Hospital wastewater treated with a novel bacterial consortium (*Alcaligenes faecalis* and *Bacillus paramycoides* spp.) for phytotoxicity reduction in Berseem clover and tomato crops

Aneeba Rashid, Safdar A. Mirza, Ciara Keating, Sikander Ali and Luiza C. Campos

ABSTRACT

Hospital wastewaters are produced in large volumes in Pakistan (~362–745 L/bed.day) and are discharged without proper treatment. They are widely used by farmers for crop irrigation and induce a phytotoxic effect on plant growth. The study was conducted to evaluate the effect of untreated and treated hospital wastewater on seed germination of a fodder crop *Trifolium alexandrinum* (Berseem clover) and a food crop *Solanum lycopersicum* (tomato). A bacterial consortium was formed with three bacterial strains, i.e., *Alcaligenes faecalis* and *Bacillus paramycoides* spp., which were individually proven efficient in previous studies. The concentrations of untreated and treated hospital wastewater (25, 50, 75 and 100%) were used to irrigate these crop seeds. To assess the efficiency of treatment, the germination percentage, delay index, germination index, stress tolerance indices, seedling vigour index and phytotoxicity index were calculated and were statistically proven significant. The seeds grown in treated wastewater concentrations showed negative values of phytotoxicity indices (tomato: −0.36, −0.47, −0.78 and −1.11; Berseem clover: −0.23) which indicate a stimulatory or non-toxic effect on seedling growth. Our work proposes that this bacterial consortium is efficient for hospital wastewater treatment before crop irrigation.

Key words | *Alcaligenes faecalis*, *Bacillus paramycoides*, biodegradation, fodder and cash crops, phytotoxicity, wastewaters

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HIGHLIGHTS

- Bacterial consortia proficiently used for hospital wastewater treatment.
- Novel combination of *Alcaligenes faecalis* and *Bacillus paramycoides* spp. promotes the efficiency of hospital wastewater treatment.
- Consortium proved to be capable of phytotoxicity reduction in two crop plants, *Trifolium alexandrinum* (Berseem clover) and *Solanum lycopersicum* (tomato), irrigated with treated hospital wastewater concentrations.

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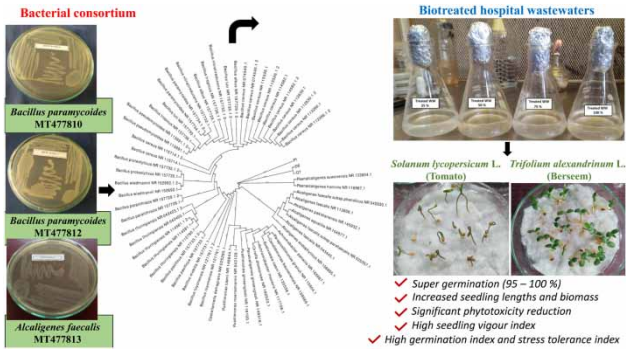
GRAPHICAL ABSTRACT



INTRODUCTION

Agriculture, a major contributing factor to the gross domestic product of Pakistan (~21%), is considered as the foundation of the country's economy (GOP 2015). The majority of the country's population (~63%) is rural and is directly or indirectly associated with the agriculture sector (TWB 2018). Almost 90% of all the country's agricultural food production is carried out using water from the Indus Basin irrigation system (Qureshi 2011), which is regarded as the world's largest natural and continuous irrigation system. It is used for various purposes (PCRWR 2019): irrigation (~60%), drinking (~90%) and industrial use (~100%). However, the system is now becoming one of the world's most overstressed and quickly diminishing natural water systems (NASA 2015). Additionally, Pakistan is predicted to face a shortage of water in the near future (Roberts 2017), with the country listed as the third most water-deficit in the world (IMF 2015) and its groundwater table is predicted to disappear by 2025 (PCRWR 2019).

Due to this freshwater shortage, the farmers are irrigating their crops with raw wastewaters coming from domestic, hospital, industrial and other waste effluent sources (Qadir *et al.* 2007). In Pakistan, the municipal wastewater production (3.06×10^9 m^3/year) is more than 70% of the total produced wastewater (4.37×10^9 m^3/year) (Murtaza & Zia 2012). The remaining 30% wastewater (1.31×10^9 m^3/year) is produced from industrial use. All of these wastewaters are discharged into combined sewers in Pakistan (Murtaza & Zia 2012) containing impurities, dyes, disinfectants, pharmaceuticals, heavy metals, solvents and toxic chemical compounds without proper treatment (Emmanuel *et al.* 2005). The hospital wastewater containing pharmaceutical contaminants, heavy metals and toxic chemical compounds is more than 10% of the municipal wastewater (Ashfaq *et al.* 2017). The rest of the municipal wastewater is produced from domestic and other sources. The hospital wastewater is produced in large volumes (~362–745 L/bed.day) and is discharged without proper treatment despite hospital waste management rules issued by the Ministry of Environment, Pakistan since 2005 (GoP 2005). This enormous amount of unsafe hospital wastewater needs special consideration (Meo *et al.* 2014). Reusing this untreated hospital wastewater for crop irrigation is extremely harmful to plants (Hamilton *et al.* 2007; Dwivedi 2018), animals and humans (Qadir *et al.* 2007; Keraita *et al.* 2008; Qadir *et al.* 2010; Contreras *et al.* 2017). It also pollutes the aquatic environment, leading to fish kills etc. (Hernando 2006). The treatment of hospital wastewater before its discharge into combined sewers would help to reduce freshwater pollution and increase its availability for safe use in crop irrigation. Previously, the biological methods using bacteria have been recognised as efficient, eco-friendly and more cost-effective than physicochemical methods for the treatment of combined wastewaters (Phugare 2011). However, their role in treatment of hospital wastewater was not confirmed. In our previous study (Rashid *et al.* 2020), we have shown the capacity of three bacterial strains (*Bacillus paramycoides* spp. and *Alcaligenes faecalis*) isolated from domestic and pharmaceutical wastewaters for the treatment of hospital wastewater. The hospital wastewater under study was characterised with pH (7.4), electrical conductivity (EC) (444 μs/cm), salinity (0.2 ppt), turbidity (51 NTU), total suspended solids (TSS) (2300 mg/L), total dissolved solids (TDS) (296 mg/L), chemical oxygen demand (COD) (396 mg/L), biological oxygen demand (BOD)

(246 mg/L), biodegradability index (0.62), chromium (1.8 mg/L), lead (0.17 mg/L) and nickel (1.8 mg/L) and it contained a mixture of emergent pharmaceutic contaminants (i.e., phenol, salicylic acid, caffeine, naproxen, octadecene and diazepam). Though most of the parameters for untreated hospital wastewater are beyond the National Environment Quality Standards (NEQS), we achieved high percentage decolourization (>93%) and degradation (100–43%) of pharmaceutic pollutants found in hospital wastewater. This work recommends the potential use of these strains as a consortium as it involves a combined mechanism of metabolism among the co-existing bacterial isolates. For this, the first step would be to demonstrate the safe use of this consortium for crop irrigation by performing a phytotoxicity analysis. Phytotoxicity is the induction of any toxic effect induced within plants due to pollutants that delay seed germination or affect plant growth parameters (length and weight) (WRAP 2002). Previously, a reduction in phytotoxicity was observed in *Lactuca sativa* (lettuce) seeds irrigated with consortia treated wastewaters (Ceretta 2018). It has also been observed that biologically treated textile wastewaters used for crop irrigation were capable of improving the growth of plants (Velayutham 2017). However, extended work is still required for a comprehensive evaluation of the phytotoxicity of untreated and treated hospital wastewaters for crop irrigation.

