Core Ideas

- Microbial risks and benefits of WTR land application were explored to inform SDG12 and 15
- Pathogen concentrations in WTR did not require pre-processing for land application
- No pathogen re-growth was evident in nutrient-poor sandy soils incubated with WTR
- Microbial competition in WTR did not limit biosolid pathogen persistence
- Both WTR and, more so, compost co-amendments increased soil microbial load and diversity

The Microbiology of Rebuilding Soils with Water Treatment Residual Co-Amendments: Risks and Benefits

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jeq2.20286.

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ABSTRACT

Water treatment residuals (WTR) are sludges from the potable water treatment process, currently largely destined for landfill. This waste can be diverted to rebuild degraded soils, aligning with the UN's Sustainable Development Goals 12 (Consumption and Production) and 15 (Terrestrial Ecosystems). Biosolids are tested against stringent pathogen guidelines, yet few studies have explored the microbial risk of WTR land application, despite anthropogenic impacts on water treatment. Here, the microbial risks and benefits of amending nutrient-poor sandy soil with WTR were explored. It was shown that the culturable pathogen load of wet and dry WTR did not warrant pre-processing before land application, according to South African national quality guidelines, with fecal coliforms not exceeding 10^4 CFU/g_{dw} in wet sludges sampled from four South African and Zimbabwean water treatment plants, and

decreasing upon drying and processing. There was no culturable pathogenic (fecal coliforms, enterococci, *Salmonella* and *Shigella*) regrowth in soil incubations amended with dry WTR. However, the competition (microbial load and diversity) introduced by a WTR co-amendment did not limit pathogen survival in soils amended with biosolids. The application of WTR to nutrient-poor sandy soils for wheat (*Triticum aestivum L.*) growth improved the prokaryotic and eukaryotic culturable cell concentrations, similar to compost. However, the compost microbiome more significantly impacted the bacterial beta diversity of the receiving soil than WTR, analyzed with ARISA. Thus, although there was a low pathogen risk for WTR-amendment in receiving soils, and total soil microbial loads were increased, microbial diversity was more significantly enhanced by compost than WTR.

INTRODUCTION

Increased strain on world-wide landfill capacities, coupled with delivery of the UN's Sustainable Development Goal 12 (re-use of waste), has promoted interest in diverting waste streams from landfills to productive applications (Lu et al., 2012). Water treatment residual (WTR) is the sludge by-product of the drinking water treatment process. Locally, a single water treatment works in the Western Cape (South Africa) produces approximately 12 700 metric tonnes of WTR per year (Clarke et al., 2019), and daily trucking to local landfill is the current disposal route of WTR in the Western Cape. Internationally, despite studies optimising its use for productive applications, WTR is still considered a hazardous waste in some jurisdictions. For example, the Environmental Protection Agency (USA) ranks WTR as having the second highest effluent environmental impact risk, contributing 10.7% of the national hazardous effluent production (EPA, 2016). Although pragmatic in terms of risk, the unquantified negative associations with the material are a barrier to use, creating administrative and regulatory obstacles in material use. Research better clarifying and

quantifying any risks involved in the land application (or other uses) of WTR would help to address this.

Water treatment residuals are essentially the concentrated sediment from terrestrial ecosystems and are removed from water bodies destined for potable water, along with key process additives including oxides and flocculants. Therefore, this material is targeted for rebuilding soils (Dayton & Basta, 2001; Mahmoud & Ibrahim, 2012; Mahdy et al., 2009; Mahdy et al., 2012) and addressing the UN's SDG 15 (sustainable terrestrial ecosystems). The reservoir characteristics, catchment geology and anthropogenic activity inevitably determine WTR characteristics. Thus, reservoir pollution or heavy metal-rich sediments will have downstream agricultural implications if WTR is diverted to productive land application, with this impact currently limited to landfill sites (Lu et al., 2012). Turner et al. (2019) state that the research gap in land applying WTR lies in determining the effects of WTR on terrestrial ecology.

In potable water treatment, reservoirs are usually not heavily contaminated according to guidelines for microbial pathogens, although this may vary. Thus, the primary land application concerns have been heavy metals and the treatment process additives. Water treatment residual is composed of either iron (Fe) or aluminum (Al) oxyhydroxide additives for flocculation and coagulation, as well as abiotic and biotic sediment particulate matter, and additives like lime for pH control and dewatering polyelectrolytes (Lu et al., 2012). It has a high BET (Brunauer–Emmett–Teller, 1938) surface area with micro- and mesopores (Chiang et al., 2012), and the consequent sorptive capacity is effective for the removal of surface water contaminants (Hovsepyan & Bonzongo, 2009). This sorptive capacity has the potential benefit of heavy metal sorption in contaminated soils and waters (McCann et al., 2018; Mahmoud & Ibrahim, 2012, Mahdy et al., 2012), but also limits soil phosphorous (P)

availability, a critical macronutrient for plant growth (Dayton & Basta, 2001, Mahdy et al., 2009).

Due to the P-sorption of WTR (Habibiandehkordi et al., 2014), it is often not ideal for plant growth as a single soil amendment (Clarke et al., 2019), and is primarily employed as a soil amendment to minimize P in agricultural runoff to rivers (Ippolito et al., 2011). However, the co-amendment of soils with WTR and compost can provide nutrient balances optimal for plant growth, often in contaminated soils (Castaldi et al., 2018; Mahmoud et al., 2015; Dao et al., 2001). The use of compost and WTR as co-amendments has received less attention than biosolids. Although compost is a costlier alternative than biosolids, and is thus less attractive in terms of amendment for land remediation, it is less complicated from a PTE (potentially toxic elements) and POP (persistent organic pollutant) point of view (Gianico et al., 2021). A recent study showed the beneficial nutrient balance afforded by a compost and WTR mixture, that promoted wheat growth in Cape Flats sandy soils, Western Cape, South Africa (Quartzipsamment; Soil Survey Staff, 2014; Clarke et al., 2019). The compost provided P and the WTR improved N availability, promoting plant growth.

Many studies have also investigated the potential of co-amending soils with WTR and biosolids (sewage sludge) for plant growth promotion (Elmi & AlOlayan, 2020). However, there are more risks associated with the agricultural application of biosolids than compost or WTR. These include a much wider variety of heavy metals, industrial contaminants and high P levels, which have wide impacts; including leaching, surface runoff, and plant uptake. This is particularly problematic in sandy soils, due to limited nutrient/contaminant immobilization (Boyd et al., 1988). Co-application of biosolids with the WTR ameliorates some of these risks, due to the latter's capacity for PTE and P sorption (Ippolito et al., 2011). The pathogenic load of biosolids poses an infection hazard during handling and application, as

well as during crop growth and produce distribution (Lu et al., 2012). The pathogenicity of sewage biosolids has been extensively explored, and stringent quality assessment is necessary for land application (Snyman & Herselman, 2006). These sludges also carry beneficial microbes and have also been extensively shown to improve microbial loads and, after beneficiation such as composting (Eastman et al., 2001), to improve the diversity of degraded or nutrient-poor soils (Bai et al., 2019).

