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Research article

Treated acid mine drainage and stream recovery: Downstream impacts on benthic macroinvertebrate communities in relation to multispecies toxicity bioassays

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ABSTRACT

The success and long term effectiveness of extensive and expensive engineering solutions to restore streams impacted by Acid Mine Drainage (AMD) is rarely tested. Concentrations of pollutants were measured in water along a longitudinal gradient from a stretch of the Tweelopie stream, South Africa, that receives pH-treated acid mine drainage (AMD) from an abandoned gold mine. The biotoxic effects of treated AMD were determined through macroinvertebrate biotic indices (SASS5) and a battery of toxicity bioassays. These included the *L. sativa, A. cepa, D. magna* toxicity and Ames mutagenicity tests, as well as an *in vitro* human liver cancer cell line HepG2. Even though the Tweelopie stream was moderately to severely degraded by multiple anthropogenic stressors, the impact of the treated AMD was masked by the improvement in the system downstream after mixing with the domestic wastewater effluent receiving stream, and subsequent further dilution as a result of the karst springs downstream. The general improvement of the system downstream was clearly shown by the decrease in the ecotoxicity and mutagenicity in relation to the in-stream macroinvertebrates. PCA multivariate analysis successfully displayed associations between the different environmental variables and the decrease in toxicity and subsequent ecosystem improvement downstream. This study indicated that environmental management of AMD remediation should consider long term assessment strategies, including multiple factors, to promote biological ecosystem recovery.

1. Introduction

Globally, land use activities and anthropogenic pollution associated with mining, agriculture and industry, have resulted in multiple pressures on freshwater ecosystems, with severe loss of biodiversity and ecological functioning. Remediation efforts to minimize the effects of AMD on stream ecosystems are occurring worldwide (Gunn et al., 2010), and has become a rather large and profitable business (Bernhardt et al., 2005). Monitoring the success of remedial actions usually involves both chemical and biological sampling (DeNicola and Stapleton, 2014), with responses of in-stream communities superior to that of chemical measurements (Adams et al., 2002; Gunn et al., 2010; Kruse et al., 2013). The reduction in diversity and abundance of macroinvertebrates by acid mine drainage (AMD) is well established and commonly used as ecological indicators (Gray, 1998; Chambers and Messinger, 2001; He et al., 2015). To date, only a few studies have examined the impacts of pH-treated AMD on macroinvertebrates, with mixed results. DeNicola and Stapleton (2002) observed reduced macroinvertebrate density as a result of AMD exposure and subsequent increase after AMD treatment system installation. Nevertheless, the increased macroinvertebrate densities observed after treatment were not comparable to controls at most sampling sites and taxa richness remained low (DeNicola and Stapleton, 2014; DeNicola and Stapleton, 2016; Gunn et al., 2010). In contrast, Perrin et al. (1992) reported no effect of treated AMD on macroinvertebrate numbers or number of taxa. In particular, treated AMD will tend to be diluted as it moves farther downstream, gradually alleviating toxic effects on biota (Oberholster et al., 2013). According to Covich et al. (1999) macroinvertebrates appeared to be more sensitive to treated AMD shown by their decline in diversity. This observation leads to dramatic changes in understanding

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organic matter processing and nutrient cycling due to the large occurrence of primarily predators. Many of the discrepancies between reported studies can be attributed to varying levels of AMD concentrations and the type of AMD treatment.

The ultimate goal when treating AMD is to improve the ecological health of a water body (Kruse et al., 2013). Traditionally, alkaline addition treatment is designed to increase the AMD to pH > 6.5 and to maintain net alkaline conditions in the stream. Yet, several studies (Cravotta and Bilger, 2001; Keener and Sharpe, 2005; McClurg et al., 2007) showed that neutral pH and net-alkaline conditions are not always successful in achieving biological recovery. Potential biological recovery and treatment success downstream are poorly understood (Gunn et al., 2010; Kruse et al., 2013; He et al., 2015). The addition of toxicity tests to evaluate stream water quality of streams affected by treated AMD will assist in assessing biological recovery of these waters. According to Gerhardt et al. (2004), this is important because rapid bioassessment methods based on macroinvertebrates represent an overall summation parameter integrating several effects on aquatic biota, such as toxic effects, habitat degradation and physical disturbance, while rapid toxicity tests can add value in the assessment and ranking of stream sites. A single bioassay is unlikely to be responsive to all possible toxicants (Toussaint et al., 1995). A multi-trophic battery of bioassays for the evaluation of complex environmental samples and toxic mixtures has been widely recommended as superior to a single bioassay (Clarke and Barrick, 1990; Rojickova-Padrtovz et al., 1998; Baran and Tarnawski, 2015). Repetto et al. (2001) as well as Kokkali and Van Delft (2014) proposed a battery of assays with a great variety of endpoints (e.g., bacterium, invertebrate, plant, and algae) as it improves the sensitivity to a variety of environmental stressors. The plant bioassays (*A. cepa*, and *L. sativa*) are fast and simple methods to assess the phytotoxicity of substances or matrices of environmental concern based on inhibition of root growth and seed germination, respectively (Roccotiello et al., 2011; Silveira et al., 2017). Geremias et al. (2012) successfully used *A. cepa* as bio-indicator to test the efficacy of treating acid mine drainage resulting from coal mining wastes. Similarly, the *D. magna* bioassay is highly sensitive to environmental changes (Fischer et al., 2011; Wojtal-Frankiewicz, 2012), especially metal toxicity (Poynton et al., 2007; Okamoto et al., 2015) and acidification resulting from anthropogenic pollution and global climate change (Locke, 1992; Locke and Sprules, 2000).

Besides concerns for the natural environment, the possible harmful effects of AMD on humans has been raised (UNEP, 2010; Steyn and Genthe, 2011; IHRC, 2016). The Ames test, making use of *Salmonella typhimurium*, is a biological assay to assess the mutagenic potential of chemical compounds in the DNA of the test organism, and by extension pose a risk of cancer in humans (Mortelmans and Zeiger, 2000). The liver is one of the main detoxifying organs of the human body. The human and/or rat primary hepatocytes or permanent human liver HepG2 or HepaRG cell lines are therefore commonly used in toxicity and clinical drug screening (Schoonen et al., 2011). For the current study, an *in vitro* bioassay making use of the human liver cancer cell line (HepG2) in addition to the aforementioned assays were used to examine the ability of this battery of tests to assess the downstream impacts of pH-treated AMD in relation to in-stream macroinvertebrates.

Around the world, ecotoxicology are increasingly used to assess impacts of mining waste or remedial activities on aquatic ecosystems or human health either through multi-species toxicity testing in the laboratory, or observing biological effects *in situ*. Short and long term studies to assess the success of such mitigation and remediation efforts are however limited. The objective of the current study was to use the Tweelopie Spruit[1] as case study to:

(a) determine the effects of pH-treated AMD and examine possible recovery of macroinvertebrate families' distribution over longitudinal distances; and
(b) in relation to (a) above, expose a battery of static bioassays to assess toxicity of the treated AMD impacted stream relative to multiple stressors.