The present study aimed to assess the phytotoxicity reduction by a novel bacterial consortium (*B. paramycoides* spp. and *A. faecalis*) (Rashid *et al.* 2020) in hospital wastewaters which are highly toxic, to allow the wastewater to be used safely for the irrigation of the main fodder crop in Pakistan i.e. *Trifolium alexandrinum* (Berseem clover), and the most popular food crop in Pakistan i.e. *Solanum lycopersicum* (tomato). Berseem clover is a vital winter fodder crop that plays an essential role in improving the dairy industry in Pakistan. Comparatively, tomato is a rapid-growing vegetable crop, providing a higher yield which is economically important. The reason for selecting these crops is that they are both considered highly valuable for the country's economy and it is the first time that these plants have been tested for phytotoxicity after wastewater irrigation. This will be determined using a range of matrices to assess plant health – seed germination percentage, delay index (DI), germination index (GI), stress tolerance indices (STIs), seedling vigour index (SVI) and phytotoxicity index (PI). These indices are predictors of phytotoxicity reduction in crop plants after irrigating with treated hospital wastewater and are compared with the irrigation with untreated hospital wastewater. The potential application of this work is to determine if the biotreatment with this consortium is a feasible method to treat highly toxic hospital wastewater and allow its safe reuse to irrigate two important crop plants, and therefore be an attractive alternative to meet the increasing demand of freshwater.

MATERIALS AND METHODS

Collection of hospital wastewater

The hospital wastewater sample (50 L) was collected from three different points of discharges from a drainage site of a local hospital in Lahore, Pakistan, according to the standard protocols (APHA 2005). The drainage site of the hospital had a combined disposal tank station containing homogenized waste from all wards. The geographical coordinates of Lahore city are 31° 34′ 55.36″ north and 74° 19′ 45.75″ east at an altitude of 217 m (712 ft). The sample was collected on 15 March 2019.

Characterization of hospital wastewater

The full characterization of hospital wastewaters is not part of present study. However, in order to assess the efficiency of biotreatment, the following parameters were investigated according to standard protocols (APHA 2005) before and after the biotreatment of hospital wastewater. These parameters were compared with the NEQS (NEQS 2000) (Table 1), i.e. physical components (colour, odour, pH, EC, TDS, TSS, salinity [ppt] and turbidity [NTU]); biological components (BOD and isolation and identification of bacterial isolates); and chemical components (COD, heavy metal estimation – arsenic, cadmium, chromium, lead and nickel, and identification of pharmaceutic contaminants). The heavy metal estimation was carried out using an Atomic Absorption Spectrophotometer (AA 7,000 F with Autosampler and Hydride Vapour Generator, Shimadzu, Japan) to access the efficiency of biotreatment. Most of the parameters for untreated hospital wastewater were beyond the range of the NEQS. The presence of pharmaceutic contaminants also highlighted the necessity of an effective biotreatment.

Characterization and development of the bacterial consortium

The bacteria used in the consortium were isolated in our previous study (Rashid *et al.* 2020). The isolates were

Table 1 | The characterization of hospital wastewater

Parameters		NEQS	Hospital wastewater
Physical components	Colour	–	Light yellow
	Odour	–	Fishy
	pH	6.6–8.5	7.4***
	EC (µs/cm)	–	444****
	TDS (mg/L)	1,000	296****
	TSS (mg/L)	<500	2,300****
	Salinity (ppt)	–	0.2**
	Turbidity (NTU)	5	51***
Biological components	BOD_5 (mg/L)	80–250	246****
	Bacterium 1	–	H-1 (*Bacillus* sp.)
	Bacterium 2	–	H-2 (*Pseudomonas* sp.)
	Bacterium 3	–	H-3 (*Bacillus* sp.)
	Bacterium 4	–	H-4 (*Bacillus* sp.)
	Bacterium 5	–	H-5 (*Pseudomonas* sp.)
	Bacterium 6	–	H-6 (*Bacillus* sp.)
	Bacterium 7	–	H-7 (*Bacillus* sp.)
	Bacterium 8	–	H-8 (*Pseudomonas* sp.)
	Bacterium 9	–	H-9 (*Bacillus* sp.)
Chemical components	COD (mg/L)	150–400	396****
	Arsenic (As)	0.05 mg/L	Nd
	Cadmium (Cd)	0.01 mg/L	Nd
	Chromium (Cr)	0.05 mg/L	1.8
	Lead (Pb)	0.05 mg/L	0.17
	Nickel (Ni)	0.02 mg/L	1.8
	Phenol (µg/L) (Aromatic)	–	876
	Salicylic acid (µg/L) (Metabolite)	–	48
	Caffeine (µg/L) (Stimulant)	–	7
	Naproxen (µg/L) (NSAID)	–	23
	Octadecene (µg/L) (Organic)	–	185
	Diazepam (µg/L) (Sedative)	–	14

Nd, Not detected; NSAID, non-steroidal anti-inflammatory drug. Significance is indicated by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

identified as *B. paramycoides* spp. and *A. faecalis* sp. (Figure 1).

Phylogenetic analysis of the strains was carried out using the top 20 BLAST hits for each isolate. This was achieved by aligning the sequences using Muscle v. 3.8.425 (Edgar 2004) and a phylogenetic tree assembled in Geneious Prime using the Tamura–Nei genetic distance method (Tamura & Nei 1993) and Neighbor-Joining tree building

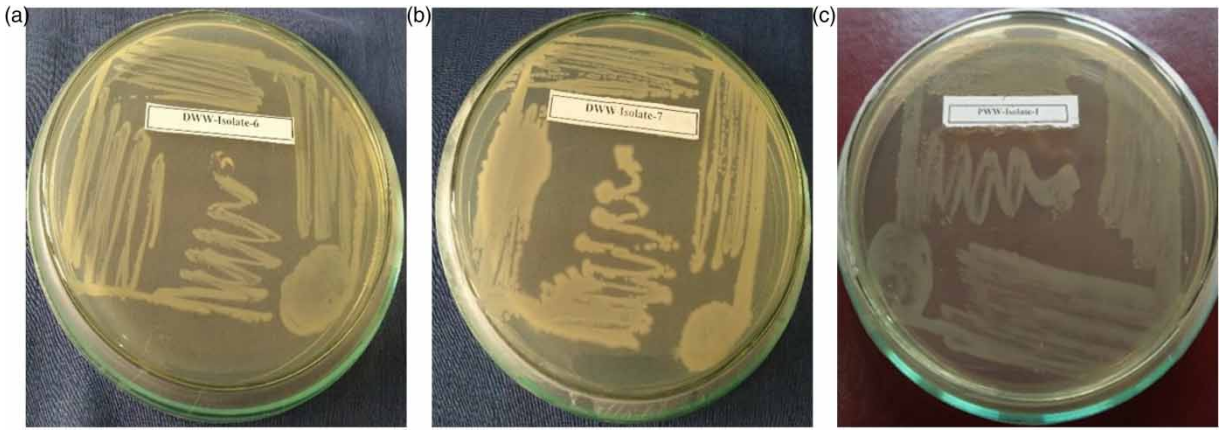


Figure 1 | Bacterial isolates for consortium development.

method (Saitou & Nei 1987). The phylogenetic tree was then imported in to the Newick file format and edited in Evolview (Zhang *et al.* 2012). Phylogenetic analysis was conducted to determine the similarity of these species to each other and their respective closely identified BLAST sequences. The three isolates were distinct from these BLAST matches. Both *B. paramycoides* spp. clustered together, demonstrating that these isolates were highly similar. The closest cluster for three of the species was identified as *Paenalcaligenes suuwonensis* and *Paenalcaligenes hominis* (Figure 2). For consortium development, one colony was picked from each of the plates of the three bacterial isolates. These were inoculated in a test tube (20 mL) containing sterilized Lysogeny broth medium (10 mL) and incubated at 37 °C in a shaking incubator. After 24 h of incubation, the consortium was ready to be used for the biotreatment experiment.

Biotreatment

One colony of the consortium was added to a test tube (20 mL) with deionized water (10 mL) to form a consortium suspension (optical density = 1). The consortium suspension (10%) was added to a conical flask (250 mL) containing hospital wastewater (100 mL) and incubated at 37 °C for 48 h in a shaking incubator for the biotreatment. This suspension was considered as treated wastewater after this period.