Yet, studies are lacking on the microbial characterization of WTR, particularly for co-application with compost, which has a low pathogenic risk. South African national land application guidelines do not require microbial analysis of WTR before agricultural application, on the foundation that "concentrations of… infectious substances (pathogens and parasites) are perceived to be low in SA WTR. However, in cases where the water treatment plants (WTP) are aware that these substances are present in the raw water, the WTR needs to be tested for these substances before land application, especially agricultural use…" (Herselman, 2013). However, we could find little evidence to support this position, and therefore we explore this perception of low pathogen risk. In addition to pathogens being added to the soil with the biosolids and WTR, these amendments can increase the total microbial biodiversity, which has been shown to enhance plant nutrient access, particularly in nutrient-poor soils (Van der Heijden et al., 2008), and limit the competitive fitness of pathogens (Van Elsas et al., 2012; Pane et al., 2020).

Therefore, the aim of this study was to explore the effect of WTR on microbial pathogen loads, persistence (risks), total microbial load and diversity (benefits) when used as a co-amendment in soil improvement techniques. The microbiology was compared for four local WTRs, from reservoirs with various geographical locations and pollution levels. Greater coliform contamination was hypothesized to be attributed to water reservoirs that have been

previously described as polluted. Differences in sludge contamination was evaluated in terms of culturable pathogens with ANOVA and Student's t-tests. The full interaction of humans in the WTR collection and transport process was investigated, remaining true to field conditions and only introducing aseptic techniques and cold storage after samples reached the laboratory. Water treatment residual characteristics were compared to pristine and polluted local river sediments and biosolids, for calibration within a range of microbial pollutants from environmental sediments. Pathogenic persistence was hypothesized to be limited through competitive exclusion due to increased microbial loads and diversity, when biosolids were co-amended with WTR in nutrient-poor sandy soil. Differences in means were assessed with two-way Student's t-tests for independent means. Finally, the amendment of plant growth trials with WTR, compost and co-applications were predicted to improve the microbial abundance and diversity in nutrient-poor sandy soils. Rhizosphere microbial loads (plate counts, ANOVA) and diversity (Automated Ribosomal Intergenic Spacer Analysis, ARISA) were quantitatively compared and qualitatively assessed (Scanning Electron Microscopy, SEM). These were tested over multiple pot trials, using a variety of crops and amendment loadings, broadening the impact of the findings.

2. MATERIALS AND METHODS

2.1. Sludge Materials

Water treatment residuals were sampled for chemical and microbiological characterization at the point of collection, prior to trucking for landfill. Samples were collected from two water treatment plants near Cape Town, South Africa (labelled CT-Fe and CT-Al); one near Johannesburg, South Africa (J-Fe); and one near Harare, Zimbabwe (H-Al). The labelling (Al and Fe) refers to the ferric and aluminum oxyhydroxide flocculants. The biosolids investigated in this study were from anaerobic digestate, collected from a wastewater

treatment plant near Cape Town, South Africa (labelled biosolids). The full process of human interaction and transport was investigated, remaining true to field conditions (non-sterile shoveling and ambient temperature transport), with aseptic techniques and cold storage introduced in the laboratory. For comparison and calibration against environmental conditions, samples of local nutrient-poor sandy soil (-33.967350 S,18.717388 E; Quartzipsamment; Soil Survey Staff, 2014; Clarke et al., 2019), unpolluted and polluted river sediments, compost, and biosolids were analysed. The Eerste and Plankenbrug rivers, used as indicators of pristine and polluted sediments, are in the Eerste River Catchment (Western Cape). Sediment samples were taken at the mountainous source (unpolluted), and after the footprint of Stellenbosch, including industry and the anthropogenic impact of an informal settlement (polluted). Locations, reservoir sources, sampling months and additives are described in Supplementary Materials (Table S1). All samples were immediately characterized within 48 hrs of collection (referred to throughout the study as 'wet sludge') with cold storage within the laboratory. Sludges and biosolids were re-characterized after drying and processing, for soil application. Water treatment residuals were air dried (to 30°C, for 1-3 weeks), crushed and passed through a 2 mm sieve. Biosolids were similarly air-dried (30°C, for 1-3 weeks), however the crushing step was not to 2 mm as with the WTR, to prevent handling risks and the production of potentially infectious dust. A pestle and mortar were used to roughly crush and break up large particles for soil application. The commercially available compost used in this study is made from municipal green waste (chipped garden refuse) and was used and analyzed without sieving, according to Clarke et al. (2019). Compost and biosolids subsamples analyzed for C, N and P were further milled prior to extractions (Supplemental Methods S1.3). All processed materials were stored at room temperature in plastic containers.

The Theewaterskloof reservoir (Western Cape, South Africa) is the source water for CT-Fe and CT-Al sludges, and is fed by a number of streams originating in the Hottentots Holland mountains, with a catchment area of 500 km^2. Runoff is received via the surrounding mountainous and agricultural areas as well as surrounding catchments via a network of shafts and tunnels (Oberholster et al., 2015).

The Vaal Reservoir (Gauteng, South Africa) is the source water for J-Fe sludge, and is mainly fed by the Vaal River, with several other feed rivers. The Vaal reservoir catchment is 38 505 km^2 (vaaldam.org, 2020), impacted by substantial mining and industrial activity (Gilbert & Avenant-Oldewage, 2014; Chinyama et al., 2016). However, the reservoir water quality has relatively low microbial pollution indicators (Randwater, 2020; Vaal Dam Catchment Forum, 2020). The Seke reservoir (Mashonaland East, Zimbabwe), the source water for H-Al sludge, lies in the upper reaches of the Manyame river with a catchment size of 748 km^2. Despite being upstream of the more populous areas of the Manyame catchment, there has been rapid expansion of semi-formal settlements and townships upstream of the Harava and Seke Reservoirs (Tendaupenyu, 2012). Both the Seke and Harava reservoirs show signs of anthropogenic enrichment, which is attributed to sewage discharge from surrounding settlements (Tendaupenyu, 2012).