2. Methods

2.1. Study area and site selection

The study area (Fig. 1) is located close to the town of Krugersdorp, west of the city of Johannesburg in the Gauteng Province of South Africa in the gold mining area of the Witwatersrand (also referred to as the Western Basin). Besides mining, land use practices in the area are predominantly agriculture (e.g., farms as well as agricultural small holdings) surrounded by peri-urban and urban land. The study area has a complex geology. The Witwatersrand Supergroup, overlain by the Ventersdorp Supergroup, is in turn covered by the Transvaal Supergroup (Durand, 2016). Hobbs and Cobbing (2007) and Hobbs (2011) explained the complicated geohydrology of Karst aquifers and quartzitic fractured rock aquifers which allows for highly variable groundwater recharge and drainage in the area. Hobbs (2011) and Van Deventer and Cho (2014) reported that dolomite springs contribute to the drainage along the reaches of the Tweelopie stream.

Sampling sites were selected along the Tweelopie stream (Fig. 1; Table 1). The Tweelopie stream, which receives the treated AMD, later confluences with the Riet stream and further downstream is joined by the Bloubank stream, a tributary of the Crocodile River. Water samples were collected at seven sites ranging from end-of-pipe pH-neutralized AMD, followed by five downstream sites (S2 – S6). A natural spring with the same geology as the Tweelopie stream, which has not been impacted by the AMD decant, was selected as the environmental reference site (S7). Site 1 is the end-of-pipe treated AMD leaving the high density sludge treatment plant, while Site 2 is where the treated AMD combines with the Tweelopie stream which later becomes the Bloubank stream. The current paper represents the treated AMD sampled in 2012 and 2013 after increasing the treatment capacity of the Short Term Intervention (STI) High Density Sludge (HDS) treatment plant from 18 ML in 2012 to 34 ML of AMD per day in 2013 (Strydom et al., 2016). The characteristics and land use activities associated with each sampling site are summarised in Table 1.

2.2. Physical and chemical water quality characteristics

Physical and chemical water quality monitoring and assessment was done over a two year period (2012–2013). The temperature, pH and conductivity at each sampling site was measured *in situ* with a Hach sension TM 156 portable multiparameter (Loveland, USA). Water samples for chemical analyses were collected with a surface grab sampler and filtered through GF/filters. The GF/F filtered water was preserved in pre-rinsed polyethylene bottles with sulfuric acid (to pH 2.0) for analysis of dissolved nutrients and metals. The samples were transported at 4 °C in a cooler box with ice packs to the laboratory. All analyses were done according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 1998).

2.3. Macroinvertebrate sampling (SASS5) and functional feeding groups

Macroinvertebrates were collected with a SASS net (25 cm diameter; 50 μm mesh), (Oberholster et al., 2009a). The macroinvertebrates were collected from sand, rocks, sediments, stones in current (SIC), stones out of current (SOOC), and marginal vegetation (Oberholster et al., 2008), according to the SASS protocol (Dickens and Graham, 2002). The organisms collected were identified to family level as listed on the South African Scoring System (SASS5) scoring sheet. Viewing and

[1] The word "spruit" is defined as: "a creek or small often dry tributary stream in southern Africa". Tweelopiespruit hereafter referred to as Tweelopie stream for international readers.

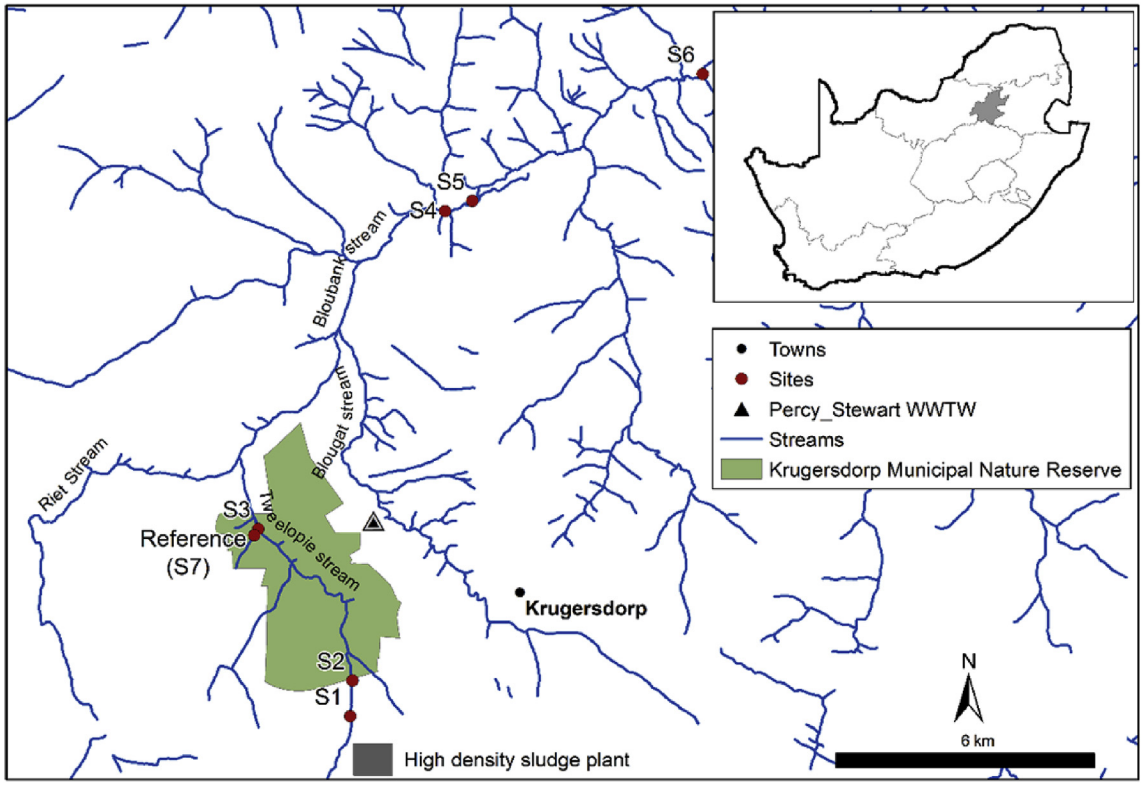

Fig. 1. Location of sampling sites within study area.

identification were done as per protocol (Gerber and Gabriel, 2002). The South Africa Invertebrate Habitat Assessment System (IHAS) Version 2, (McMillan, 1998), was also included in this study.

An additional classification system, involving the analysis of macroinvertebrate feeding groups based on their morpho-behavioural mechanisms of food acquisition, was employed (Wallace and Anderson, 1995). The use of functional feeding groups to describe community structure was justified by Mihuc (1997). This classification system comprises the following functional feeding groups: shredders (SH); scrapers (SC); collector gatherer (GC); collector-filterer (FC) and predators (PR). The total SASS5 score, and the Average score per taxon (ASPT) according to the method developed by Dallas (2007a,b) was used to categorize results into ecoregions (Kleynhans, 1999) ranging from Class An unmodified or natural water body to highly impacted and severely modified water body (Class E/F).

2.4. Bioassays

A battery of tests making use of five different bioassays including the commercially available human liver cancer cell line (HepG2) were selected for the study. These bioassays were selected to represent many trophic levels and different endpoints. Additionally, their sensitivity to a wide spectrum of aquatic pollution (mainly AMD) and reproducibility were added advantages. Mining, agriculture and urban land use practices and the associated water extraction and use in the study area supported bioassay selection.