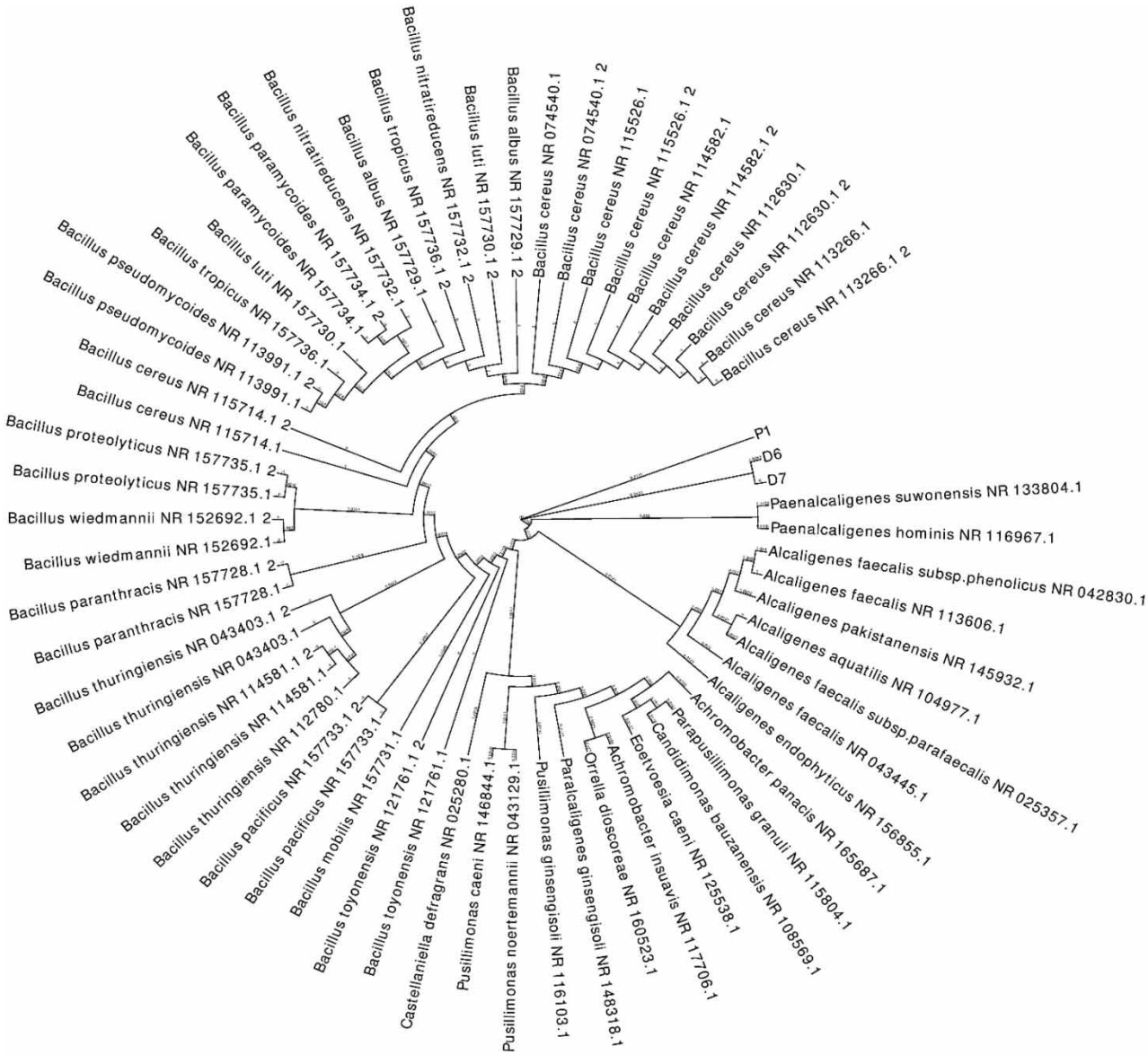


Figure 2 | Phylogenetic relationship between the three isolates and associated BLAST reference sequences.

Phytotoxicity experiments

Seed selection and sterilization

The fodder crop (*T. alexandrinum*, Berseem clover) and food crop (*S. lycopersicum*, tomato) seeds were generously provided by the Seed Certification Department, Lahore, Pakistan. The names of the certified variety for the crop seed were Berseem clover seed (Anmol) and Seminis Hybrid Tomato ONYX (Ori-Thailand). The germination experiments were conducted within a randomized complete block design with three replications. Fifteen Berseem clover seeds were sterilized in a laminar fume hood with 4% (v/v) bleach solution. Then, 50 μL of Tween 20 detergent was added to the bleach solution to avoid any contamination during germination.

Preparation of wastewater concentrations and germination experiment

A broad selection of dilutions of untreated and treated hospital wastewater concentrations, i.e. 25, 50, 75 and 100%, were chosen to simulate different levels of contaminant concentrations (Figure 3). These concentrations were diluted with deionized water to assess the differential extent of phytotoxicity (Ceretta 2018). The sterilized seeds were placed on cotton (1 g) placed in sterilized glass Petri dishes (dimensions 100 × 15 mm). The cotton was wetted with a similar volume of the different dilutions of untreated and treated hospital wastewater (20 mL). Tap water (TW, 20 mL) and deionized water (20 mL) were used for the control treatment, separately (Kaushik *et al.* 2005). In total, there were 30 Petri dishes, including controls. Seeds were germinated under continuous white fluorescent tube lights with the light intensity of 2,500–3,500 lx/m^2/s inside a growth room at 25 °C.

Measurements of length and weight

The observations of seeds and growth parameters were recorded daily up to 1 week and germination indices were calculated. A seed was considered as germinated when its root was visible and measurable (i.e. >0.5 mm); the root

Figure 3 | Concentrations of untreated and treated hospital wastewater (WW, wastewater; HWW, hospital wastewater).

lengths of ungerminated seeds were considered as zero. The length of root and shoot of the germinated seeds were measured from each experimental set. The shoot length was measured from the base of the primary leaf to the base of the hypocotyl in centimetres. Root length was measured from the tip of the primary root to the base of the hypocotyl in centimetres. By adding the root length and shoot length, the seedling length was calculated and expressed in centimetres. Both shoots and roots were oven-dried at 65 °C overnight and the dry weights were determined.

Germination percentage (GP)

The GP is the ratio between the total number of germinated seeds to the total number of viable seeds. This parameter is an indicator to assess the viability of seeds and to see whether the treatment condition is suitable for the seeds to grow or not. The range of desirable GP for seeds irrigated with treated hospital wastewater is between 80 and 100%; whereas, less than 75% GP indicates poor germination (Solomon 2013). The GP was calculated using Equation (1) (Amin *et al.* 2013):

$$\mathrm{GP}(\%) = \frac{\text{no. of germinated seeds}}{\text{total no. of viable seeds}} \times 100 \quad (1)$$

Delay index (DI)

DI is the ratio between delay in germination time over control to germination time for control. This parameter is an indicator to see the delay in seeds that are germinated in different dilutions of untreated and treated hospital wastewater compared to the time required by those seeds germinated in control (DW). The desirable DI for seeds irrigated with treated hospital wastewater should be the same as the control. The higher the DI, the poorer the ability of seeds to germinate. DI was calculated using Equation (2) (Kaushik *et al.* 2005):

$$\mathrm{DI} = \frac{\text{delay in germination time over control (X)}}{\text{germination time for control (Y)}} \quad (2)$$

Germination index (GI)

The GI is the product of relative seed germination (RSG) and relative root growth (RRG). This is the parameter associated with the two different characteristic features of any wastewater, i.e. EC and heavy metals. The increase in GI values in crop plants irrigated with treated wastewater may indicate the efficiency of the treatment in removing or reducing the concentration of heavy metals and may cause an increase in the EC value. This efficacy also promotes the increase in RSG as well as the RRG. GI values are used to elucidate possible differences in phytotoxicity induced in seeds in the presence of different concentrations of untreated and treated hospital wastewater. The desirable GI value for seeds irrigated with treated hospital wastewater is 100% larger than the GI value for the control (Hoekstra *et al.* 2002). The RSG, RRG and the GI of the plants were calculated using Equations (3)–(5) (Hoekstra *et al.* 2002):

$$\mathrm{RSG} = \frac{\text{no. of seeds germinated in sample}}{\text{no. of seeds germinated in control}} \times 100 \quad (3)$$

$$\mathrm{RRG} = \frac{\text{mean root length in the sample}}{\text{mean root length in control}} \times 100 \quad (4)$$

$$\mathrm{GI} = \frac{\mathrm{RSG} \times \mathrm{RRG}}{100} \quad (5)$$

Stress tolerance indices (STIs)

