Water Air Soil Pollut (2012) 223:2525–2539
DOI 10.1007/s11270-011-1045-9

Comparing the Tolerance Limits of Selected Bacterial and Protozoan Species to Vanadium in Wastewater Systems

I. Kamika · M. N. B. Momba

Received: 25 June 2011 / Accepted: 8 December 2011 / Published online: 27 December 2011

Abstract This study compared the tolerance limits of selected bacterial (*Bacillus licheniformis*, *Brevibacillus lactosporus* and *Pseudomonas putida*) and protozoan (*Aspidisca, Trachelophyllum* and *Peranema*) species to V^{5+} in wastewater systems. The isolates were exposed to various concentrations of V^{5+} (from 10 to 240 ppm), and their tolerance limits to this heavy metal were assessed at different temperatures (25, 30, 35 and 40°C) and pHs (4, 6, 7, 8 and 10) for 5 days. Chemical oxygen demand (COD), dissolved oxygen (DO) and die-off rate of the isolates were measured using standard methods. The results indicated that test isolates were tolerant to V^{5+}, with a gradual decrease in their colony/cell counts when V^{5+} concentration gradually increased. Bacterial species were found to be more significantly tolerant (MIC: 110–230 ppm V^{5+}) to V^{5+} than protozoan species which showed an earlier total inhibition/die-off rate (100%) at 60–100 ppm V^{5+} (MIC) ($p<0.001$). *P. putida* was the most tolerant bacterial species (MIC: 230 ppm V^{5+}) and *Aspidisca* sp. the most sensitive protozoan species (MIC: 60 ppm V^{5+}). An increase in COD and DO removal was observed throughout the experimental period. The highest COD increase (up to 237.11%) and DO removal (almost 100%) were observed in mixed liquor inoculated with *P. putida* after exposure to 10 ppm V^{5+}. Changes in pH and temperature affected the tolerance limits of all isolates. This study suggests the use of these tolerant bacterial and protozoan species in the bioremediation of V^{5+} from domestic and industrial wastewater under the control of pH and temperature.

Keywords Heavy metals · Vanadium · Bacteria · Protozoa · Wastewater treatment · Tolerance

I. Kamika · M. N. B. Momba (✉)
Department of Environmental, Water and Earth Sciences, Faculty of Science, Tshwane University of Technology, Arcadia Campus, Private Bag X680, Pretoria 0001, South Africa
e-mail: mombamnb@tut.ac.za

1 Introduction

Water is regarded as the most important and indispensable of all the natural resources. It plays a central and critical role in all aspects of life—in the national environment, in economies, in food security, in production and in politics. Human, industrial and agricultural wastes have been identified as the major sources of water pollution. Uncontrolled wastes and poorly managed wastewater have led to the release of heavy metals into water sources. The presence of heavy metals in the environment at concentrations above critical values stipulated by national and international regulatory bodies is considered as unacceptable. Due to their toxicity to living organisms, heavy metals have become a global issue, although some of them are essential for the growth of microorganisms, while others do not demonstrate any biological proprieties (Roane and Peper 2000).

Vanadium is regarded as an essential element and is used as an electron accepter by certain microorganisms such as the *Pseudomonas* species (Antipov et al. 2000; Rehder 2008). It is also a growth factor and can be involved in nitrogen fixation and accumulation by some bacteria (Mukherjee et al. 2004). Vanadium is considered to be a dietary supplement and therapeutic agent and can be used in the treatment of cancer and osteoporosis (Evangelou 2002). Despite these beneficial attributes, vanadium is toxic when present in high concentrations (Silva et al. 2009). Its toxicity is related to its oxidation state, as it is commonly present in various oxidation states (−1 to +5) (ATSDR 2009). Mannazzu (2001) reported that vanadium (V) is usually toxic and stable in aqueous environments. According to Klaassen (2008), an occupational exposure to airborne vanadium can lead to bronchitis and bronchopneumonia and increases the risk of lung cancer.

A number of investigators have provided evidence that vanadium is toxic to living organisms (Owusu-Yaw et al. 1990; Migliore et al. 1993; Kadiiska et al. 1997). High concentrations of heavy metals such as vanadium have noxious effects on microorganisms by inhibiting their growth in wastewater systems, which is observed in the reduction of both cell densities and species richness (Cheremisinoff 1995), and they therefore affect the effectiveness of biological processes in wastewater treatment plants (Madoni 2000). In his study, Chandy (1999) classified several heavy metals in terms of their toxicity and reported that vanadium toxicity was similar to that of nickel when tested on chromogenic and non-chromogenic marine bacteria.