2.4.1. Daphnia magna assay (Daphnia test)

The 48 h static renewal *Daphnia* test originally described by Dutka (1989) was conducted with a stock of *D. magna* initially cultured in dechlorinated tap water at ambient temperature conditions in our laboratory. The stock conformed to the specified quality parameters (e.g., no ephippia, < 10% mortality, time to first brood (10 days), and average number per brood 10). Three replicates of 5 organisms per vessel (24 h old neonates) were used for each test sample and for the control. Exposures were conducted in 250 ml glass beakers containing 50 ml of solution. This was used as a screening test (100% concentration). Mortality (defined as lack of movement after gentle prodding) was recorded at 24 h and 48 h.

2.4.2. Allium cepa test (onion test)

The 72 h root growth inhibition of *A. cepa* (onion bulbs) was tested according to Fiskejo (1993), with slight modifications. For each sample site, 4 onion bulbs were exposed and samples were kept at a constant volume in each tube. This static test was conducted without dilution (100% concentration) of contaminated water from each sampling site. Tap water was used as a control after standing 24 h prior to the experiment. Mean root growth (mm) was recorded and compared to the tap water control after 72 h.

2.4.3. Lactuca sativa assay (seed test)

The short term (120 h) static *L. sativa* (seed germination) test was performed in accordance with the protocol described by Dutka (1989). The test was carried out in 90 mm petri dishes without soils or sediments. Baby leaf lettuce mix seeds were provided by Starke Ayres (PTY) Ltd. Gauteng, South Africa and stored in its original packaging at room temperature. Twenty seeds, similar in size, shape and colour, were placed in a petri dish on Whatman filter paper moistened with 6–7 ml water from each sampling site or control (tap water left to stand for 24 h). Petri dishes were covered with aluminium foil and kept in the dark at room temperature for the duration of the test. After 120 h the number of germinated seeds was counted and compared to the control.

2.4.4. Ames Test

A 96-well microplate version of the *Salmonella typhimurium* Ames Test (Ames et al., 1975), the Environmental Bio-detection Products Inc. (EPBI) Muta-Chromo Plate™ was used to test for mutagenicity in the test samples. The strain *S. typhimurium* TA98 with and without S9 was used to screen the AMD impacted waters of the study area. The test was performed according to the manufacture's protocol. Bacteria were

Table 1
Characteristics of sampling sites.

Parameters	S1	S2	S3	S4	S5	S6	S7*
Coordinates	26.11514°S 27.72489°E	26.1075°S 27.72231°E	26.0762°S 27.69957°E	26.00874°S 27.74136°E	26.00647°S 27.74765°E	25.97877°S 27.80054°E	26.07692°S 27.69928°E
Site description	End of pipe treated AMD (34 ML/day) from HDS Treatment Plant	Treated mine water discharged from the HDS Plant into the Tweelopie stream	Inlet to Aviary Dam on the Tweelopie stream before confluence with Riet Stream	Bloubank stream after confluence of Riet stream with Blougat stream; downstream of WWTW	Bloubank stream carrying a similar water as occurs at S4 with the addition of the Zwartkrans Spring water	Bloubank stream carrying a similar water to S5 with the addition of the Kromdraai Spring water	Stream fed by pristine karst groundwater
Land use activity	HDS treatment plant	Mine impacted	Mine impacted (inside Krugersdorp Municipal Game Reserve)	Urban and industrial runoff mixed with domestic sewage effluent from WWTW	Urban/agriculture	Urban/agriculture/Industry	Draining agricultural runoff from small holdings West of Krugersdorp Nature Reserve
Geology	N/A	Dolomite	Dolomite	Dolomite	Dolomite	Dolomite	Dolomite
Bottom substrate type	N/A	Clay, cobbles	Clay, cobbles	Sand, cobbles	Sand, cobbles	Sand, cobbles	Sand, cobbles
Canopy cover	N/A	0%	40%	30%	30%	80%	20%
Bank stability#	N/A	Good	Good	Good	Good	Good	Good
Stream channel width	N/A	1 m	1.5 m	3.2 m	4 m	2.9 m	0.78 m
Water depth within channel	N/A	0.010 m	0.012 m	0.026 m	1.1 m	0.016 m	0.011 m
Stream flow speed	Not done	0.200 $m^3 s^{-1}$	0.263 $m^3 s^{-1}$	0.365 $m^3 s^{-1}$	0.500 $m^3 s^{-1}$	0.938 $m^3 s^{-1}$	0.0142 $m^3 s^{-1}$
Water column dilution from other sources	N/A	No	No	Wastewater treatment works effluent via upstream tributary	Karst spring upstream of site	Karst spring upstream of site	No
In-stream macrophytes	N/A	No	No	No	No	No	No

N/A = Not applicable; * = Reference site; # = The degree of bank erosion was done according to Spencer et al. (1998).

grown overnight (16–18 h) at 37 °C. Water samples were filtered through 0.22 µm syringe filters prior to use. Positive (2-Nitrofluorene) and negative (sterile water) controls were included and the measurement of the background reverse mutation rate was compared to the rates following exposure to the test samples. A single 96 well plate was used for each test sample or control. The 96 well plates were incubated at 37 °C.in Ziploc bags to prevent dehydration and examined after 5 days. Samples with double the number of reverse mutations compared to the background mutation rate (turning the wells on the plate from purple to yellow), were considered mutagenic.

2.4.5. HepG2 cell line and real-time cell analyser (RTCA)

2.4.5.1. Cell culture. Human HepG2 hepatoma cells (ATCC) were cultured and maintained in Eagle's Minimum Essential Medium (EMEM from Merck) supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% penicillin-streptomycin (Merck), in T-75 cell culture flasks (Corning) at 37 °C in a humidified atmosphere of 5% CO_2. The media was renewed every two days, and confluent layers of cells were sub-cultured every 5–6 days using a 0.05% trypsin-0.53 mM ethylene-diamine-tetra-acetic acid (EDTA) Solution (Merck). After each passage, cell concentration and viability were determined using the Countess™ Automated Cell Counter (Invitrogen) and the trypan blue dye (Invitrogen) exclusion test assay (Meli et al., 2012).

2.4.5.2. Real time cell analyser (RTCA). The xCELLigence® System from ACEA Biosciences, Inc., a powerful and reliable tool for toxicity and pharmacology studies (Atienzar et al., 2011), makes use of special tissue culture E-plates. The E-plates have integrated microelectrodes on the bottom of the plates (Solly et al., 2004) which allows the xCELLigence® System to measure electrical impedance across these microelectrodes. This provides quantitative information on the biological status of the cells, including cell number, viability, and morphology. The optimum cell density of 15 000 HepG2 cells (per well) (as determined according to Atienzar et al. (2011) and Gerets et al. (2012)), were added to the plate and allowed to attach at room temperature for 30 min. The plate was placed in the reader in the incubator for continuous recording of impedance for 48 h for HepG2. The cells were then exposed to EMEM media (see section on *Cell culture* above) prepared from the test water (S1 – S7 filtered through 0.22 µm syringe filters) and monitored for over 40 h. Each sample was measured in triplicate. The cells were monitored in real time, at 37 °C in a humidified 5% CO_2:95% air atmosphere, using the single plate xCELLigence® platform. Intervals for data collection were every 15 min for 24 h followed by every 60 min for 72 h. From the cell-electrode impedance measurements, a Cell Index (CI) is derived according to Abassi et al. (2009):

$$CI = \max\left[\frac{Rcell(fi)}{Rb(fi)} - 1\right]$$
$$i = 1,\ldots,N \qquad \text{Eq. 1}$$

Where:

Rb(f) = the frequency-dependent electrode resistances (a component of impedance) without cells;
Rcell(f) = the frequency-dependent electrode resistances (a component of impedance) with cells present;
N = the number of the frequency points at which the impedance is measured.

