacter* (Figure 3(a)), the genus showed higher abundance in July and August relative to March and May. *Aliarcobacter* is normally found in untreated and treated sewage and the genus includes several pathogenic species (Chieffi *et al.* 2020). Some *Aliarcobacter* species remain viable in the water environment as they are aerotolerant (do not require oxygen for growth but can tolerate its presence) and can survive in low water temperatures (Chieffi *et al.* 2020). For genes from faecal indicator bacteria (Figure 3(b)), the genus *Prevotella* predominated in the 16S rRNA gene amplicon libraries of the CW influent samples in March, May, and July. *Prevotella* is also reported to be highly abundant in sewage (Fisher *et al.* 2015).

Several mechanisms could be responsible for the removal of these bacteria in the CW including sedimentation, filtration, adsorption, oxidation, solar disinfection, root exudation of biocides, and predation by protozoa (Wu *et al.* 2016). *P. australis*, the plant established in the CW, produces bactericidal substances such as phenolic compounds and secondary metabolites,

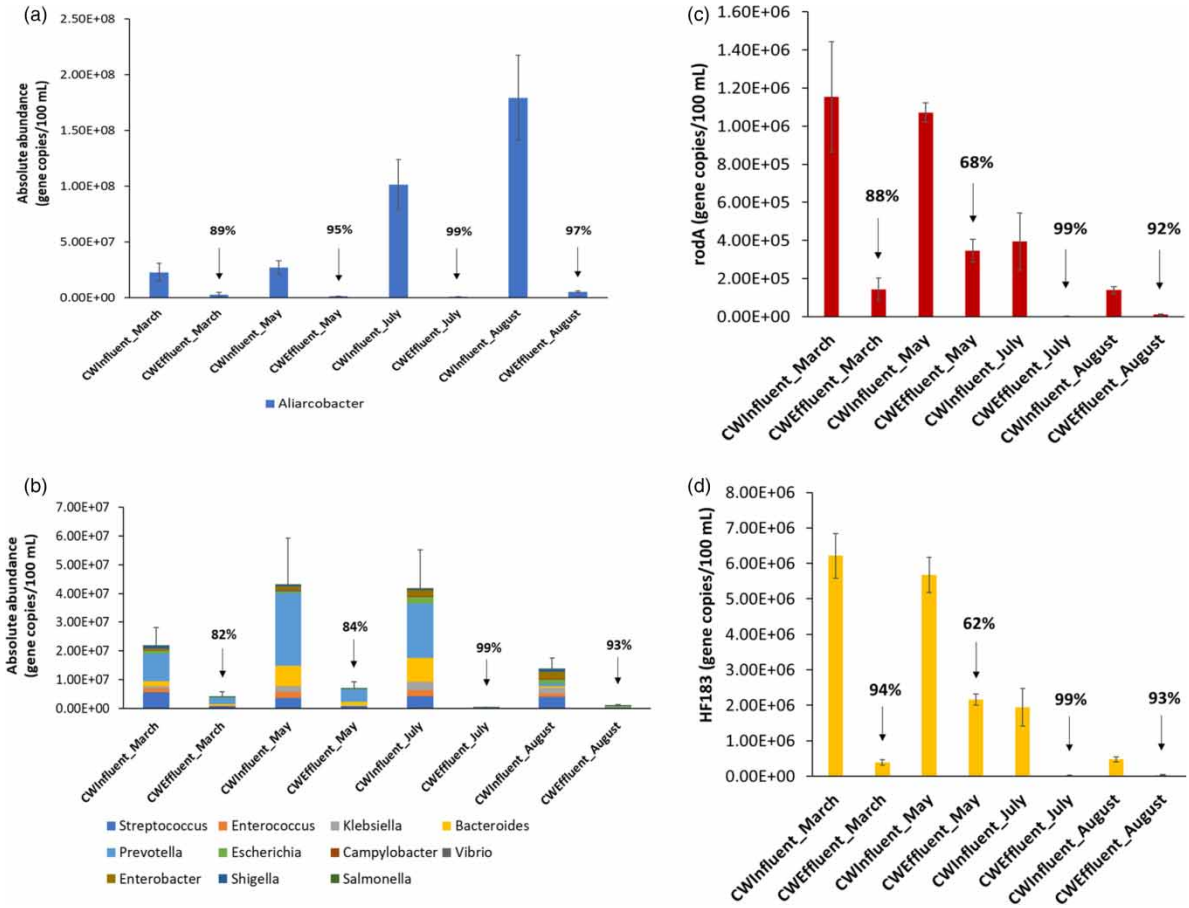


Figure 3 | Absolute abundance (gene copies/100 mL) of selected genera containing a putative human pathogen (*Aliarcobacter*) (a), faecal indicator bacteria (b), and qPCR data for *rodA* (*E. coli*) (c), and HF183 (*Bacteroides*) (d). (a) and (b) were obtained by multiplying relative abundance from MinION 16S rRNA gene sequencing with qPCR quantification of 16S rRNA gene copy numbers in each sample of the CW influent and CW effluent of four sampling events (March, May, July, August). (c) and (d) were obtained from a specific gene by qPCR. Error bars were calculated as a standard deviation of the total absolute abundance of the selected bacteria of triplicate samples in each month. The percentage above each bar indicates the overall % removal by the CWs in each sampling event.

e.g., tannins, terpenoids, alkaloids, and flavonoids that kill pathogenic/faecal indicator bacteria (Cowan 1999; Shingare *et al.* 2019; Dan *et al.* 2020). Pathogen removal in CWs can vary with seasons and weather conditions, being higher in the summer than the colder periods, which could be attributed to the increased temperature and UV radiation (Wu *et al.* 2016; Shingare *et al.