STI is a valuable parameter to determine the high yield and stress tolerance capability in crop plants. STIs are comprised of six indices, namely the root length stress tolerance index (RLSTI), shoot length stress tolerance index (SLSTI), root fresh weight stress tolerance index (RFSTI), shoot fresh weight stress tolerance index (SFSTI), root dry weight stress tolerance index (RDSTI) and shoot dry weight stress tolerance index (SDSTI). These values determine the difference between the stress tolerance potential in shoots and roots between plants irrigated with untreated and treated hospital wastewater in terms of their lengths and fresh and dry weights. The desirable range of all STIs for seeds irrigated with treated hospital wastewater is larger than the STI values for the control and seeds irrigated with untreated hospital wastewater. These were calculated

using Equations (6)–(11) (Amin *et al.* 2013):

$$\text{RLSTI} = \frac{\text{root length of stress plant}}{\text{root length of control plant}} \times 100 \quad (6)$$

$$\text{SLSTI} = \frac{\text{shoot length of stress plant}}{\text{shoot length of control plant}} \times 100 \quad (7)$$

$$\text{RFSTI} = \frac{\text{root fresh weight of stress plant}}{\text{root fresh weight of control plant}} \times 100 \quad (8)$$

$$\text{SFSTI} = \frac{\text{shoot fresh weight of stress plant}}{\text{shoot fresh weight of control plant}} \times 100 \quad (9)$$

$$\text{RDSTI} = \frac{\text{root dry weight of stress plant}}{\text{root dry weight of control plant}} \times 100 \quad (10)$$

$$\text{SDSTI} = \frac{\text{shoot dry weight of stress plant}}{\text{shoot dry weight of control plant}} \times 100 \quad (11)$$

Seedling vigour index (SVI)

This is the property of seed that determines the level of performance of seed to germinate and emerge as a seedling. It is a measurable parameter to describe the germination characteristics linked with the seed performance. The desirable SVI values for seeds irrigated with treated hospital wastewater are larger than the SVI values for control and seeds irrigated with untreated hospital wastewater (Amin *et al.* 2013). These were calculated according to Equation (12), where the higher the SVI, the more dynamic the growth (Amin *et al.* 2013):

$$\text{SVI} = \text{germination percentage} \times \text{seedling length} \quad (12)$$

Phytotoxicity index (PI)

The PI indicates any delay in seed germination, plant growth inhibition or any side effect on seedlings caused by toxic pollutants. It also specifies any danger happening to the plant growth. The possible range of PI values is between 0 and 1, in which a positive PI value indicates a toxic effect on seedlings, whereas a negative PI value demonstrates a stimulatory or non-toxic effect (Tiquia *et al.* 1996). It was calculated based on germination and root elongation according to Equation (13) (Rusan *et al.* 2015):

$$\text{PI} = 1 - \frac{\text{root length of sample}}{\text{root length of control}} \quad (13)$$

Statistical analysis

Experiments were statistically analyzed through one-way analysis of variance (ANOVA) in which the factor was the concentration of hospital wastewater from biotreatment and the effects were the indices. The obtained data were statistically analyzed using GraphPad Prism® 2020. The results were presented as means ± standard deviation (SD). The data were compared using a t-test with Welch's correction and two-tailed *p*-value calculation using GraphPad Prism software. The comparisons included the control (DW) with each treatment (tap water, TW, R25, T25, R50, T50, R75, T75, R100 and T100) and the raw wastewater within each concentration level with the corresponding treated wastewater (e.g. R25 with T25). For all comparisons, differences were considered significant when the probability level was less than 0.05.

RESULTS AND DISCUSSION

Characterization of hospital wastewater

The biotreatment was highly effective to show reduction in the values of TSS (79%; 2,300 to 483 mg/L), TDS (26%; 296 to 220 mg/L), COD (34%; 396 to 260 mg/L), BOD (68%; 246 to 78 mg/L), EC (40%; 444 to 267 μs/cm) and heavy metals nickel (86%; 1.8 to 0.25 mg/L), lead (100%; 0.17 mg/L to non-detected) and chromium (100%; 1.8 mg/L to non-detected) after the treatment of hospital wastewater. In addition, the pharmaceutic contaminants, i.e. phenol (100%; 874 μg/L to non-detected), salicylic acid (85%; 48 to 7 μg/L), caffeine (100%; 7 μg/L to non-detected), naproxen (74%; 23 to 6 μg/L), octadecene (90%; 185 to 19 μg/L) and diazepam (100%; 14 μg/L to non-detected) were degraded after the biotreatment (Figure 4(a)–4(c)).

Characterization of the bacterial consortium

The bacteria used in the consortium were isolated from our previous study (Rashid *et al.* 2020). The isolates were identified as two *B. paramycoides* species and *A. faecalis* sp. Phylogenetic analysis was conducted to determine the similarity of these species to each other and their respective closely identified BLAST sequences. The three isolates were distinct from these BLAST matches. Both *B. paramycoides* spp. clustered together, demonstrating that these isolates were highly similar. The closest cluster for three of the species was identified as *P. suuwonensis* and *P. hominis*.

Phytotoxicity experiments

Germination percentage

According to Solomon (2013), seeds grown in the laboratory showing more than 90% GP (e.g. super germination) are