CI therefore represents a quantitative measure of the status of the cells in an electrode-containing well. A higher CI reflects a larger Rcell (f) value, which indicates higher numbers of cells attaching onto the electrodes. A decrease in the CI therefore points to possible lifting of cells and resultant cell death. For this paper, the CI for each site compared to the control (HepG2 cells in growth media; no sample addition) was used to indicate cytotoxicity due to treated AMD downstream.

2.5. Statistical analyses

The Spearman Rank Order Correlation was used as a non-parametric statistical method to determine the strength of association between the physical and chemical water quality concentrations (data failed normality) and the distance from the HDS treatment plant. Statistically significant difference was set as $p = < 0.05$. A Kruskal-Wallis Analysis of Variance on Ranks (ANOVA) was used to compare the physical-chemistry data for all sites, while the all pairwise multiple comparison Tukey test isolated the group/groups that differed from the rest. A one-way analysis of variance (ANOVA) was performed to determine the statistical significant differences (data passed normality) between the different bioassays and its significance downstream. For the Ames TA98 assay, test sites exhibiting double reverse mutation rates compared to the background mutation were identified and values recorded. The real time Cell Index (CI) of the HepG2 cells exposed to each downstream site was recorded and compared to the CI of the control cells. The above mentioned statistical analyses were carried out using SigmaStat 4.0 statistical package (Systat, 2017).

The multivariate analytical tool, PCA (Principal Component Analyses) bi-plot was constructed by means of XLSTAT (2017) statistical package for Windows which is based on a linear model to identify possible associations between multiple variables (Van den Brink et al., 2003) such as water chemistry, toxicity test results, and SASS5 data. Data was log-transformed and samples were mapped on a two-dimensional basis where the placements of the samples reflect the (dis)similarities (associations) between the sample sites. The method of Ter Braak and Smilauer (2002) was used for interpretation of the bi-plot, where the ordination of the sample sites downstream is related to the environmental variables and the angle between these variables. An angle close to 0° indicates a positive correlation between samples and, an angle close to 90° indicates that variables are uncorrelated. Variables are negatively correlated when the angle is close to 180°.

3. Results and discussion

3.1. Physical and chemical water quality

The mean physical and chemical water quality (2012–2013) data is summarised in Table 2 (Supplementary Figure S1 shows the mean chemical composition of the water samples from the different study sites in a Piper diagram). Sites 1–3 represent water directly impacted by AMD. As described by Akcil and Koldas (2006), the alkaline treatment (neutralisation) process does not affect sulphate levels; evident from the high sulphate (SO_4^{2-}) concentrations (2721 mg l^{-1}) still present at S1 to S3 (Table 2; Supplementary Figure S1). An overall gradual improvement in water quality (e.g., decrease in metal concentrations) is seen from S4 downstream to S6. The unimpacted profile of the water from the reference site (S7) is clear.

Site 1 had an average pH of 5.2 while near neutral pH values were observed for all other sampling sites, ranging between pH 7.01–7.99 (Table 2). Most constituents decreased from S1 downstream. Electrical conductivity decreased significantly downstream ($p = 0.003$) from where it left the treatment plant (S1) to the most downstream site S6. Similarly, the decrease in sulphate (SO_4^{2-}) concentrations downstream is evident $P = 0.002$ (Table 2). Notable is the decrease in metal contents from S1 – S3 and further downstream (S4–S6). Oberholster et al. (2013) reported Al concentrations of 3908 µg l^{-1} in the untreated AMD sampled between 2011 and 2012 in the same study area. For this study, a mean Al concentration of 1900 µg l^{-1} was present in the end–of-pipe treated AMD at S1 and hardly detected further downstream (ranging from 3 to 9 µg l^{-1}). The alkaline treatment process increased the pH with resultant Al precipitation, with additional reduction once the treated AMD mixed with the Tweelopie stream (Site 2) with a higher pH. This could lead to further degradation of the habitat (Younger et al., 2002). According to work by Farley et al. (2004) and Borch (2005), the stretch of the river immediately following alkaline treatment where the river still acts as a settlement pond is known as the "sacrifice zone" or the "impaired zone" (Kruse et al., 2013). Fe precipitated out between S1 and S2 (Fig. 2), resulting in the 'yellow boy'[2] and possibly responsible for the lack of macroinvertebrates at these sites (Jarvis and Younger, 2000). Manganese on the other hand only decreased gradually downstream as it did not precipitate out. This is similar to what Truter et al. (2014) reported in the same study area. This was also the case for B, Mo, Ni, Sr, Zn and Ti.

There was a statistically significant correlation between most metals (e.g., Ni, Co, Fe, Mo, Zn and Mn) and the distance from the HDS treatment plant downstream (Fig. 2). Similarly, the concentration of salts (TDS, SO4, Na, Mg, Ca, Total N) differed significantly with distance from the HDS treatment plant. As the distance increased, the metal and salt concentrations decreased. The opposite was found for Nitrate and Nitrite (N) increasing significantly with an increase in distance (Fig. 2). This was also the case with ortho Phosphate (P) but not statistically significant. Site 3–6 had increased concentrations of N. It is hypothesised that the increased N at Site 3, situated in the Krugersdorp Municipal Nature Reserve, possibly resulted from urine and faeces of the resident Hippopotamus (Oberholster et al., 2009b) or surface runoff from the lion enclosure at sites upstream from Site 3. The even higher N concentrations at Site 4, 5 and 6 is likely associated with the wastewater effluent discharged upstream of Site 4 as well as the agricultural land use in the area with possible runoff of fertilisers into the Bloubank stream. Zongo et al. (2017) observed that eutrophic waters were mostly found outside reserves resulting from human activities and that protected areas with high land cover, especially forestry reserves, protected water quality and ecosystem health.

The improvement of water quality in terms of ecotoxicity and metal content downstream from site S1 to S6 as well as the quality of the environmental reference site (S7) is evident (Table 2). However, the quality of the treated AMD still exceeded (values in bold) the International Water Quality Guidelines for Ecosystems (UNEP, 2016) a) Class I (Natural) – b) Class IV (Extremely Impacted), and the South African water quality guideline concentrations for c) aquatic ecosystems; d) irrigation, and e) livestock watering (DWAF, 1996a,b,c). Farmers in the downstream areas are extracting water from this stream for irrigation. This comparison therefore provides some insight into the possible long term impacts to not only agricultural production and a possible decrease in crop/livestock quality or quantity, but also potential human health impacts associated with the bioaccumulation of metals in agricultural produce consumed from the area. TDS concentrations for example exceeded the South African aquatic water quality guidelines at all sites except the reference site and the irrigation water guideline at all sites. Yang et al. (2017) reported that long term exposure of the aquatic animal *S. constricta* (a molluscan bivalve) to high suspended solid concentrations, could impact enzyme activity, disturb osmoregulation and nutrient absorption, cause oxidative damage and eventually impact the gills, in turn affecting food intake and ultimately resulting in systems failure and death.