* 2019). Moreover, CW showed higher pathogen removal in the dry/warm weather than in the wet weather (Alufasi *et al.* 2017; Shingare *et al.* 2019). Similarly, this study found higher removal in the summer (July and August) than in spring (March and May), and the lowest removal of genetic markers attributed to faecal bacteria was found in May (Figure 3(c) and 3(d)) when the weather was at the wettest. The high removal of *rodA* in August was different from the low removal of faecal coliform obtained from culturing in August (Table S13 in SI). But such low removal of faecal coliforms could be artefactual because faecal coliform counts in August were unusually low in the CW influent. Bacterial abundance estimates typically differ between culturing and qPCR methods and are generally higher by qPCR. This is because the culturing methods demonstrate the viability of the cells, although not all bacteria can be cultured, while in genomic methods the targeted genes could be from both viable and damaged cells or extracellular DNA (Figueroa-González & Pérez-Plasencia 2017; Acharya *et al.* 2020b). The *rodA* and HF183 results aligned with a previous study by Bunce *et al.* (2020) who found a similarly high abundance of these two genes (10^6–10^7 gene copies/100 mL) in small STP influents in the UK. In that study, the small STPs showed mean removal of around 98 and 95% for *rodA* and HF183, respectively. In summary, the CW could efficiently remove pathogens and faecal bacteria across all sampling events, but with reduced efficiency due to rainfall in May.

3.7. Impacts of the CW discharge on water quality in the receiving river

Table 2 shows the water quality of the River Team upstream and downstream of the CW discharge in relation to the WFD standards for rivers. The combined mine water and STP effluents contribute substantially to the flow in the River Team in dry weather conditions ((Welsh 2005) and Table S1 in SI), meaning that there is limited dilution of the discharge. From Table 2, there was no significant difference between NH_4^+-N concentration in the river upstream and downstream of the discharge (t-test, p-value > 0.05). The NH_4^+-N concentration was indicative of moderate status for the upstream, and of good status for the downstream, of the discharge meaning that the discharge may even have improved the river water quality in terms of ammonium concentrations.