2.2. Local Sludge Characterization: Pathogen Risks

2.2.1. Microbiological analysis

All WTR and biosolids, as well as sediments, were analyzed in triplicate pre-drying (stored at 4°C for a period of up to 48hrs) and post-drying (dried to a constant mass at 30°C, for a period of up to 3 weeks). Chemical characterization is described in Supplementary Materials. Microbiological characterization included cell-matrix disruption and plating on selective

media. Colony forming units were determined by vortexing samples for 3 minutes in phosphate buffered saline with Tween20 (PBST; 8 mM Na_2HPO_4, 0.15 M NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.5% Tween20, pH 7.4, to a total liquid volume of 15 mL), and 100 μL of a dilution series plated on the respective media (Table 1). Total prokaryotes and eukaryotes were quantified after incubation at 26°C (72 hrs), whereas pathogenic species (fecal and total coliforms, enterococci and *Salmonella* and *Shigella*) were quantified after incubation at 37°C (24 hrs).

Table 1. Selective media components, for isolating general and pathogenic microbial populations.

Microbes	Media
Total Prokaryotes	Tryptic Soy Agar (Tryptic Soy Broth, 3 $g.L^{-1}$; Agar, 15 $g.L^{-1}$).
Total Eukaryotes	Yeast Malt Agar (Peptone, 5$g.L^{-1}$; Yeast Extract, 3$g.L^{-1}$; Malt Extract, 3$g.L^{-1}$; Dextrose, 10 $g.L^{-1}$; Agar, 15 $g.L^{-1}$)
Fecal Coliforms	m-FC Agar (52 $g.L^{-1}$; 10 mL 1% rosolic acid in 0.2N NaOH; boil).
Total Coliforms	MacConkey Agar (MacConkey-Boullioun Broth, 40$g.L^{-1}$; Agar, 15 $g.L^{-1}$).
Enterococci	*Enterococcus* Selective Agar (42 $g.L^{-1}$; boil).
Salmonella Shigella	SS Agar (60 $g.L^{-1}$; boil).

The suite of microbial parameters was analyzed again after a month of dry storage for CT-Al, and a year of dry storage for CT-Fe, prior to utilization in further experiments and in order to assess the impact of long-term storage. All media were purchased from Sigma Aldrich and prepared according to manufacturer's instructions. All media were autoclave sterilized (121°C, 15 psi, 15 minutes), unless otherwise indicated.

2.2.2. Pathogen Persistence in Sandy Soil

Microcosm incubations were assessed for metabolic turnover of nutrients and pathogen persistence. Amendments were added to nutrient-poor sandy soil, including (1) Fe-WTR (CT-Fe), (2) Al-WTR (CT-Al), (3) anaerobic digestate (biosolids), and (4) a 1:1 co-

amendment of each of these WTRs with biosolids. Sludges were prepared and stored as described in Section 2.1. Fe-WTR was stored for 1 year before use, and biosolids and Al-WTR were used once dried (within one month of collection). It was hypothesized that the additional microbial concentrations and diversity of the WTR amendments added to nutrient-poor sandy soils would limit the persistence of the total pathogen load of the biosolids, through competitive exclusion. Microcosms contained 30 g total soil weight (including amendments), with single amendments of 20% (w/w) each, and co-amendments of 20% (w/w) each (total 40% w/w). Amendment loads higher than agronomic rates were selected for proof-of-principle, increasing the likelihood of data resolution. Moisture (non-sterile tap water) was added to field water capacity (FWC), after calculating the dry weight of each mixture. Jars were covered with pierced lids, to allow aerobic conditions but prevent moisture loss. Mass was monitored weekly and non-sterile tap water added to FWC. Ammonium and nitrate were assessed in microcosms at time 0 and after 21 days of incubation, as described in Supplementary Materials (chemical characterization). Total prokaryotes, eukaryotes, coliforms and fecal coliforms, as well as enteric bacteria and *Salmonella* and *Shigella*, were also assessed in microcosms at time 0 and after 21 days of incubation, as described above (2.2.1).

2.3. Microbiology of a Sandy Soil Amended with Different Sludges

2.3.1. Microbiological Dynamics in a Wheat Growth Trial: Pot Trial Design

The amendment of a nutrient-poor sandy soil with (1) WTR (CT-Fe), (2) compost and (3) a 1:1 co-amendment of WTR and compost was explored in terms of wheat (*Triticum aestivum L.*) growth, as detailed in Clarke et al. (2019). Chemistry and plant growth are detailed in the previous study (briefly discussed in this study in Results, and Supplementary Information), whereas the microbiological dynamics in the soil are described in this study. Compost was

selected as a co-amendment for these plant trials, as it is less complicated than biosolids in terms of PTEs and POPs. The microbial loads of these bulk soils, rhizosphere soils, as well as alpha and beta diversity, are reported. Bulk soils were sampled against the edge of each pot, and rhizosphere soils collected by removing the roots from the soils, and shaking the soil particles attached to the roots into sterile 100 mL beakers. The control (sandy soil, zero amendment) and 12.5% (w/w) application rate are compared, for the single compost and WTR treatments, and 25% (w/w) for the 1:1 WTR-Comp co-amendment. Amendments are described in Supplemental Materials (Table S2).

All treatments were prepared in triplicate. Pots (5L) were packed to a bulk density of 1500 $kg.m^{-3}$. Six wheat seeds (*Triticum aestivum* L.) were planted per pot and thinned to 3 plants after germination. Pots were weighed and watered twice a week, maintaining FWC. Greenhouse pot placement was randomized, and randomly re-organized twice during the 3-month trial. Pots were fertilized using the wheat recommendation of the Fertilizer Society of South Africa (FSSA, 2007) for Western Cape sandy soils (N = 130, P = 50, K = 75, Ca = 40, Mg = 13 and S = 40 $kg.ha^{-1}$). The 500 mL fertilizer concentrate was added as three applications over the 3-month trial period, the first day of each month.

2.3.2. Soil Microbial Load

At termination of the 3-month trial, bulk soil was collected closest to the pot edge. Rhizosphere soil was collected by removing the roots, and shaking the attached soil from the roots into sterile 100 mL beakers. Separate soil samples were stored overnight (4°C), dry weights (dried at 105°C to a constant mass) calculated, and the total culturable prokaryotes and eukaryotes per gram dry weight were analyzed as described above (2.2.1), in triplicate from 3 separate pots per treatment.