3.2. Macroinvertebrate functional feeding groups and SASS5

Long term impacts of repeated AMD decant into the study environment since 2002 was still evident in the area in 2013 after an upgrade of the High Density Sludge treatment plant from 18 mL to 34 mL/day in 2012. Macroinvertebrates were sampled on a number of occasions ($n = 3$) during 2012 and 2013 to represent high and low flow conditions (Supplementary Table S1). Site 1 represents the end of the pipe treatment at the HDS treatment plant and was therefore not

[2] Yellow boy is the thick, impermeable layer of ferrichydrite precipitate that often covers the river sediment and which gives a yellow-orange appearance to the river bed (Durand, 2012).

Table 2
Mean pH, electrical conductivity (EC), total dissolved solids (TDS) and element concentrations (ug l^{-1}) for 7 sites (2012–2013) in the Tweelopie/Bloubank stream receiving treated AMD (Values in bold exceed guideline values).

Constituent ug l^{-1}	Sampling sites						
	S1	S2	S3	S4	S5	S6	Ref (S7)
pH	**5.20**$_{a,c,d}$	7.01	7.48	7.81	7.51	7.44	7.99
EC (uS/m)	**442 500**$_{d}$	**380 000**$_{d}$	**184 000**$_{d}$	**94 000**$_{d}$	**89 000**$_{d}$	**72 000**$_{d}$	25 000
Total Dissolved Solids (TDS)	**2832 000**$_{c,d,e}$	**2432 000**$_{c,d,e}$	**1177 600**$_{c,d,e}$	**601 400**$_{c,d}$	**569 600**$_{c,d}$	**460 600**$_{c,d}$	**159 800**$_{d}$
Potassium (K)	9735	8212	4540	5384	3403	2506	908
Sodium (Na)	**80 013**$_{d}$	**87 701**$_{d}$	54 767	47 686	42 190	27 603	2217
Calcium (Ca)	489 808	635 267	286 879	78 634	76 260	57 750	21 917
Magnesium (Mg)	184 398	142 175	71 852	36 425	42 735	35 011	14 595
Ammonia (NH_3)	**6 130**$_{a,b,c}$	**4 790**$_{a,b,c}$	**1 027**$_{a,b,c}$	**1 029**$_{a,b,c}$	**272**$_{a,b,c}$	**53**$_{a,c}$	**47**$_{a,c}$
Sulphates (SO_4^{2-})	**2721 852**$_{e}$	**2280 255**$_{e}$	970 632	223 276	203 390	119 566	27 575
Chloride (Cl)	**42 800**$_{d}$	**42 150**$_{d}$	**35 600**$_{d}$	**49 900**$_{d}$	**47 850**$_{d}$	**35 650**$_{d}$	**3 385**$_{d}$
Nitrate and Nitrite (N)	**46**$_{a,b}$	**303**$_{a,b}$	**3 255**$_{a,b}$	**7 765**$_{a,b,d}$	**9 700**$_{a,b,d}$	**5 720**$_{a,b,d}$	**2 510**$_{a,b}$
Ortho Phosphate (P)	**51**$_{a}$	**38**$_{a}$	**279**$_{a,b}$	**416**$_{a,b}$	**225**$_{a,b}$	**126**$_{a}$	**44**$_{a}$
Fluoride (F)	236	207	104	109	53	54	175
Mercury (Hg)	**2**$_{a,b,c}$	**1**$_{a,c}$	**2**$_{a,b,c}$	**2**$_{a,b,c}$	**2**$_{a,b,c}$	**1**$_{a,c}$	**2**$_{a,b,c}$
Uranium (U)	**13**$_{d}$	3	0	0	0	0	0
Antinomy (Sb)	0	1	1	0	0	0	0
Arsenic (As)	2	1	0	0	1	1	1
Cadmium (Cd)	**17**$_{a,b,c,d,e}$	**2**$_{a,b,\ c,\ d,\ e}$	**1**$_{a,c,d,e}$	**2**$_{a,b,c,d,e}$	**4**$_{a,b,c,d,e}$	**4**$_{a,b,c,d,e}$	**4**$_{a,b,c,d,e}$
Lead (Pb)	**22**$_{a,b,c}$	**7**$_{a,b,c}$	**8**$_{a,b,c}$	**3**$_{a,c}$	**3**$_{a,c}$	**3**$_{a,c}$	**2**$_{c}$
Selenium (Se)	**7**$_{c}$	**3**$_{c}$	**10**$_{c}$	2	**6**$_{c}$	**3**$_{c}$	2
Aluminium (Al)	**1 962**$_{a,\ b,\ c}$	3	9	7	5	9	7
Boron (B)	273	68	41	53	34	20	4
Barium (Ba)	19	12	10	14	9	9	4
Beryllium (Be)	7	3	2	5	3	1	3
Cobalt (Co)	**561**$_{d}$	39	15	5	4	3	5
Chromium (Cr)	**44**$_{a,b,c}$	3	3	2	5	3	1
Copper (Cu)	**24**$_{a,b,c}$	**4**$_{a,b,c}$	**2**$_{a,c}$	**2**$_{a,c}$	**3**$_{a,b,c}$	**4**$_{a,b,c}$	**2**$_{a,c}$
Iron (Fe)	**421 264**$_{c,d,e}$	**246**$_{c}$	**50**$_{c}$	**29**$_{c}$	17	15	14
Manganese (Mn)	**71 390**$_{c,d,e}$	**13 526**$_{c,d,e}$	**5 532**$_{c,d}$	**440**$_{c,d}$	**138**$_{d}$	6	7
Molybdenum (Mo)	**230**$_{d,e}$	**125**$_{d,e}$	**107**$_{d,e}$	**42**$_{d,e}$	**40**$_{d,e}$	**32**$_{d,e}$	**15**$_{d,e}$
Nickel (Ni)	**920**$_{a,b,d}$	**134**$_{a,b}$	**61**$_{a,b}$	**28**$_{a,b}$	14	5	3
Strontium (Sr)	511	462	225	110	75	48	14
Vanadium (V)	3	3	2	4	2	3	2
Zinc (Zn)	**393**$_{a,b,c}$	**42**$_{a,c}$	**50**$_{a,c}$	**7**$_{c}$	**5**$_{c}$	**3**$_{c}$	**3**$_{c}$
Titanium (Ti)	351	102	89	5	19	13	4

a - High Integrity – International Water Quality Guidelines for Ecosystems (UNEP, 2016); b - Extreme Impairment – International Water Quality Guidelines for Ecosystems (UNEP, 2016); **c** – SA Aquatic Water Quality Guideline (DWAF, 1996a); **d** – SA Irrigation Water Quality Guideline (DWAF, 1996b); **e** – SA Livestock Water Quality Guideline (DWAF, 1996c).