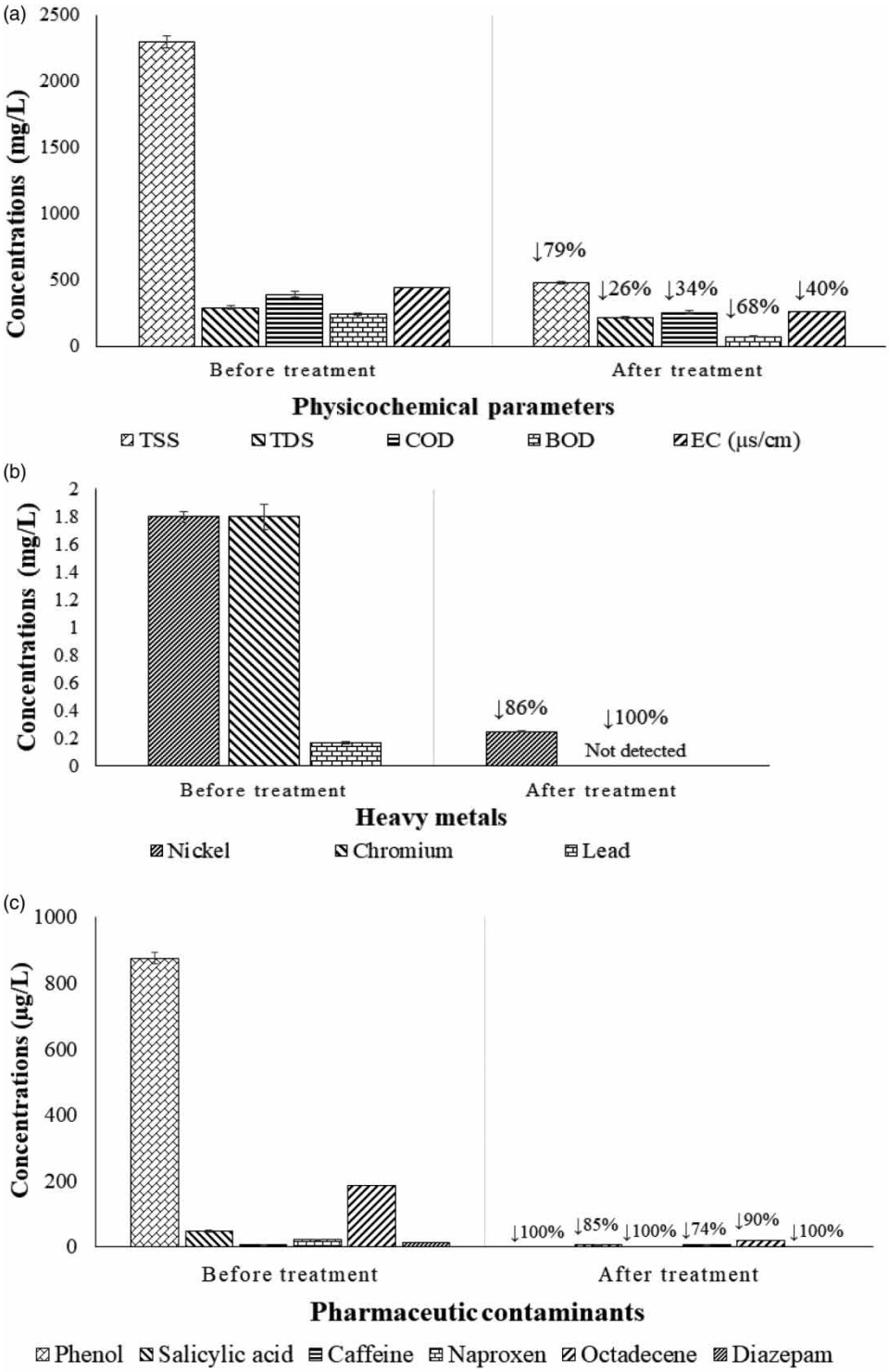


Figure 4 | (a) Physicochemical parameters, (b) heavy metals and (c) pharmaceutic contaminants in hospital wastewater before and after treatment.

expected to grow *ex situ* with a GP of 65% or higher. Similarly, seeds with 85% GP in the laboratory (e.g. good germination) are expected to possess more than 50% GP. Finally, seeds with 75% or less GP in the laboratory (e.g. legal germination) are expected to have at least 15% practical GP in fields. Both Berseem clover and tomato seeds

showed 60–80% growth in all untreated hospital wastewater concentrations (R25, R50, R75 and R100), while the seeds tested showed super germination (95–100%) in different concentrations of treated hospital wastewater concentrations (T25, T50, T75 and T100).

Delay index

The Berseem clover plant was strongly influenced by irrigation with untreated wastewater at all concentrations as it showed a higher value of DI (2) for all untreated hospital wastewater concentrations. The tomato showed a high value of DI (1.25) at all concentrations of untreated hospital wastewaters. The order of DI among these two crop plants followed the trend: Berseem clover < tomato.

Measurements of length and weight

The effects of treated (T) and untreated (R) wastewater concentrations (25, 50, 75 and 100%) were compared for the seedling growth of Berseem clover and tomato. The associated statistical measurements indicated that the means of lengths and weights were statistically significant. The comparisons included the control (DW) with each treatment (TW, R25, T25, R50, T50, R75, T75, R100 and T100) and the raw wastewater within each concentration level with the corresponding treated wastewater (e.g. R25 with T25). It was observed that the seedling length of Berseem clover (shoot and root) was longer (shoot: 1.6–2.7 cm; root: 2.9–4.6 cm) in treated wastewater concentrations than in untreated wastewater concentrations (shoot: 1–1.8 cm; root: 1.2–2.9 cm). Likewise, the root and shoot weights of Berseem clover (fresh and dry) were found to be greater in treated wastewater concentrations than in untreated wastewater concentrations (Table 2).

It was also observed that the seedling lengths of tomato (shoot and root) were longer (shoot: 3.7–4.4 cm; root: 4.9–7.6 cm) in treated wastewater concentrations than in untreated wastewater concentrations (shoot: 1.6–2.8 cm; root: 1.7–3.3 cm). Similarly, the root and shoot weights of tomato (fresh and dry) were found to be greater in treated wastewater concentrations than in untreated wastewater concentrations (Table 3).

Germination index

In our previous study, we showed the presence of organic pollutants (phenol, salicylic acid, caffeine, naproxen, octadecene and diazepam) in the raw/untreated hospital wastewater (Rashid *et al.* 2020). In this present study, the GI values for Berseem clover and tomato seeds irrigated with four concentrations of untreated hospital wastewater (R25, R50, R75 and R100) were increased to 60–150% and 189–278% in treated hospital wastewater (T25, T50, T75 and T100), respectively. Compared to seeds irrigated with tap water (as control), the GI values for tomato seeds irrigated with treated hospital wastewater were higher (Figure 5(a)). Although the GI values of Berseem clover seeds irrigated with treated hospital wastewater were lower than control (TW) they were higher than the GI values of seeds irrigated with raw hospital wastewater (Figure 5(b)). This result also indicates the sensitivity of tomato seeds compared to the Berseem clover seeds.

Zucconi & De Bertoldi (1987) previously mentioned that the 60% increase in GI values compared to distilled water is an indication of improved germination. Our results indicate 100% increase in GI values compared to distilled water. The low values of GI in both crop seeds irrigated with untreated wastewater concentrations reflect the presence of organic pollutant compounds that inhibited the RSG as well as the RRG. Our results agree well with the previously reported work by Tiquia *et al.* (1996). The increase in GI values in Berseem clover and tomato seeds irrigated with treated hospital wastewater concentrations suggest the efficiency of the treatment in removing or reducing the concentrations of organic pollutant compounds. The present study also confirms the presence of three heavy metals: nickel, chromium and lead (Figure 4(b)) in untreated hospital wastewater with low GI and high EC values (Figure 5(a)). However, the treated hospital wastewater showed high GI and EC values due to the negligible amounts of heavy metals (Figure 5(b)). This efficacy indicates the increase in RSG as well as the RRG (Selim *et al.* 2012). The high GI values in seeds irrigated with treated hospital wastewater also elucidate the decrease in phytotoxicity (Tiquia *et al.* 1996).

Stress tolerance indexes

Each of the six stress tolerance indices (RLSTI, SLSTI, RFSTI, SFSTI, RDSTI and SDSTI) for Berseem clover and the tomato crop plant were compared with the treated (T) and untreated (R) wastewater concentrations (25, 50, 75 and 100%) (Tables 4 and 5).

The values of six STIs (RLSTI, SLSTI, RFSTI, SFSTI, RDSTI and SDSTI) for Berseem clover seeds grown in untreated wastewater concentrations were increased to 276, 170, 733, 185, 14,800 and 539%, respectively in treated wastewaters (Table 4). Zvobgo *et al.* (2018) noted that the

Table 2 | Effect of treated (T) and untreated (R) wastewater concentration (25, 50, 75 and 100%) on seedling growth of Berseem clover plant (*T. alexandrinum*) ± SD in brackets