2.3.3. Alpha and Beta Soil Microbial Diversity

The soil DNA (250 mg wet weight, per sample) was extracted from bulk and rhizosphere soils within 2 hrs of sampling, using a Zymo Soil DNA (Zymo Research, USA) extraction kit according to manufacturer's instructions. Automated Ribosomal Intergenic Spatial Analysis (ARISA) was used to analyse bacterial diversity, with ITSReub and FAM (carboxy-fluorescein)-labelled ITSF according to Cardinale et al. (2004). Electropherograms were generated from amplicons on an ABI PRISM 2010XL genetic analyzer (Applied Biosystems, USA), in order to assess fragment length and fluorescent intensity, against an LIZ1200 size standard. Fragment lengths were interpreted from fluorescence peaks using Genemapper 5 software, generating operational taxonomic units (OTU's). The relative abundance of the fragments per sample was indicated by peak heights. A best-fit curve of the size standards allowed for calculation of fragment lengths (Slabbert et al., 2010). Fragment sizes of OTU's were filtered to include 100 - 1000 base pairs and peak heights higher than 150 fluorescent units, as well as a bin size of 3 bps. Diversity (alpha and beta indices) were analyzed using the Vegan package in R (Version 2.5-7; R Core Team, 2013).

2.4. Statistics

Descriptive statistics were generated in Microsoft Excel. The Q-Q plots and Shapiro-Wilk assessed normal distribution, and mean and median were compared, as well as skewness and kurtosis. For differences between treatments, an analysis of variance (ANOVA with a confidence level of 95%, $p<0.05$, Microsoft Excel) was followed by a Tukey's Honest Significant Difference (HSD) post-hoc test for an equal number of samples (Statistica, StatSoft, Tulsa, OK, USA). Differences between individual treatments (pre- and post-incubations, as well as bulk soil and rhizosphere) were assessed in Excel with Student's t-tests for differences in independent means, with a confidence interval of 95% ($p< 0.05$).

Similarly, the chemistry of the sludge samples (pH, EC, C, N; Table S3 and S4) were compared with a Student's t-tests for differences in independent means, with a confidence interval of 95% ($p< 0.05$).

3. RESULTS

3.1. Local Sludge Characterization: Pathogen Risks

3.1.1. Microbiological Analysis

The total microbial and pathogenic loads of wet WTR sludges were compared to sandy soils, biosolids and local pristine and polluted river sediments (Figure 1). Total prokaryotic and eukaryotic populations were significantly higher for all wet sludge samples (WTR and biosolids) than the nutrient-poor sandy soil and pristine river sediment (Figure 1A) ($p<0.05$). Total prokaryotic and eukaryotic populations in the sludge samples were between 6.3 and 7.2 log(CFU.gdw^{-1}), with J-Fe and H-Al slightly higher than CT-Fe and CT-Al. Total coliforms were exponentially (100 fold) higher in the biosolids and polluted river sediment than in the WTR, which were consistent (Figure 1B). Total coliforms were also exponentially less prevalent in the sandy soil and pristine river sediment, with no evidence of fecal coliforms in these samples. Although total coliforms were consistently between 3.14 and 3.9 log(CFU.gdw^{-1}) across WTR samples, the percentage of fecal coliforms in relation to total coliforms was significantly higher for the H-Al samples (10 fold higher), which are from treated water sourced from a reservoir with anthropogenic influence (Masere et al., 2012; Ruhonde, 2017). However, all of the wet WTR was within the South African land application guidelines (Herselman, 2013) for unrestricted use (10^4 CFU.gdw^{-1}; Figure 1B). Wet H-Al sludge was at the threshold between unrestricted use and general use, but still within safe general use standards even before drying. Biosolids were far closer to the threshold of

general/restricted use quality (10^6 CFU.gdw^{-1}; Figure 1B). The associated chemical characteristics of the sludges are included in Supplemental Information (Table S3).

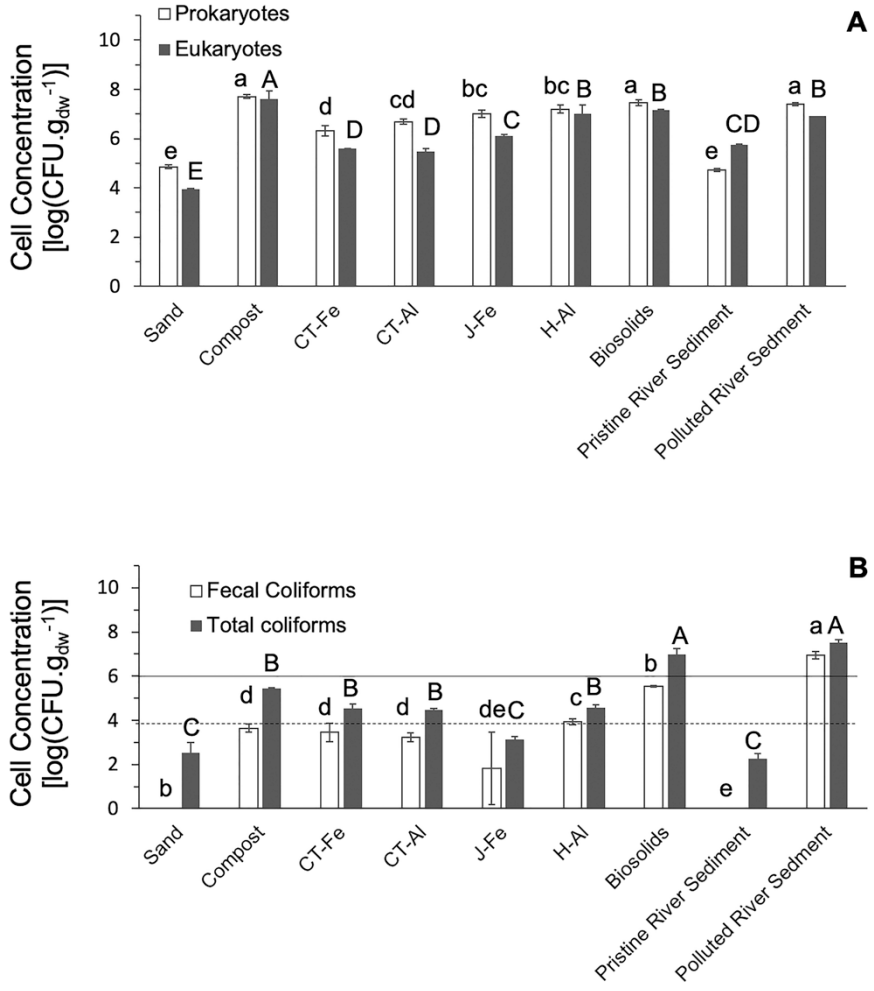

Figure 1. Microbial load of the wet water treatment residuals, contextualized with nutrient-poor sandy soil, local municipal compost and pristine and polluted river sediments. Microbial loads were quantified as general populations (A), and pathogenic indicators (B). South African National Guideline limits for land application are indicated, including general use (10^6 CFU.g^{-1}, solid line) and maximum permissible unrestricted use (10^4 CFU.g^{-1}, dashed

line). Results are expressed as means of triplicate samples. Error bars indicate standard deviation (SD). Significance lettering (p<0.05) is applied to each data range separately.