applicable for macroinvertebrate sampling. Macroinvertebrates were completely eliminated at Site 2 and Site 3 throughout the study duration, but were present from Site 4 downstream. Of the macroinvertebrate orders and families represented at S4 to S6 and the reference site, 39.45% were highly tolerant to pollution while more than half of the invertebrates were moderately tolerant to pollution (54.6%). The remaining 6% of the macroinvertebrates were found at predominantly Site 6 and the reference site. Even though some families of the orders Ephemeroptera, Plecoptera, and Trichoptera (EPT orders) were found to be sensitive to metal pollution and are often absent from metal polluted streams (Casper, 1994; Gower et al., 1995; Beasley and Kneale, 2003) or urban impacted areas (Lenat and Crawford, 1994; Bacey and Spurlock, 2007; Hepp et al., 2010), some of the moderately metal tolerant families such as the *Baetidiae* (Beasley and Kneale, 2003) were found at S4 – S7. A significant number of the family *Simuliidae* (450 of the 566 individuals) were found at Site 4. This was possibly due to the wastewater effluent discharged upstream of this site and the resultant availability of organic pollutants and nutrients; also from the agricultural land use activities. *Baetidae* was the most abundant at all sampling sites.

The collector-gatherers dominated the functional feeding groups for all sites. Shredders and scrapers were not well represented at all sites (Fig. 3; Supplementary Table S1). The loss of shredder biomass as a result of metal toxicity and the resultant direct impact on leaf litter breakdown was reported by Niyogi et al. (2001) and later corroborated by Carlisle and Clements (2005), Rasmussen et al. (2008) as well as Gunn et al. (2010). According to Wesolek et al. (2010) recovery of the shredder populations will possibly require reestablishment of riparian forest cover. Zongo et al. (2017) reported the importance of forestry reserves and high land cover in sustainable watershed management. Simmons et al. (2005) reported on both the shredders and scraper populations being low in numbers, as well as the abundance of collector-gatherers in AMD impacted streams. There was an increase in the SASS5 score downstream (Site 4–6), with the highest score represented by the reference site (Fig. 3).

Site 6 had a higher SASS5 score than Site 4 and 5, but less taxa and not much diversity. This was also reflected by the IHAS (Fig. 3). While the reference site had a lower density of organisms, it had higher diversity and showed greater taxonomic richness (reflected by the Shannon Diversity Index) compared to Site 4, 5 and 6. Taxa found exclusively at the reference site included *Philopotamidae, Elmidae/Dryopidae*, *Athericidae*, *Muscidae*, and the Gastropoda *Ancylidae.*

3.3. Responses of the bioassays to environmental samples

The end of pipe treated AMD (S1) was found to have the biggest negative impact in all static bioassays compared to the other downstream sites. The percentage of lethality of the daphnids in undiluted sample water is indicated in Fig. 4. The survivor percentages at S4 – S6 increased downstream. Similarly, for the onion and lettuce seed assays, there was an increase in germination or root length percentages further downstream. Significant increases in seed germination and root length

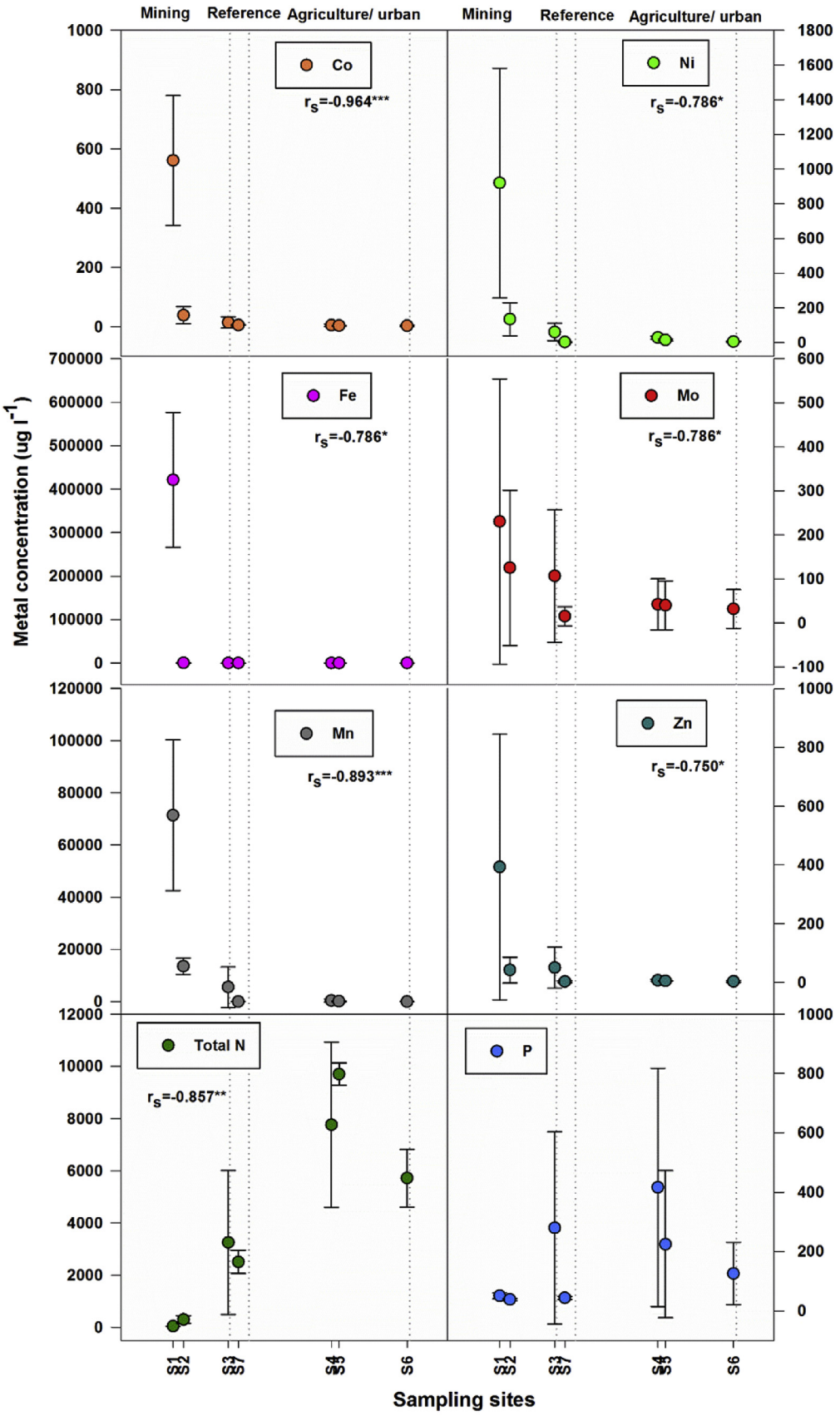

Fig. 2. Spearman correlation (r_s) of mean metal and nutrient concentrations in water with distance from HDS Treatment Plant. *P < 0.05, **P < 0.005, ***P < 0.0005.