PO_4^{3-}-P concentration in both upstream and downstream river samples indicated poor status with respect to this nutrient. Overall, there was no significant difference of TN and TP concentration in the river upstream and downstream samples (t-test, p-value > 0.05 for both TN and TP) implying no significant impact of the CW discharge on the nutrient status in the receiving river. High PO_4^{3-}-P levels were already noted in the river upstream of the discharge, and therefore attributed to upstream sources, which include an STP at the East Tanfield. If the STP effluent from Birtley had been discharged directly into the river, it would have further augmented P levels in the river as there was higher PO_4^{3-}-P concentration in the STP effluent (Table 1) than in the river upstream (Table 2). Cotreatment of STP effluent and mine water thus demonstrated a clear benefit of P removal that will benefit the River Team in terms of reduced P loading.

The pH of both the upstream and downstream samples was in the desired range. The DO as % saturation at the 10th percentile was indicative of the high status of the river in terms of its oxygenation. The water temperature in both upstream and downstream samples was also indicative of high status. There was significantly higher alkalinity, salinity, conductivity, and TDS in the river downstream relative to the river upstream (t-test, p-value < 0.01, for all). This is a consequence of the mine water characteristics and the poor removal of the main soluble ions in water like calcium, magnesium, chloride, and sulphate in the CW (Table S12 in SI).

In terms of heavy metals, the Fe, Pb, Cu, and As concentrations were below the standards (z-test, p-value < 0.01, for all) for both river upstream and downstream samples, while the mean values of Zn and Mn were only marginally below the limit without statistical significance (z-test, p-value > 0.05, for both). This indicates a potential risk of detrimentally affecting the receiving river because of the bioavailable levels of some metals (Mn from the mine water and Zn from the sewage) that are poorly removed in the CW.

There are currently no standards for bacteria in UK rivers except for two sites that are regulated as designated bathing waters (WFD 2017; Defra 2022a). However, this may change in the future as the public and the authorities become increasingly concerned about sewage impacts on recreation in rivers (DOHSC 2022; EAC 2022). The numbers of faecal coliform were high in both the river upstream and downstream samples, but lower in the downstream. They were converted to estimated numbers of *E. coli* to compare with the Bathing Water regulations. These estimated *E. coli* numbers exceeded the limit for sufficient bathing water status (900 CFU/100 mL as 90th percentile) by an order of magnitude.

Environmental DNA analysis may provide a powerful tool for comprehensive and real-time monitoring of wastewater treatment systems and freshwater quality in the future (Werner *et al.* 2022). To illustrate this concept, a PCA of 16S rRNA gene sequencing data (Figure 4) compares the bacteriology of the CW influent and effluent samples with the river water samples upstream and downstream of the CW discharge. The first three principal components (PCs) accounted for approximately 60% of the observed variance between these samples. The 15 most notable bacterial genera (i.e., variables) explaining the variance in the three-dimensional space were illustrated by the purple arrows. For the CW influent, these included notable genera-containing putative human pathogens that can be isolated from sewage samples such as *Aliarcobacter* (Chieffi *et al.* 2020) and other genera that dominate bacterial communities within sewerage systems, such as *Acinetobacter* and *Trichococcus* (VandeWalle *et al.* 2012). Interestingly, the genus *Acinetobacter* contains denitrifying bacteria that use Mn^{2+} as an electron donor (Su *et al.* 2015). These bacteria may be involved in the denitrification that occurred in the CW based on the chemical evidence in Table 1. The PCA plot also highlighted many genera-containing freshwater bacteria such as *Aquirufa*, *Polynucleobacter*, *Flavobacterium*, *Rhodoferax*, and *Orrella* (Newton *et al.* 2011; Pitt *et al.* 2019; Sheu *et al.* 2020; Hiruy *et al.* 2022). As expected, these bacteria were most notable in the river samples. PC1 separated the wastewater from the surface water samples (empty versus filled symbols), which had negative loadings of putative human pathogens such as *Aliarcobacter,* and positive loadings of freshwater bacteria such as *Aquirufa* and *Polynucleobacter*. The CW effluent samples in March, July, and August were clearly shifted in a positive sign direction relative to the CW influent samples in these months, which

Table 2 | Conventional water quality parameters of the river upstream and downstream of the CW discharge relative to the Water Framework Directive's standards