Less typical pollution indicators were also assessed. Enterococci were not present in any of the sludges or sediments, except the polluted river sediment [4.48±0.15 log(CFU.gdw^{-1})] and the biosolids [3.3±0.14 log(CFU.gdw^{-1})]. *Salmonella* and *Shigella* were not present in any of the samples except the polluted river sediment [3.12±0.02 log(CFU.gdw^{-1})], the biosolids [4.1±0.42 log(CFU.gdw^{-1})], and at very low concentrations in H-Al [1.2±0.13 log(CFU.gdw^{-1})].

The microbial loads after drying, as well as after long-term storage for CT-Al and CT-Fe, were analysed. As there was a decrease in pathogenic populations to negligible concentrations, these results are reported in-text. After drying, the total microbial load of each sludge and soil dropped approximately 10-fold, consistently, for both eukaryotes and prokaryotes. Post-drying total coliform counts were between 0 and 100 CFU.gdw^{-1} for all WTRs, well within unrestricted use guidelines. Post-drying fecal coliforms dropped approximately 10 fold (CT-Fe, CT-Al, J-Al, biosolids) to 100 fold (H-Al), also well within unrestricted use guidelines. After long-term storage (one month for CT-Al, one year for CT-Fe), and the associated limited access to water, fecal coliforms did not persist at all in these WTRs.

3.1.2. Pathogen Persistence

Chemical and microbial turnover were assessed in microcosm incubations, consisting of nutrient–poor sandy soil amended with CT-Fe (20% w/w), CT-Al (20% w/w), biosolids (20% w/w), as well as co-amendments of each of the WTRs (CT-Fe and CT-Al) with biosolids (20%:20% w/w). Upon ammonium and nitrate consumption (Table S4, potentially due to mobility, nitrification or mineralization), the total microbial load remained consistent for all

amendments (Figure 2A and B; $p<0.05$), whilst the standard pathogenic indicator, fecal coliforms, was significantly lower post-incubation, dropping to near zero (Figure 2C; $p<0.05$). However, less commonly measured pathogenic indicators like enterococci, *Salmonella* and *Shigella* persisted after 21 days (Figure 2D and 2E) in samples amended with biosolids. In almost all biosolid-amended samples, there was no significant difference in these pathogens pre- and post-incubation, except a slight, yet statistically significant, decrease in *Salmonella* and *Shigella* in some treatments (Figure 2E). There was no evidence of these pathogens in soils amended with WTR in this study, pre- or post-incubation.

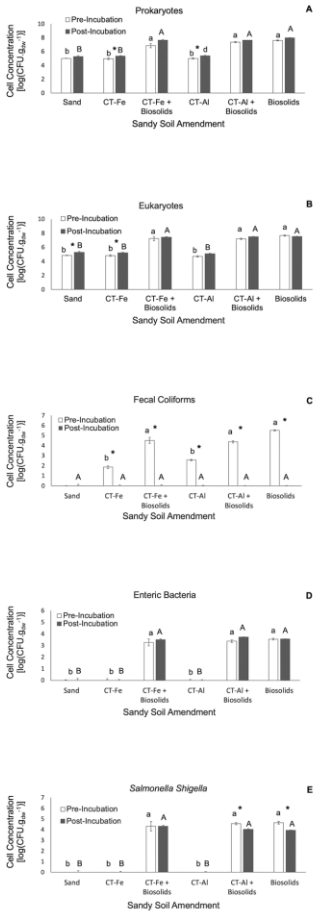

Figure 2. Microbial persistence in 21-day incubations (FWC) in nutrient-poor sandy soil. Sandy soil microcosms were amended with 20% CT-Fe, CT-Al, or biosolids, and 1:1 co-incubations of each WTR with biosolids (20%:20%). Total prokaryotic (A) and eukaryotic

(B) populations were assessed, along with pathogens, including total coliforms (C), fecal coliforms (D) and *Salmonella* and *Shigella* (E). The results are the means of triplicate samples. Error bars indicate standard deviation (SD). Significance lettering (p<0.05) is applied to each data range separately. Differences between pre- post-incubation means (p<0.05) are indicated with an asterisk (*).

3.2 Microbial Load and Diversity of Sandy Soil Amendments: Pot Trials

The microbiome of pot trials (wheat growth in nutrient-poor sandy soils) was assessed, upon amendment with (1) WTR (CT-Fe), (2) compost, and (3) a co-amendment with the WTR and compost. All amendments significantly increased the microbial load of both the bulk and rhizosphere soil (Figure 3, p<0.05) in comparison to the control soil, for both prokaryotes and eukaryotes. The microbial load of the receiving nutrient-poor sandy soil was exponentially increased by all amendments, between 1 and 2.5 log(CFU.gdw^{-1})]. The alpha diversity (within-treatment diversity) indices, although not statistically significant, indicated a trend towards greater diversity in the amended soils than the nutrient-poor sandy soil (Figure 4A). The beta diversity plot (between-treatment diversity) shows, in both the compost and co-amended treatments, that compost has a greater effect on species diversity than the WTR, which did not shift the between-groups microbial diversity from the control as dramatically as compost, and soils co-amended with compost (Figure 4B).