	Root length	Shoot length	Seedling length	Root fresh weight	Shoot fresh weight	Root dry weight	Shoot dry weight
Treatment	**cm**			**g**			
1. DW	3.2 (±0.031)^{****2,3,5,6,7,9,10}	2.7 (±0.041)^{****2,3,4,5,7,8,9}	5.9 (±0.072)^{****2,3,4,5,6,7,8,9}	0.006 (±0.0003)^{****2,4,6,8,10}	0.023 (±0.0009)^{***2,8****3,4,5,6,9,10}	0.001 (±0.0003)^{*3,5****2,4,6,8,10}	0.009 (±0.0008)^{**2,6****3,4,5,9,10}
2. TW	4.8 (±0.0895)^{****1}	2.1 (±0.065)^{****1}	6.9 (±0.1545)^{****1}	0.017 (±0.0006)^{****1}	0.027 (±0.0003)^{***1}	0.008 (±0.0007)^{****1}	0.011 (±0.0003)^{**1}
3. R25	2.1 (±0.041)^{****1,4}	1.6 (±0.101)^{****1}	3.7 (±0.142)^{****1,4}	0.004 (±0.0007)^{****4}	0.029 (±0.009)^{****1}	0.0001 (±0.0001)^{*1****4}	0.013 (±0.0004)^{**4****1}
4. T25	3.1 (±0.0322)^{****3}	1.6 (±0.038)^{****1}	4.7 (±0.0702)^{****1,3}	0.012 (±0.0007)^{****1}	0.031 (±0.0003)^{****1}	0.003 (±0.0003)^{****1,3}	0.019 (±0.001)^{**3****1}
5. R50	2.9 (±0.028)^{****1,6}	1.7 (±0.0455)^{****1,6}	4.6 (±0.0735)^{****1,6}	0.004 (±0.0001)^{****6}	0.033 (±0.002)^{****1,6}	0.0001 (±0.00)^{*1}	0.0106 (±0.0007)^{****1,6}
6. T50	4.6 (±0.051)^{****1,5}	2.5 (±0.122)^{****5}	7.1 (±0.173)^{****1,5}	0.019 (±0.0012)^{****5}	0.016 (±0.0007)^{****1,5}	0.008 (±0.0004)^{****1,5}	0.007 (±0.0002)^{****5}
7. R75	1.2 (±0.0431)^{****1,8}	1.0 (±0.001)^{****1,8}	2.2 (±0.0441)^{****1,8}	0.006 (±0.0005)^{****8}	0.022 (±0.0007)^{*8****1}	0.001 (±0.0002)^{****8}	0.01 (±0.0003)^{**8}
8. T75	3.2 (±0.054)^{****7}	2.2 (±0.102)^{****1,7}	5.4 (±0.156)^{****1,7}	0.02 (±0.0011)^{****1,7}	0.019 (±0.0012)^{*7****1}	0.009 (±0.0001)^{****1,7}	0.008 (±0.0006)^{**7}
9. R100	2.2 (±0.022)^{****1,10}	1.8 (±0.055)^{****1,10}	4.0 (±0.077)^{****1,10}	0.007 (±0.0002)^{****10}	0.012 (±0.0007)^{****1}	0.001 (±0.0002)^{****10}	0.003 (±0.0003)^{****1,10}
10. T100	2.9 (±0.021)^{****1,9}	2.7 (±0.1325)^{****9}	5.6 (±0.1535)^{****9}	0.033 (±0.0021)^{****1,9}	0.034 (±0.0008)^{****1,9}	0.014 (±0.0001)^{****1,9}	0.016 (±0.0008)^{****1,9}
F-value	1,700	138.9	461.9	302.5	177.5	754.3	195.9

The associated statistical measurements are calculated from one-way ANOVA and Sidak multiple comparison testing. The comparisons included the control (DW) with each treatment (TW, R25, T25, R50, T50, R75, T75, R100 and T100) and the raw wastewater within each concentration level with the corresponding treated wastewater (e.g. R25 with T25). *Denotes the *p*-value *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ within different treatments. The F-value is the ratio of two mean square values. A large F ratio means that the variation among group means is higher than expected by chance.

Table 3 | Effect of treated (T) and untreated (R) wastewater concentration (25, 50, 75 and 100%) on seedling growth of tomato plant (*S. lycopersicum*) ± SD in brackets

	Root length	Shoot length	Seedling length	Root fresh weight	Shoot fresh weight	Root dry weight	Shoot dry weight
Treatment	**cm**			**g**			
1. DW	3.6 (±0.141)^{****2,3,4,5,6,7,8,9,10}	3.7 (±0.048)^{*4****2,3,5,7,8,9,10}	7.3 (±0.189)^{****2,3,4,5,6,7,8,9,10}	0.0240 (±0.002)^{*2***4****3,5,7,8,9,10}	0.037 (±0.0025)^{***2,6****3,5,7,8,9,10}	0.012 (±0.0004)^{****2,3,4,5,7,8,9,10}	0.021 (±0.0015)^{***2,6,10****3,4,5,7,8,9}
2. TW	2.9 (±0.025)^{****1}	3.4 (±0.033) ^{**1}	6.3 (±0.058)^{****1}	0.027 (±0.0006)^{*1}	0.032 (±0.0012)^{***1}	0.013 (±0.0007)	0.019 (±0.0006)^{***1}
3. R25	2.5 (±0.035)^{****1,4}	2.8 (±0.025)^{****1,4}	5.3 (±0.006)^{****1,4}	0.009 (±0.0007)^{****1,4}	0.011 (±0.0006)^{****1,4}	0.002 (±0.0004)^{****1,4}	0.003 (±0.0007)^{****1,4}
4. T25	4.9 (±0.025)^{****1,3}	3.8 (±0.033)^{*1****3}	8.7 (±0.058)^{****1,3}	0.028 (±0.0015)^{***1****3}	0.03 (±0.00012)^{****3}	0.015 (±0.0007)^{****1,3}	0.017 (±0.0003)^{****1,3}
5. R50	1.7 (±0.0541)^{****1,6}	1.6 (±0.005)^{****1,6}	3.3 (±0.0591)^{****1,6}	0.012 (±0.0007)^{****1,6}	0.006 (±0.0011)^{****1,6}	0.003 (±0.0009)^{****1,6}	0.001 (±0.0001)^{****1,6}
6. T50	5.3 (±0.0745)^{****1,5}	3.7 (±0.025)^{****5}	9 (±0.0995)^{****1,5}	0.024 (±0.012)^{****5}	0.032 (±0.012)^{***1****5}	0.012 (±0.0009)^{****5}	0.019 (±0.0005)^{***1****5}
7. R75	2.2 (±0.0115)^{****1,8}	1.9 (±0.035)^{****1,8}	4.1 (±0.0465)^{****1,8}	0.006 (±0.007)^{****1,8}	0.009 (±0.0006)^{****1,8}	0.001 (±0.0001)^{****1,8}	0.002 (±0.0002)^{****1,8}
8. T75	6.4 (±0.044)^{****1,7}	4.4 (±0.021)^{****1,7}	10.8 (±0.065)^{****1,7}	0.034 (±0.0004)^{****1,7}	0.046 (±0.0006)^{****1,7}	0.022 (±0.0005)^{****1,7}	0.033 (±0.0007)^{****1,7}
9. R100	3.3 (±0.041)^{****1,10}	1.6 (±0.055)^{****1,10}	4.9 (±0.096)^{****1,10}	0.006 (±0.0009)^{****1,10}	0.007 (±0.0003)^{****1,10}	0.001 (±0.0001)^{****1,10}	0.001 (±0.0002)^{****1,10}
10. T100	7.6 (±0.011)^{****1,9}	4.4 (±0.0325)^{****1,9}	12 (±0.0435)^{****1,9}	0.038 (±0.0007)^{****1,9}	0.03 (±0.0003)^{****1,9}	0.024 (±0.0008)^{****1,9}	0.018 (±0.0003)^{***1****9}
F-value	3812	2600	3492	427.7	523.2	645.5	651.3

The associated statistical measurements are calculated from one-way ANOVA and Sidak multiple comparison testing. The comparisons included the control (DW) with each treatment (TW, R25, T25, R50, T50, R75, T75, R100 and T100) and the raw wastewater within each concentration level with the corresponding treated wastewater (e.g. R25 with T25). *Denotes the *p*-value *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ within different treatments. The F-value is the ratio of two mean square values. A large F ratio means that the variation among group means is higher than expected by chance.