	River upstream	River downstream	Standard[a]
NH_4^+-N (mg/L) *90th percentile*	0.65 ± 0.24 *0.96*	0.33 ± 0.12 *0.48*	Quality 0.3 = high, 0.6 = good, 1.1 = moderate, 2.5 = poor
NO_2^--N (mg/L)	0.17 ± 0.09	0.10 ± 0.03	N/A
NO_3^--N (mg/L)	6.69 ± 1.96	5.52 ± 1.09	N/A
TN (mg/L)	9.09 ± 2.92	7.20 ± 1.70	N/A
PO_4^{3-}-P (mg/L)	0.46 ± 0.19	0.31 ± 0.14	Quality[b] 0.04 = high, 0.08 = good 0.19 = moderate, 1.03 = poor
TP (mg/L)	0.50 ± 0.19	0.33 ± 0.15	N/A
Fluoride (mg/L)	0.39 ± 0.15	0.53 ± 0.20	N/A
Alkalinity (mg/L $CaCO_3$)	130.75 ± 34.64	312.00 ± 77.49	N/A
Salinity (mg/L)	414.00 ± 56.26	987.25 ± 168.81	N/A
pH *5th and 95th percentile*	8.02 ± 0.30 *7.54–8.02*	7.64 ± 0.21 *7.30–7.64*	6–9
Conductivity (μS/cm)	852.25 ± 111.36	1,960.50 ± 305.28	N/A
TDS (mg/L)	595.25 ± 88.94	1,371.75 ± 206.80	N/A
DO (% saturation) *10th percentile*	83.15 ± 6.08 *75.36*	72.59 ± 5.20 *65.93*	Quality 70 = high, 60 = good 54 = moderate, 45 = poor
COD (mg/L)	25.53 ± 9.45	20.31 ± 3.24	N/A
DOC (mg/L)	9.24 ± 2.25	7.22 ± 1.09	N/A
Temperature °C *98th percentile*	11.98 ± 5.02 *22.30*	12.15 ± 4.81 *22.00*	Quality 25 = high, 28 = good 30 = moderate, 32 = poor
Faecal coliform (log_{10}CFU/100 mL) *90th percentile*[c] *95th percentile*[c]	3.38 ± 0.48 *10147.10 (8554.00)*[d] *15265.47 (12868.79)*[d]	3.31 ± 0.39 *6508.83 (5486.95)*[d] *9062.40 (7639.60)*[d]	Quality[e] (CFU/100 mL) 500 = excellent[f] 1,000 = good[f] 900 = sufficient[g]
Heavy metals (μg/L)[h]			
Fe	17.50 ± 5.00	25.00 ± 12.91	<1,000
Mn	140.00 ± 92.01 *106.50 ± 94.47*[i]	435.00 ± 250.13 *121.51 ± 61.27*[i]	<123 bioavailable
Pb	5.00 ± 5.77 *0.18 ± 0.19*[i]	5.00 ± 4.08 *0.19 ± 0.19*[i]	<1.2 bioavailable
Zn	35.00 ± 12.91 *9.14 ± 4.20*[i]	33.75 ± 4.79 *10.80 ± 1.50*[i]	<12.3 bioavailable
Cu	4.10 ± 1.04 *0.16 ± 0.04*[i]	2.53 ± 1.16 *0.08 ± 0.04*[i]	<1 bioavailable
As	0.34 ± 0.23	0.26 ± 0.20	<50

Notes: Results were reported to two decimal places as Mean ± *SD* or percentile (in italics) of four sampling events. Values in percentile were provided for comparison with the standard as required by the directive.

The River Team is currently not a designated bathing river.

[a]The WFD Standards (England and Wales) 2015 for river.

[b]Based on the standard for river upstream.

[c]CFU/100 mL. Estimated numbers of *E. coli* (CFU/100 mL) are shown in parentheses after the faecal coliform numbers.

[d]Not all but most faecal coliforms (about 75–93%) are *E. coli* (Hamilton *et al.* 2005). Hachich *et al.* (2012) recommended 84.3% for the conversion.