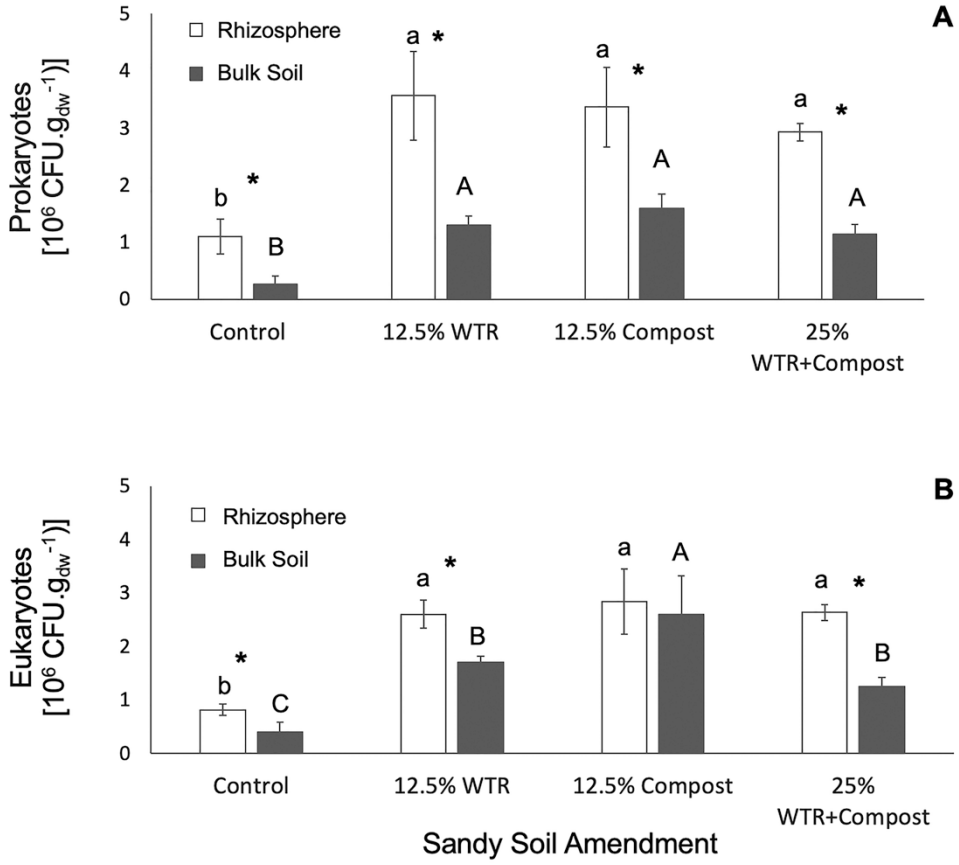

Figure 3. Post-harvest microbial load in bulk and rhizosphere soil in single and co-amendments. Both WTR and compost amendments, as well as the co-amendment, increased the microbial prokaryotic (A) and eukaryotic (B) concentrations in the bulk and rhizosphere soil, after a 3-month wheat trial. Results are the mean of triplicate samples. Error bars indicate standard deviation (SD). Significance lettering (p<0.05) is applied to each data range separately. Differences between bulk soil and rhizosphere means (p<0.05) are indicated with an asterisk (*).

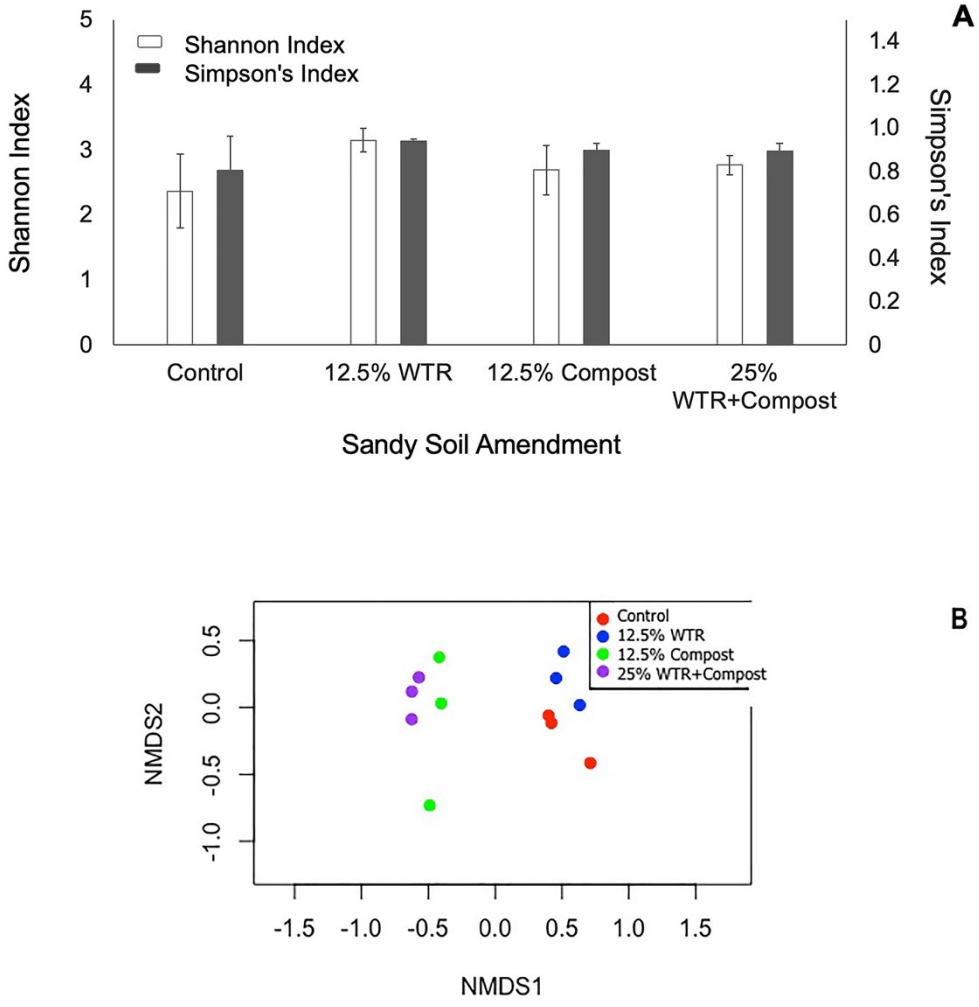

Figure 4. Post-harvest bacterial diversity in single amendments (12.5% WTR, 12.5% Compost) and a co-amendment (25% WTR-Compost). (A) The α diversity within treatments is quantified with the Shannon Diversity Index (H), as well as the Simpson's Diversity Index (D), describing the abundance and evenness of the species in the groups (B), with no significant differences (p<0.05). The β diversity (NMDS ordination plot) represents the change in species diversity between treatments.

4. Discussion

4.1. Microbiological Safety of WTR for Land Application: A Case Study

4.1.1. Microbiological WTR Characterization

Land-applied wastes can contain industrial contaminants and pose a microbial risk during handling, application, crop care and food consumption (Lu et al., 2012). In contrast to biosolids, few studies have analysed the pathogenic risk of WTR, particularly in relation to reservoir geography and pollution impact. This study showed the limited microbial risks of wet water treatment residuals from four reservoir catchments in Southern Africa, impacted by varying levels of pollution (Gilbert & Avenant-Oldewage, 2014; Chinyama et al., 2016). After drying, the pathogen loads decreased even further, demonstrating the limited microbial risk of land amendment with the WTRs analyzed here.