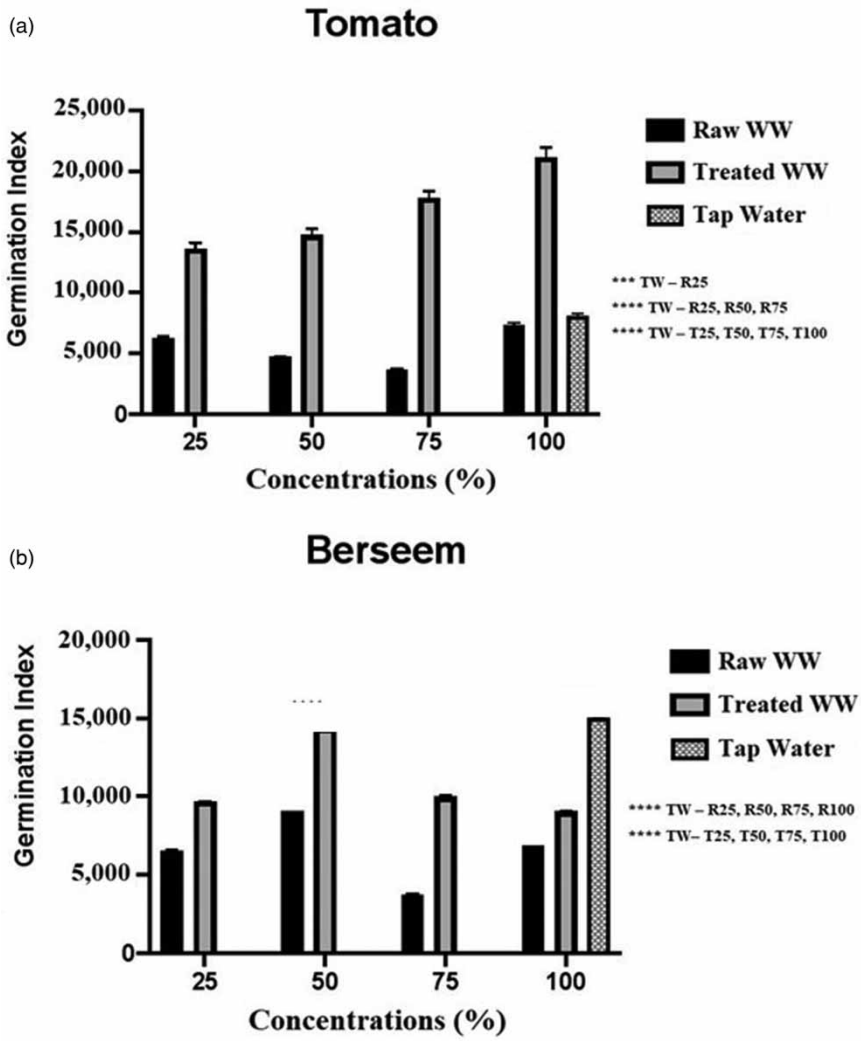


Figure 5 | Germination indices for (a) tomato and (b) Berseem clover. *Denotes the *p*-value $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

higher the tolerance index, the more tolerant the genotype. This higher percentage in STIs for Berseem clover seeds irrigated with treated wastewaters therefore indicates the high yield and stress tolerance capability in Berseem clover. These high values determine the stress tolerance potential in plant shoots and roots in terms of lengths and fresh and dry weights. The values of the six STIs (RLSTI, SLSTI, RFSTI, SFSTI, RDSTI and SDSTI) for tomato seeds irrigated with untreated wastewater concentrations were also increased to 349, 177, 536, 675, 2,400 and 3,040%, respectively, in treated wastewaters (Table 5). This percentage increase is indicative of high yield and stress tolerance capacity in the tomato plant. It also supports the efficacy of hospital wastewater treatment that enhanced the extent of stress tolerance capability in both crop plants.

Seedling vigour index

The SVI values of Berseem clover seeds irrigated with untreated hospital wastewater concentrations (R25, R50, R75 and R100) were increased to 27, 54, 145 and 40% in treated wastewater concentrations (T25, T50, T75 and T100), respectively. Similarly, the SVI value of tomato seeds irrigated with untreated wastewater concentrations (R25, R50, R75 and R100) were increased to 82, 173, 339 and 206% in treated wastewater concentrations (T25, T50, T75 and T100), respectively. These increased SVI values of Berseem clover and tomato plants germinated at different concentrations of treated hospital wastewater demonstrate that the growth is more dynamic in these treated wastewaters. Previously, Rusan *et al.* (2015) showed that the SVI

Table 4 | Stress tolerance indexes (RLSTI, SLSI, RFSTI, SFSTI, RDSTI and SDSTI) of Berseem clover plant (*T. alexandrinum*) in the treated (T) and untreated (R) wastewater concentration (25, 50, 75 and 100%) ± SD in brackets

Treatment	RLSTI	SLSTI	RFSTI	SFSTI	RDSTI	SDSTI
1. TW	149.991 (±1.344)^{****2,3,4,5,6,7,8,9}	77.765 (±1.227)^{****2,3,4,5,6,8,9}	283.473 (±4.179)^{****2,3,4,5,6,7,8,9}	117.477 (±3.295)^{**2****3,4,5,6,7,8,9}	837.363 (±189.595)^{*9***2,4,6,8}	122.672 (±7.601)^{**2****2,3,4,5,6,8,9}
2. R25	65.621 (±0.646)^{****1,3}	59.230 (±2.842)^{****1,3}	66.388 (±8.358)^{****1,3}	126.114 (±1.023)^{**1,3}	9.890 (±8.583)^{**1}	144.946 (±8.473)^{**1****3}
3. T25	96.875 (±0.068)^{****1,2}	59.254 (±0.508)^{****1,2}	199.944 (±1.672)^{****1,2}	134.886 (±3.977)^{**2****1}	313.187 (±66.916)	211.568 (±7.725)^{****1,2}
4. R50	90.625 (±0.003)^{****1,5}	62.956 (±0.729)^{****1,5}	66.722 (±1.672)^{****1,5}	143.398 (±3.087)^{****1,5}	10.659 (±3.346)^{**1****5}	178.257 (±8.099)^{****1,5}
5. T50	143.749 (±0.201)^{****1,4}	92.561 (±3.113)^{****1,4}	316.527 (±4.179)^{****1,4}	69.557 (±0.322)^{****1,4}	843.956 (±223.053)^{**4}	78.058 (±4.735)^{****1,4}
6. R75	37.494 (±0.984)^{****1,7}	37.042 (±0.526)^{****1,7}	99.889 (±3.343)^{****1,7}	95.670 (±0.701)^{****1,7}	102.198 (±11.153)^{**1***7}	111.502 (±6.604)^{**7****1}
7. T75	99.995 (±0.719)^{****1,6}	81.456 (±2.541)^{****6}	333.278 (±1.672)^{****1,6}	82.557 (±1.988)^{****1,6}	957.143 (±289.968)^{***6}	88.963 (±1.246)^{**6}
8. R100	68.750 (±0.021)^{****1,9}	66.656 (±1.025)^{****1,9}	116.750 (±2.507)^{****1,9}	52.148 (±1.004)^{****1,9}	102.198 (±11.153)^{**1****9}	33.311 (±0.374)^{****1,9}
9. T100	90.62 (±0.222)^{****1,8}	99.966 (±3.390)^{****1,8}	549.749 (±7.522)^{****1,8}	147.886 (±2.310)^{****1,8}	1490.110 (±457.258)^{*1****8}	178.191 (±6.978)^{****1,8}
F-value	9166	254.7	3737	658	19.79	232.7

The associated statistical measurements indicated are calculated from one-way ANOVA and Sidak multiple comparison testing. *Denotes the *p*-value *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ within different treatments. The F-value is the ratio of two mean square values. A large F ratio means that the variation among group means is higher than expected by chance.