[e]The Bathing Water Regulations 2013 for *E. coli* in inland surface waters.

[f]Based upon a 95th percentile evaluation.

[g]Based upon a 90th percentile evaluation.

[h]Dissolved metal concentration (μg/L).

[i]Bioavailable concentration (μg/L) was calculated using the UKTAG tool. 'Bioavailable' means the fraction of the dissolved concentration of such metal is likely to result in toxic effects as determined using the UKTAG Metal Bioavailability Assessment Tool.

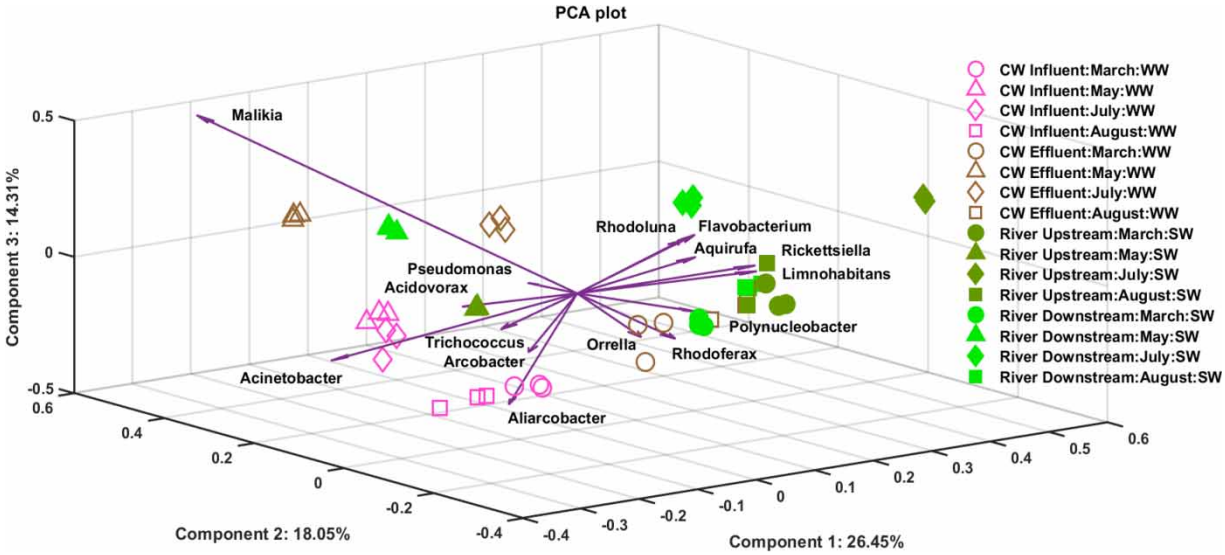


Figure 4 | PCA plot of the microbial community dissimilarity among CW influent, CW effluent, river upstream, and river downstream of four sampling events (March, May, July, August). Wastewater (WW) and surface water (SW) indicate the type of water samples, which are wastewater (empty symbols) and surface water (filled symbols), respectively. The three PCs (components 1, 2, and 3) were plotted showing the scores (circles, triangles, diamonds, and squares) and the top 15 loadings (genera), (arrows) explaining the variance in the three-dimensional space. The percentage of variation accounted for by each PC is shown with the axis label.

demonstrates the treatment benefit. This outcome aligned with our previous study of this CW to demonstrate on-site sequencing in April 2019 (Acharya *et al.* 2020a). The same trend applied in May (pink and brown triangles), although with a much smaller shift than in the other months, presumably due to the shorter HRT in the CW following rainfall. In May, the upstream and downstream river samples (green triangles) were all shifted significantly in a negative sign direction along PC1, having a much stronger sewage signature as compared with the samples from March, July, and August. This is also evidenced by the qPCR data for HF183 (*Bacteroides*) and *rodA* (*E. coli*) for the river upstream and downstream samples (Table S18 in SI). This is highly likely due to faecal pollution of the river in the upstream via discharge from combined sewer overflows and other rainfall-related inputs. PC2 separated March samples from the samples in the other months, with a reduced influence in March of *Acinetobacter* and a greater influence of *Aliarcobacter* for the wastewater bacteria, and a reduced influence of *Flavobacterium* and a greater influence of *Rhodoferax* for the river bacteria. Finally, PC3 highlighted a further shift in the community of CW influent and effluent samples. The sequencing data thus revealed how the CW could convert sewage and mine water microbiomes into those more akin to a freshwater microbiome.