The evidence of coliform microbial contamination in all of the WTR samples (Figure 1) may have come from the reservoirs, from the water treatment process, or from handling and transport. This study investigated the full process of human interaction with the WTR without aseptic sampling and cold transport, remaining true to field conditions. A local Water Research Council study reported elevated coliforms in the drinking water treatment process, particularly in filter backwash water (Mokonyama et al., 2017), another potential source of anthropogenic contamination.

Fecal coliforms in the wet Zimbabwean sludge (H-Al) were statistically higher than the South African sludges, but well within the unrestricted handling and application regulations (Figure 1B) (Herselman, 2013). The rivers feeding the Seke reservoir in Harare exceed national water quality guidelines for many chemical parameters, including turbidity, nitrates and phosphates, although microbial parameters are not reported (Masere et al., 2012; Ruhonde, 2017). Zimbabwean plant operations are also vulnerable to the ongoing precarious financial climate.

For example, currency instability prevented access to treatment chemicals as recently as 2019 (IOL, 2019).

Yet, despite variation in wet WTR quality, even the reservoir sources with higher anthropogenic influence in this study, like the Seke Reservoir in Harare (Zimbabwe) or the Vaal Reservoir in Gauteng (South Africa), generated wet WTR quality well within South African national microbial regulations for unrestricted handling, without the pretreatment necessary for sewage sludge land application. The drying of sludges before application further reduced the already low microbial risk associated with wet sludges. In addition to the pathogenic risks, the Fe- and Al-oxyhydroxides, and heavy metals occurring naturally in sediments (Carstens et al., 2020) carry a potential bio-accumulation risk. However, most studies show that bioremediation or heavy metal sorption is more likely than bioaccumulation (Lombi et al., 2010). This has been studied in much greater depth than the microbial risk (reviewed in Garau et al., 2021), and is thus not the focus of this article. However, Clarke et al. (2019) previously analyzed the CT-Fe WTR used in this study for an extensive suite of heavy metals. In this case, the WTR also promoted plant access to growth-limiting micronutrients and heavy metals, rather than bioaccumulation in plants near risk thresholds. Similarly, these PTE's have been extensively quantified in local WTRs (Titshall and Hughes, 2005), and elegant studies have explored the response (or lack thereof) of ecological indicators to theses PTEs (Howell et al., 2018).

4.1.2. Pathogen Persistence in Soils

Potential pathogen proliferation is a risk upon soil amendment with contaminated sludges, depending on the competitive microbial dynamics under moist conditions (Zaleski et al., 2005). In this study, the potential persistence and regrowth of pathogens was analyzed in moist, amended soil microcosms, measured before and after 21 days of incubation. The

increased diversity of the WTR microbiome as compared to the nutrient-poor sandy soil was proposed to provide competitive inhibition of the biosolid pathogens, and this hypothesis tested with pathogen survival rates in incubation microcosms. The persistence of the fraction of pathogens (fecal coliforms, enteric bacteria and *Salmonella* and *Shigella*) was evaluated, compared to the persistence of the total prokaryotic and eukaryotic microbial populations. Under these laboratory-based conditions, the eukaryotes and prokaryotes remained consistent (Figure 2A-B) upon nitrogen consumption (Table S4), and WTR showed no coliform regrowth after wetting to FWC (Figure 2C). Increased diversity and microbial load has been shown to have an inverse correlation with the survival of invasive pathogens (Van Elsas et al., 2012; Pane et al., 2020). The addition of the WTR microbiome had no competitive influence on biosolid pathogen persistence (Figure 2D-E), likely due to the low impact of WTR on the microbial diversity of the receiving soil (Figure 3 and 4), and the high pathogen loads of the biosolids (Figure 1). The fate of pathogens in soils upon biosolid application is influenced by the sludge to soil ratio (Ellis et al., 2018), as well as temperature, soil texture and soil water content (Park et al., 2016).

For both pathogen persistence studies (2.2.2) and the pot trials (2.3.1), the application rates selected exceed the typical agronomic rates suggested by the US Environmental Protection Agency (EPA, 1994). These rates were selected as proof of concept, as the more realistic microbial loads of environmental application rates carried the risk of lower resolution for statistical analyses. Since these were laboratory-based trials, higher application rates were selected with the aim of shifting to agronomic application rates in future field trials. In addition, as this is a risk assessment, the study leaned towards a cautionary analysis. It provided worst-case scenario simulation data, more representative of the risk of repeat sludge applications with high amendments. Considering the mass of sludges produced world-wide (Clarke et al., 2019; Lu et al., 2012) and predicted increases in the global human population

and urbanization (Leeson, 2018), studies assessing repeat applications and high amendments are realistic strategic considerations for future sludge management and governance. In addition, the aim of co-amendment with WTR is to sorb many of the contaminants, potentially increasing the mass of sewage sludge that can be disposed of at the same agronomic rates. Thus, these lab trials were executed with higher application rates, with the aim of proving principles and optimizing environmental rates for future field trials.

In this study, despite the competitive reduction in fecal coliforms to negligible concentrations post-incubation, less typical pathogenic indicators like enterococci, *Salmonella* and *Shigella* remained relatively consistent during incubation with biosolids (Figure 2D-E). This supports the pre-processing of biosolids for land application (Lu et al., 2012). Although this study did not explore non-culturable pathogens, the suite of pathogens was broadened to include *Salmonella* and *Shigella* as well as enteric bacteria, since there is evidence that species persistence depends on soil type, temperature and moisture content (Underthun et al., 2018). In this study, all the indicators, other than the fecal coliforms, persisted during incubation of soils at field water capacity, although they were only associated with the biosolids and not the WTR. Thus, this study promotes the safe application of WTR, in terms of microbial pathogen persistence, but suggests a broader suite of pathogens is necessary for pathogen survival proxies in soil microbial studies. Field trials exploring agronomic application rates would contribute to a more realistic understanding of the impact of WTR on sandy soil microbiology.