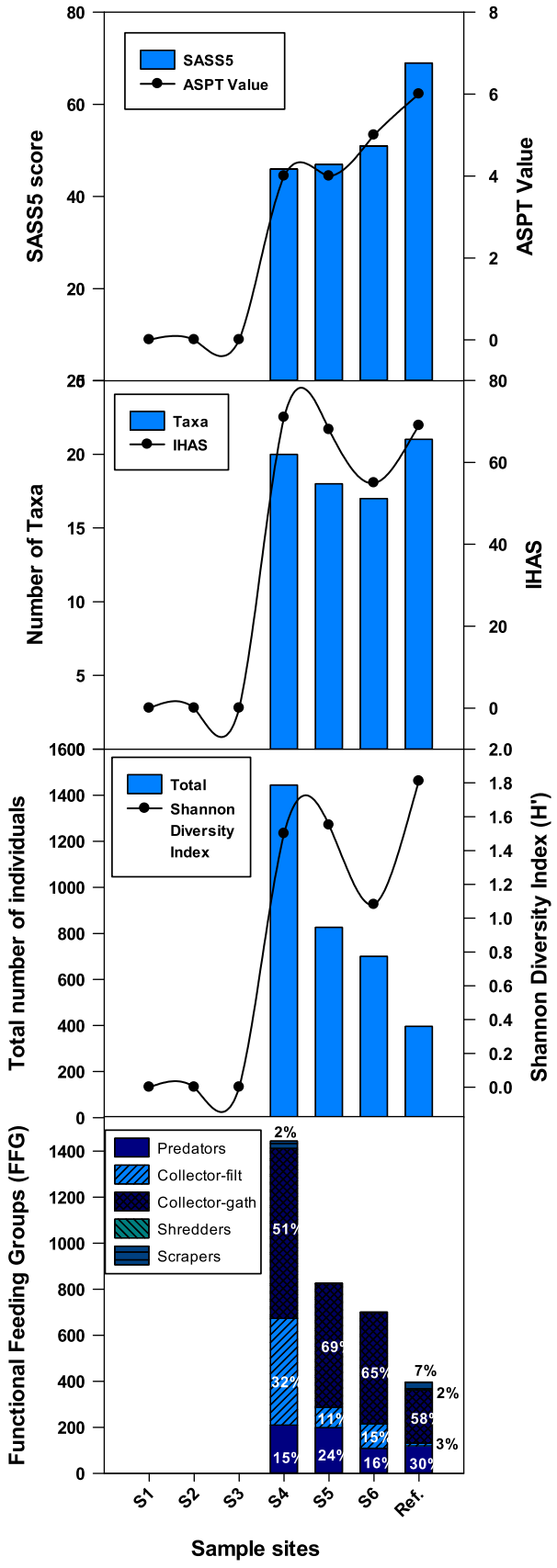

Fig. 3. Mean response of the different bioassays at sample sites downstream from AMD treatment, 2012–2013 (n = 4).

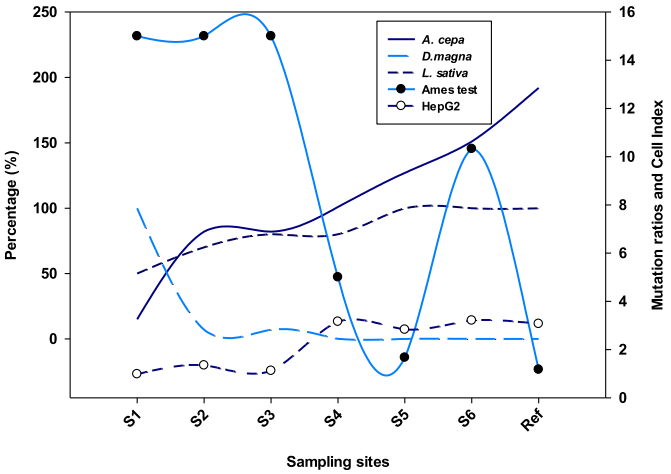

Fig. 4. Summary graphs of the SASS5 scores, habitat integrity, diversity, abundance and Functional Feeding Groups (FFG) of macroinvertebrates in the study area.

was observed from S4 further downstream to S6 as well as for the reference site (S7).

Although the seeds at S1 germinated, there was a visible difference in the growth at this site compared to any of the other sites. The greatest percentage of root growth inhibition and the lowest percentage seed germination was shown for S1 and S2, although not significant at all sites. The root tips of onion bulbs grown in the treated AMD polluted water from S1 were characterised by twists and crochet hooks (roots bent upwards resembling hooks). Induction of root malformations in *A. cepa* has been shown to be useful signs of toxicity by other studies (Babatunde and Bakare, 2006; Bakare et al., 2009; Olorunfemi et al., 2011a, 2011b). Although slightly improved, the surface water from Site 3 was still inhibitory to growth in the *A. cepa*, *L. sativa*, *D. magna* and HepG2 bioassays, with high mutation ratios. Except for the increased mutation ratios at S6, there was an overall improvement from Site 4 downstream and for the reference site.

3.4. Principle components

Multivariate statistical methods have been widely applied in environmental data reduction and interpretation of multi-parameter chemical, physical and biological measurements (Razmkhah et al., 2010; Kebede and Kebedee, 2012; Dabrowski and de Klerk, 2013; Truter et al., 2014). The multivariate statistical analysis, Principle Component Analysis (PCA), was performed on the macroinvertebrate communities, the static toxicity tests, and the physical chemical properties of water for each site. The actively pH-treated AMD, with a pH ranging between 5.2 and 7.9, contributed high concentrations of Fe, Mn, Cd, Pb and Zinc in the Tweelopie stream.

Fig. 5 shows the PCA biplot (mean data for 2012 and 2013) and the associations between the water chemistry (metals, pH and salts), the *A. cepa*, *L. sativa*, *D. magna*, human liver cancer cell (HepG2) toxicity and mutagenicity (AMES) test results and the in-stream macroinvertebrates at each sampling site. The first two axis explained 78.88% of the variation in the data, with 64.24% and 14.64% of the variation explained by Axes 1 and 2, respectively. Clear associations existed between Site S2 with the metals (Pb, Mn, Co, Zn, Fe) in the water as well as Daphnia lethality. Site S2 and to some extend Site 3 with high sulphates, electrical conductivity, Sr, Mo, Ca, and Mg concentrations in the water correlated well with the inhibition of seed germination and onion root lengths, cytotoxicity in HepG2 cells as well as mutagenicity by means of the Ames test. No macroinvertebrates were present at Site 2 and Site 3 indicating the remaining toxicity of the treated AMD at these sites. Toxicity decreased from Site 4 downstream (Fig. 4; Fig. 5), while