3.8. Relationships between chemical and microbial water quality

Figure 5 shows a PCA and cluster analysis of physicochemical parameters and microbial parameters derived from culturing (faecal coliforms) and qPCR of bacterial marker genes (16S for total bacteria, *rodA* for *E. coli*, and HF183 for human host-associated *Bacteroides*). In the PCA plot (Figure 5(a)), PC1 showed the impact of the CW treatment by clearly separating CW influent samples (pink empty symbols) from the CW effluent samples (light brown empty symbols). Along component 2, the river upstream samples (dark green filled symbols) were clearly separated from the CW effluent samples (light brown empty symbols) with the river downstream samples (light green filled symbols) from different sampling months scattering in between the two groups. This illustrates that the river downstream samples are a mixture of the CW effluent and river upstream samples, in terms of their biogeochemistry. The four types of samples were a significant factor when considering the similarities of the biogeochemical characteristics (one-way ANOSIM, p-value < 0.01 and $R = 0.44$), while sampling month overall had a lower, but still significant effect (p-value < 0.05 and $R = 0.19$). The 17 variables explaining the variance in the PC1 and PC2 spaces are illustrated by the purple arrows in Figure 5(a). The CW influent samples were characterized by positive loadings of the nutrient parameters including TP, phosphate-P, ammonium-N, and nitrite-N, along with microbial parameters indicative of faecal matter, obtained from both plate count (faecal coliforms) and qPCR (*rodA*, HF183) methods.