4.2. How does WTR affect rhizosphere microbiology?

This study explored the shift in the microbial dynamics in a previously reported wheat growth trial, in-nutrient poor sandy soils amended with WTR, compost and a co-amendment of these materials (Clarke et al., 2019). The previous study showed that the co-amendment

promoted plant biomass, related to the N:P ratios. Along with the chemical benefits of co-amendment reported in the previous study, this work showed the improvement of prokaryotic and eukaryotic concentrations in the nutrient-poor receiving soil. Both groups are beneficial to soil structure and functionality, with prokaryotes often associated with metabolic turnover (Luo et al., 2018) and eukaryotes shown to play a role in drought tolerance (de Vries et al., 2018), cellulolytic humification (Tortosa et al., 2020), and plant root access to nutrients via mycorrhizae (Ren et al., 2020). Local studies explored the alpha diversity of agricultural soils (Shannon = 2.8±0.3, Simpson = 0.76±0.01), pristine soils (Shannon= 2.58±0.12, Simpson = 0.8±0.03) (Dube et al., 2019), and wheat rhizosphere soils (Shannon = 3.45) (Gqozo et al., 2020). Although the diversity in this study fell within the range of these local studies, between-treatment resolution is challenging (Figure 4A). The clearer shift in bacterial beta diversity with single and co-amendment of compost indicated a more species-rich compost microbiome than WTR microbiome. Both compost (Wu et al., 2016) and biosolid amendments (Cytryn et al., 2011) were shown to increase the microbial diversity and species richness in soil, which has been positively linked to many soil functions (Van der Heijden et al., 2008; Pane et al., 2020; Cytryn et al., 2011; Delgado-Baquerizo et al., 2016). It is well-established that soil bacterial diversity increases with soil pH (Rousk et al., 2010), which was shown in certain studies to have a greater effect than mineral N or P (Zhalnina et al., 2015). Furthermore, soil texture (clay content and pore sizes) was also shown to affect microbial diversity (generally increasing with increasing clay content), although soil pH is dominant (Xia et al., 2020). The enhancement in bacterial diversity could be explained by the treatments' effects on soil pH and texture, as compost (pH 7.5-7.6 in KCl; Table S3) increased the sandy soil pH (4.3-5.6, in KCl) significantly more than the CT-Fe WTR used in this trial (pH 5.8-6.6, in KCl), correlating with the shift in diversity (Figure 4B). The greater impact of compost on the sandy soil bacterial diversity than WTR thus supported Rousk's

(2010) association between pH and bacterial diversity. The particle size distribution and particle texture of WTR was previously shown to increase the heterogeneity and pore size distribution (water holding capacity) of these nutrient-poor sandy soils (Steytler, 2021), also supporting Xia et al. (2020). This shift in microbial diversity based on soil texture appeared to be species-dependent, particularly linked to access to organic compounds in finer textured soils, such as the more heterogeneous WTR amendment. It is challenging to extricate the effects of diversity from the effects of specific microbial species. Functional redundancy has been assumed to overwhelm the function-diversity relationship of the soil microbiome (Van der Heijden et al., 2008). Improved microbial diversity has also been shown to enhance bioremediation functionality, upon WTR-compost co-amendment to heavy metal contaminated soils (Garau et al., 2019).

Because WTR is the source of the bio-available ammonium and nitrate in the compost-amended soils (Table S3) (Clarke et al., 2019), and nitrogen-fixing and nitrogen-mineralizing bacteria are particularly relevant in the rhizosphere (Töwe et al., 2010), functional nitrogen-mineralizing and nitrogen-fixing microbial populations in the WTR are of interest for future studies. Describing the plant growth parameters and chemistry of this study, Clarke et al. (2019) showed that the WTR-compost co-amendment promoted wheat growth in sandy soils, partly due to the optimal N:P balance, with compost providing the P and WTR the N necessary for agricultural productivity. Thus, the microbiome facilitated by WTR might have metabolic potential in nitrogen cycling. Töwe et al. (2010) showed that the evolution of the soils in the presence of nitrogen availability is quantitatively linked to the presence of genes associated with nitrogen cycling in the microbial population. Thus, although WTR facilitates less total diversity than compost (Figure 4B), it may facilitate critical microbial functionality due to the functional genes present in the original sediment microbiome, contributing to the benefits of the compost-WTR co-amendment. This holds interesting potential for a follow-up

study. Since microbial symbioses facilitate plant root access to limiting nutrients, and promote soil nutrient turnover, microbial diversity is suggested to functionally contribute most at low soil nutrient availabilities (Van der Heijden et al., 2008). This has particular relevance for enriching nutrient-poor Cape Flats sandy soil with WTR amendments, both rich in nutrients and microbes, explored here and in Clarke et al. (2019).

In addition to improved microbial load and diversity, there was qualitative evidence of microbe-root associations for compost, WTR and co-amendments. Microbial cells were not evident in the control samples. In the amended samples, cells were evident and microstructures were clearly visible, linking the cells to the roots (Supplemental Materials, Figure S1), which were likely pili or fimbriae. These have been shown to be important mediators of rhizosphere microbe-root interactions, facilitating twitching mobility, attachments and endophytic associations in roots and nodules (Vesper & Bauer 1986; Timmusk & Nevo, 2011; Böhm et al., 2007). Such bacterial-root associations mediated by pili have been shown to lessen heavy metal stress responses in plants (Wright et al., 2016). This is qualitative evidence to support the benefits of microbial load and diversity that the sludge amendments facilitated in the nutrient-poor sandy soil.

5. Conclusions

This study showed that, despite variation in culturable pathogens in local WTR related to anthropogenic activity, even the most contaminated WTR did not pose a handling or agricultural application risk, in terms of the human pathogens assessed. Pathogens from dry WTR did not regrow in microcosm incubations, but the microbial load and diversity introduced by WTR co-amendment had no limiting influence on pathogen survival in soils co-amended with biosolids. In this case, this study indicated that WTR processing is not necessary for pathogen reduction, prior to land application, and in fact, the co-amendment of

WTR and compost increased both microbial concentrations and microbial diversity in receiving, nutrient-poor sandy soils. Compost had a greater influence than WTR on the receiving soil microbiome diversity. Evidence contributing to the safety of waste re-use supports the sustainable consumption and production patterns encouraged by SDG12, as well as the sustainable use of terrestrial ecosystems encouraged by SDG15. Information encouraging the use of wastes assists in interrupting the funneling of valuable nutrients to landfill sites, instead promoting soil health, productivity and biodiversity.

Acknowledgements

The authors would like to acknowledge Casper Brink (Sporatec, Stellenbosch, South Africa) for assistance with ARISA and Lydia Marie Joubert (Electron Microscopy Unit, Central Analytical Facility, Stellenbosch University) for expert support in electron microcopy. We are grateful to the City of Cape Town's Scientific Services and Randwater (Johannesburg) for access to sludge samples. The project formed part of the Biogeochemistry Research Infrastructure Platform (BIOGRIP), which is funded by the Department of Science and Innovation of South Africa, and Lynsay Blake was supported by the Daphne Jackson Fellowship. Johnson and Quinton's contributions were funded by N8 pump priming fund awards, administered by both Durham and Lancaster Universities. The authors declare no conflict of interest, and all conclusions are the views of the authors, independent of the funding bodies.

Conflict of Interest

The authors declare no conflict of interest.

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