Table 5 | Stress tolerance indexes of tomato plant (*S. lycopersicum*) in treated (T) and untreated (R) wastewater concentration (25, 50, 75 and 100%) ± SD in brackets

Treatment	RLSTI	SLSTI	RFSTI	SFSTI	RDSTI	SDSTI
1. TW	80.620 (±2.465)^{***8****3,4,5,6,7,9}	91.894 (±0.300)^{****2,3,4,5,6,7,8,9}	112.885 (±6.931)^{****2,4,6,7,8,9}	86.604 (±2.614)^{****2,4,6,7,8}	108.284 (±2.225)^{*5****2,3,4,6,7,8,9}	90.649 (±3.627)^{****2,4,6,7,8}
2. R25	69.490 (±1.751)^{****3}	75.678 (±0.306)^{****1,3}	37.512 (±0.210)^{****1,3}	29.747 (±0.389)^{****1,3}	16.605 (±2.781)^{****1,3}	14.175 (±2.327)^{****1,3}
3. T25	136.232 (±4.645)^{****1,2}	102.706 (±0.427)^{****1,2}	116.861 (±3.501)^{****2}	81.182 (±2.247)^{****2}	124.963 (±1.669)^{****1,2}	81.161 (±4.380)^{****2}
4. R50	47.231 (±0.347)^{****1,5}	43.247 (±0.426)^{****1,5}	50.070 (±1.260)^{****1,5}	16.131 (±1.887)^{****1,5}	24.852 (±6.675)^{****1,5}	4.755 (±0.137)^{****1,5}
5. T50	147.358 (±5.192)^{****1,4}	99.999 (±0.135)^{****1,4}	100.396 (±7.141)^{****4}	86.690 (±4.516)^{****4}	99.963 (±1.669)^{*1****4}	90.489 (±0.274)^{****4}
6. R75	61.165 (±2.078)^{****1,7}	51.349 (±0.280)^{****1,7}	24.953 (±0.840)^{****1,7}	24.325 (±0.022)^{****1,7}	8.321 (±0.556)^{****1,7}	9.511 (±0.274)^{****1,7}
7. T75	177.928 (±5.751)^{****1,6}	118.927 (±0.975)^{****1,6}	142.234 (±10.222)^{****1,6}	124.631 (±6.815)^{****1,6}	183.377 (±1.947)^{****1,6}	157.521 (±7.938)^{****1,6}
8. R100	91.731 (±2.456)^{****9}	43.235 (±0.926)^{****1,9}	24.907 (±1.680)^{****1,9}	18.940 (±0.470)^{****1,9}	8.321 (±0.556)^{****1,9}	4.733 (±0.616)^{****1,9}
9. T100	211.319 (±7.977)^{****1,8}	118.925 (±0.664)^{****1,8}	158.908 (±10.362)^{****1,8}	81.292 (±4.693)^{****8}	200.000 (±0.000)^{****1,8}	85.939 (±4.722)^{****8}
F-value	532.2	8802	220.6	396.2	2276	635

The associated statistical measurements indicated are calculated from one-way ANOVA and Sidak multiple comparison testing. *Denotes the *p*-value *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ within different treatments. The comparisons include between the control (DW) and six stress tolerance indexes (RLSTI, SLSTI, RFSTI, SFSTI, RDSTI and SDSTI). The F-value is the ratio of two mean square values. A large F ratio means that the variation among group means is higher than expected by chance.

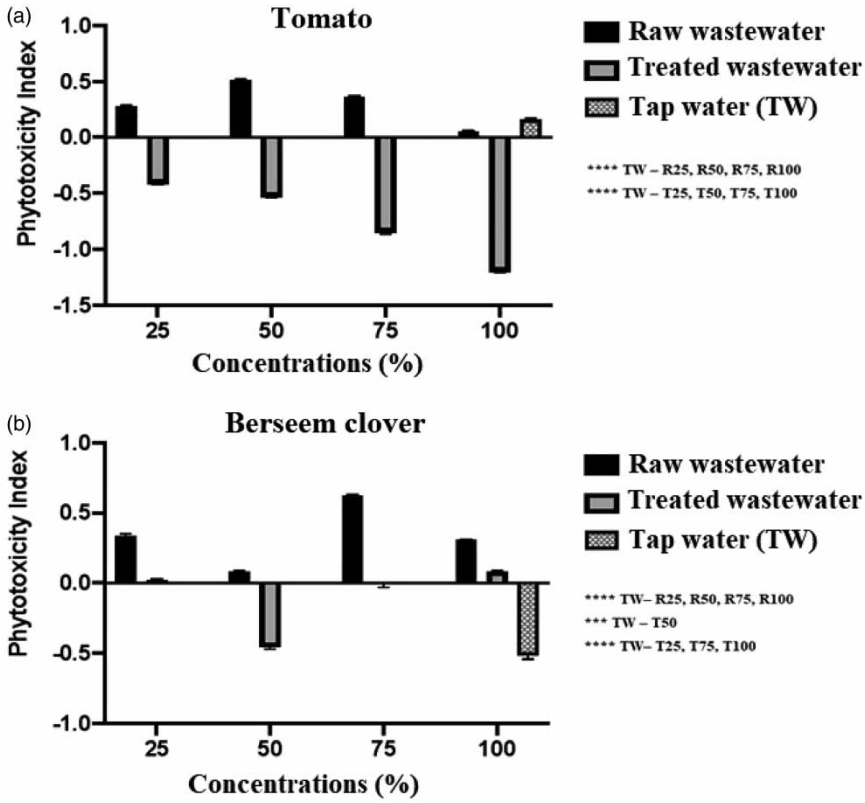


Figure 6 | Phytotoxicity indices for (a) tomato and (b) Berseem clover. *Denotes the *p*-value $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

for barley plant was highest for the control treatment (TW). Similarly, the higher the SVI, the more dynamic the growth (Amin *et al.* 2013). Our results agree well with these as we obtained much higher SVI values. This indicates the level of performance of these seeds, which have been irrigated with treated hospital wastewater, to germinate and emerge as a seedlings.

Phytotoxicity index

In the present study, the PI values in seeds irrigated with treated hospital wastewater were statistically proven significant compared to the PI values of seeds irrigated with untreated hospital wastewater for both crop plants. For tomato seeds grown in tap water (TW) and untreated hospital wastewater concentrations (R25, R50, R75 and R100), the PI values were positive (0.15, 0.31, 0.53, 0.39 and 0.08), which indicates an extremely toxic effect on seedling growth (Figure 6(a)). In contrast, the seeds irrigated with all treated hospital wastewater concentrations (T25, T50, T75 and T100) showed negative PI values (−0.36, −0.47, −0.78 and −1.11), which indicates a strong stimulatory or non-toxic effect on seedling growth. For Berseem clover seeds grown in untreated hospital wastewater concentrations (R25, R50, R75 and R100), we observed positive values of PI (0.60, 0.27, 0.42 and 0.42) (Figure 6(b)). This highlights a highly toxic effect induced by the raw wastewater even when diluted. However, the seeds irrigated with the T75 treated hospital wastewater and tap water showed negative PI value (−0.23 and −0.51), which indicates a stimulatory non-toxic effect on seedling growth. Referring to the previous literature, the low PI value in seeds irrigated with tap water is attributed to the presence of higher concentrations of nitrogen, phosphorous and potassium (Rusan *et al.* 2015). After irrigation with treated hospital wastewater, it may be considered that the reduction in the phytotoxicity value is due to the reduction in heavy metal concentration, phenolic and other toxic organic compounds, and stress tolerance (Aviani *et al.* 2009; Ben-Gal *et al.* 2009; Kopittke *et al.* 2010).

CONCLUSIONS

In this present study, we used a novel consortium made of three bacterial strains (two *B. paramycoides* spp. and one *Alcaligenes faecalis*) for irrigating Berseem clover and

tomato that were individually proven efficient in our previous studies (Rashid *et al.* 2020). The germination percentages for both crop plants irrigated with treated hospital wastewater concentrations (T25, T50, T75 and T100) were between 95% and 100%, which is considered as super germination. The seedling lengths and weights of Berseem clover and tomato (shoots and roots) were higher in treated wastewater concentrations than in the untreated wastewater concentrations. The values of six STIs (RLSTI, SLSTI, RFSTI, SFSTI, RDSTI and SDSTI) for Berseem clover and tomato seeds irrigated with treated wastewater concentrations were increased from 170% to 14,800%. The seeds grown in all treated wastewater concentrations (T25, T50, T75 and T100) showed negative values for phytotoxicity indices (tomato: −0.36, −0.47, −0.78 and −1.11; Berseem clover: −0.23), which indicates a strong stimulatory or non-toxic effect on seedling growth. Our work endorses that the biotreatment with this consortium is a feasible method to treat hospital wastewater before irrigation of tomato and Berseem clover crop plants and therefore is an attractive alternative to meeting the increasing demand for freshwater. However, if fruits and nuts are produced by plants irrigated by treated hospital wastewater, the authors recommend that these produces should be tested to make sure they are safe for consumption.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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