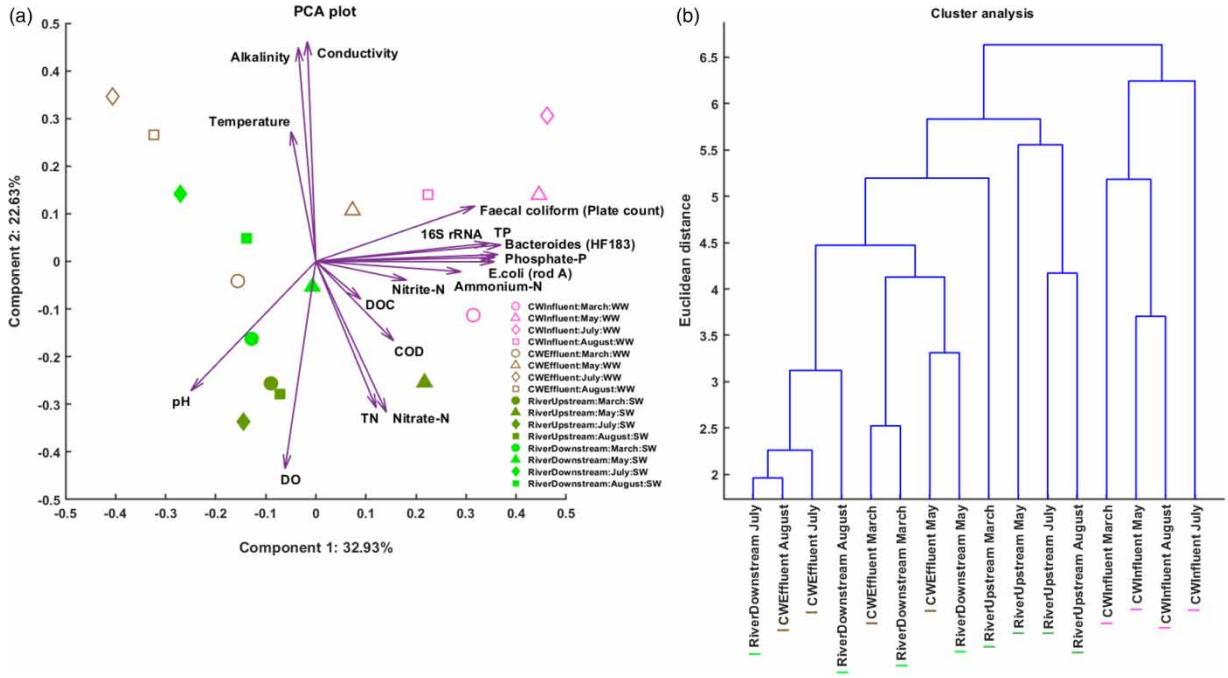


Figure 5 | (a) PCA and (b) cluster analysis combining chemical with microbial parameters to assess dissimilarity among CW influent, CW effluent, river upstream, and river downstream samples from four sampling events (March, May, July, August). WW and SW indicate the type of water samples, which are wastewater (empty symbols) and surface water (filled symbols), respectively. For the PCA, the first 2 PCs (components 1 and 2) were plotted showing the scores (circles, triangles, diamonds, and squares) and 17 loadings (arrows) of the 13 physicochemical parameters and 4 microbial parameters obtained from plate counting and the qPCRs of specific genes explaining the variance in the two-dimensional space. The percentage of variation accounted for by each PC is shown with the axis label.

This demonstrates a clear link between these nutrients, the overall size of the microbial community as measured by 16S rRNA gene copies, and the abundance of faecal bacteria (*rodA*), including from human hosts (HF183). The removal of such nutrients and faecal bacteria is then shown by the reduced or negative loadings of these parameters for the CW effluent samples. In comparison with the river upstream samples, the CW influent and effluent samples were characterized by positive loadings of alkalinity and conductivity due to the minerals dissolved in the mine water, and the higher temperature. The river upstream samples were characterized by a positive loading of DO saturation, which was likely due to the faster and more turbulent flow characteristics of the river as compared with the CWs. Cluster analysis (Figure 5(b)) shows that the greatest dissimilarities across chemical and microbial parameters were between the CW influent samples from all the sampling months and the other samples. Compared with the CW influent, the CW effluent was more similar to the river water samples in all months, which further illustrates the benefit of the CW water treatment. Nonetheless, the river downstream samples clustered more closely with the CW effluent than the river upstream samples, which shows that the CW discharge still had an impact on the river water, mainly in terms of its physicochemical characteristics (conductivity, alkalinity, pH, temperature, DO) and total and nitrate-nitrogen.

Additionally, Table S19 in SI shows that the variables PO_4^{3-}-P, pH, DO, and the temperature can best explain the dependency of the microbial community composition on the environmental conditions.

4. CONCLUSIONS

CWs designed for cotreatment of secondary-treated STP effluent and mine water in an average ratio of 1:4 had lower effluent than influent COD, nutrient, Fe, and faecal coliform levels and consistently achieved all compliance limits. However, levels of manganese, lead, copper, and zinc were not significantly reduced by the CW. The CW effectively removed the micropollutants acetaminophen, caffeine, and sulpiride, and to a lesser extent DEET, carbamazepine, sulfapyridine, venlafaxine, and cetirizine. The CW discharge did not detrimentally alter the nutrient status of the receiving river but could detrimentally

affect the river due to the bioavailable concentration of manganese and zinc, which were near the respective guidance limits in the WFD. From the molecular microbiology data, the CW treatment resulted in the removal of putative human pathogen and faecal indicator bacteria, and consequently reduced the impacts of the STP effluent on the recreational value of the receiving river. CWs are a suitable nature-based treatment option to polish effluent from small STPs in rural areas by synergistic cotreatment with effluent from abandoned mines in a single system.

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DATA AVAILABILITY STATEMENT

16S sequencing data generated in this project has been submitted to the NCBI Sequence Read Archive (SRA) with BioProject accession number PRJNA837409. Additional data created during this research are openly available (https://doi.org/10.25405/data.ncl.24937038). Please contact Newcastle Research Data Service at rdm@ncl.ac.uk for access instructions.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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