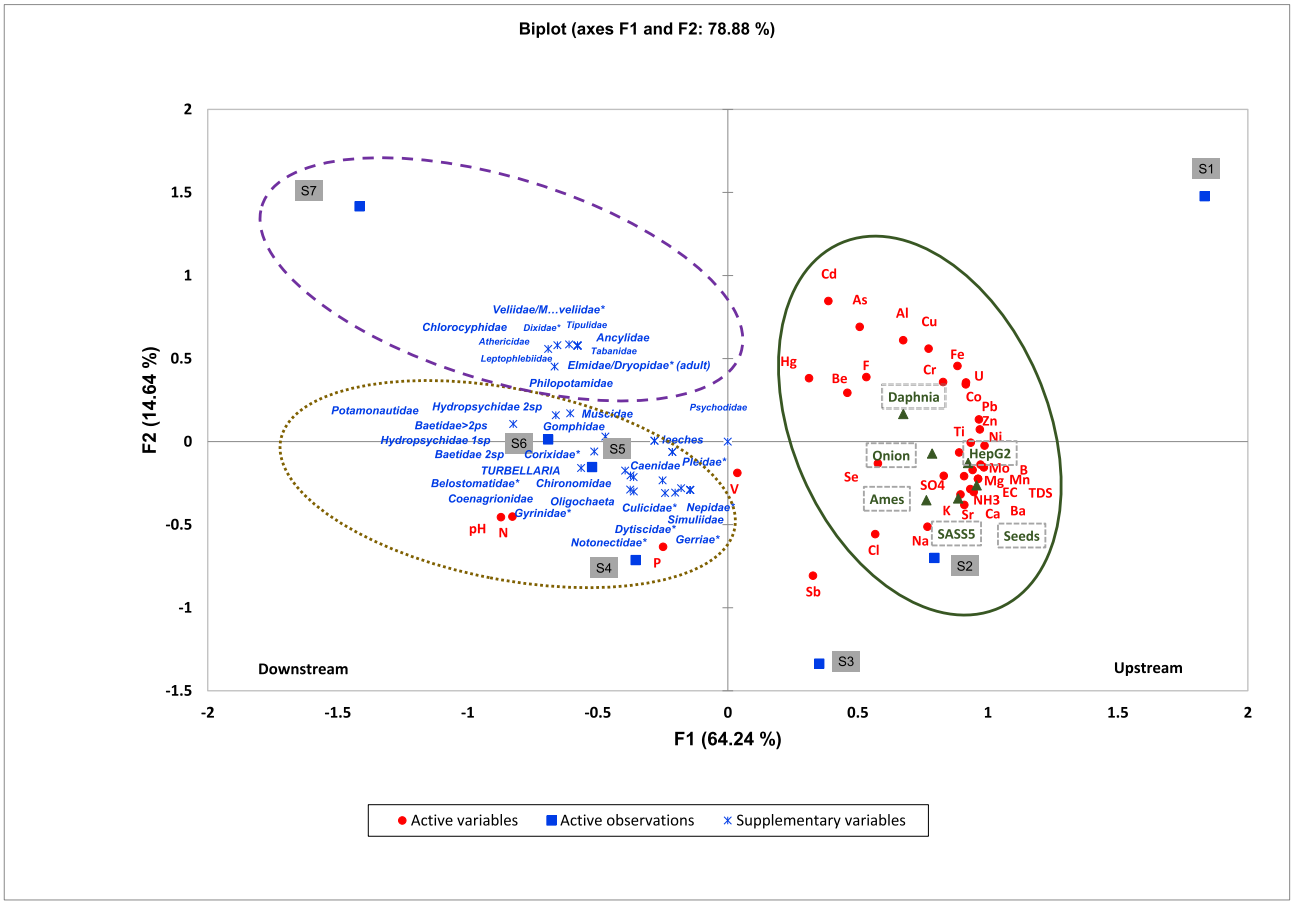

Fig. 5. Principle Component Analysis biplot showing association between variables.

macroinvertebrate diversity and sensitivity increased from Site 4 onwards. Mutagenicity was high at S1 – S3 as well as S6. There was also an increase in the N and P concentrations at Site 4 following the upstream discharge of domestic wastewater effluent. The downstream improvement of the water quality at Sites 4–6, the resultant decrease in toxicity and mutagenicity and the increase in macroinvertebrates at these downstream sites, also correlated well with the reference site, showing the improvement in water quality downstream.

According to the ecosystem classification system (A – E/F) (DWAF, 2008), the results indicate that the first 7 km after receiving alkaline treated AMD, the Tweelopie stream was severely degraded (Class E/F). There seem to have been little to no recovery of the sites (Site 2–3) directly following alkaline treated AMD. Gunn et al. (2010) reported rapid recolonization of many macroinvertebrate families, but that the sites remained significantly impaired even after 8 years after remediation. DeNicola and Stapleton (2016) concluded after following macroinvertebrate communities for 11 years post remediation, that substantial recovery of the sites below remediation did not occur and that factors other than more time is needed. From this and work by Kruse et al. (2013), it is clear that multiple factors (e.g., stream velocity, different recovery and response rates of different types of macroinvertebrate communities) influence the recovery of stream ecology following AMD remediation. While Site 4, 5 and 6 was impacted by multiple environmental stressors (e.g., treated AMD from upstream sites, agricultural runoff, urban run-off and domestic waste water effluent) the ecological impact of receiving treated AMD at Site 4, 5 and 6 was less severe and could not be directly linked to only AMD. Site 4 was largely modified (Class D), while Site 5 and Site 6 was moderately modified (Class C). The reference site was mostly natural and unimpacted by pollution with no evidence of AMD inputs (Class A). The battery of static bioassays was successful in indicating the toxicity of treated AMD in relation to macroinvertebrate families downstream.

4. Conclusion

While the Tweelopie stream was still severely impacted for kilometres following active alkaline treatment, the treatment was effective in reducing the physical-chemical water quality of the stream. However, there seems to be a delay in biological recovery of the system directly downstream. Providing conclusive results on impacts associated solely from alkaline treated AMD was unfortunately not possible. The Tweelopie/Bloubank stream was moderately to severely degraded by multiple anthropogenic stressors and the impact of the treated AMD was surpassed by the improvement in the system downstream after mixing with a stream that receives domestic wastewater effluent, and possibly due to further dilution as a result of the karst springs downstream. While the study had multiple limitations (e.g., limited biological sample size, short assessment duration after remediation (2 years), no sediment analysis), the overall results are however still important from an environmental ecological and human health risk management perspective as it provides insight into the complex interaction between physical, chemical and biological recovery of stream ecosystems downstream of alkaline AMD treatment. The downstream improvement of the ecosystem was also clearly shown by the decrease in toxicity by means of the ecotoxicological screening tools in relation to the in-stream macroinvertebrates. PCA multivariate analysis successfully displayed associations between the different environmental variables and the decrease in toxicity and subsequent ecosystem improvement downstream. The results seem to agree with previous studies in that multiple factors play a role in the recovery of ecosystems following remediation. The AMD alkaline treatment, in absence of mixing and dilution with the Tweelopie Stream downstream, did not provide

sufficient treatment to effectively protect ecosystem health from acid mine drainage. The alkaline AMD treatment is therefore not recommended as a long term solution for the study area and it is recommended that additional treatment of the AMD should be considered by officials responsible for managing the health of the environment. Due to the uncertainty of long term health impacts associated with the downstream extraction and use of this AMD impacted water (e.g. for agricultural crop production), further research on the long term impacts of alkaline treated AMD on the ecosystem and human health is recommended. It is therefore suggested that future work focus on long term monitoring and assessment programs (e.g., earth observation studies (Anderson et al., 2017) which includes multiple environmental factors (e.g., sediment analysis, stream velocity), as well as management interventions focused on stream-bed recovery, as well as re-establishment and recolonization of benthic species immediately downstream of the treatment plant.

Competing interests

The authors declare that we have no competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2019.01.051.

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