Of the microorganisms present in wastewater systems, bacteria, fungi and protozoa are generally the first category to be exposed to heavy-metal toxicities and serve as very constructive models for investigating the dangerous effects of metals at the cellular level (Avery 2001). These microorganisms, especially bacteria, have demonstrated their ability to resist heavy metals by developing several resistance mechanisms (i.e. efflux, complexation, etc.) (Madoni et al. 1996; Shirdam et al. 2006). Through the acquisition of these specific resistance mechanisms, they can play a significant role in the bioremediation of heavy metals in highly polluted wastewater (Madoni et al. 1996; Gosh et al. 1997; Shirdam et al. 2006; Rajbanshi 2008; Ezzouhri et al. 2009).

Although protozoan species are part of the dynamic population of wastewater systems, their ability in terms of tolerance against and removal of heavy metals has not been fully documented. Therefore, the purpose of this study was to compare the tolerance limits of bacterial species to vanadium in wastewater systems to that of protozoan species in order to determine which of these two groups might play a major role in the removal of vanadium at high concentrations. The effectiveness of the selected microorganisms in the detoxification process of the contaminants was tested in laboratory-scale reactors that operated in batches.

2 Materials and Methods

2.1 Test Organisms

Six different isolates were used in this study. Three bacterial species (*Bacillus licheniformis*-ATCC12759, *Brevibacillus laterosporus*-ATCC64 and *Pseudomonas putida*-ATCC31483) were purchased from Quantum Biotechnologies (Strydompark, Randburg, South Africa). These bacterial species have been reported for metal tolerance or removal (Clausen 2000; Shirdam et al. 2006) and antibiotic resistance (Choopan et al. 2008). The three protozoan species (*Aspidisca* sp., *Trachelophyllum* sp. and *Peranema* sp.) used in this study were obtained from our laboratory stock cultures. These protozoan species were previously isolated from wastewater mixed liquors collected from the aeration tanks of the Daspoort Wastewater Treatment Plant (Pretoria, South Africa). They have demonstrated the ability to successfully remove nitrate and phosphorus in modified mixed liquor (Akpor et al. 2008). The preparation of these protozoan species was done as specified by these authors.

2.2 Sample Collection and Preparation of the Culture Medium

Wastewater samples were collected on a monthly basis between August and October 2010 from the effluent (before disinfection) of the Daspoort Wastewater Treatment Plant in Pretoria. To remove biomass and other suspended solids, samples were allowed to settle for 2 h prior to filtration (using Whatman No. 1 filter papers). The profile of the filtered samples was determined in

terms of COD, DO, pH and vanadium. The COD concentration was measured using closed reflux methods as described in standard methods (APHA 2001), while the vanadium concentration was determined using the Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Other parameters, such as pH and DO, were analysed using a pH probe (Model: PHC101, HACH) and DO probe (Model: LDO, HACH). D-Glucose anhydrate (2.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and KNO_3 (0.18 g/L) were added to the filtrate to serve as a carbon source and nutrient supplement in the mixed liquor (Momba and Cloete 1996; Akpor et al. 2008). The experimental study was performed in triplicate for each sample.

The test metal used in the experimental study was analytical grade and was purchased from Sigma Aldrich (Cape Town, South Africa). Sodium metavanadate anhydrous ($NaVO_3$) was used as a source of vanadium ions. The stock solution of vanadium at a concentration of 1000 ppm was prepared in deionised water. Various concentrations of vanadium (from 10 to 250 ppm increased at a scale of 10 ppm) were prepared from the stock solutions of V^{5+}.

Each of the vanadium concentrations was added to a 250-mL flask containing wastewater mixed liquor medium to obtain a final volume of 150 mL, and the pH was adjusted at 7.2±0.3 using 1.0 M HCl and 1.0 M NaOH (Merck, SA). ICP-OES was used to confirm the metal concentrations in the wastewater mixed liquor medium. The culture medium was autoclaved and cooled down to room temperature before use. To check the sterility of this medium, 1 mL aliquot was plated onto the sterile bacteriological agar and incubated at 37°C for 24 h. Only flasks containing the sterile media were inoculated with a known population of the respective test organisms.

2.3 Vanadium Tolerance Experimental Study

2.3.1 Tolerance Limits of Test Organisms

The tolerance limit experiments were conducted in 250-mL Erlenmeyer flasks containing 150 mL of the modified mixed liquor. Separate flasks were aseptically inoculated with fresh culture of bacterial isolates (100 cfu/mL) or protozoan cells (100 cells/mL). One positive control and one negative control were also included in the experimental study. The positive control flask contained the mixed liquor without vanadium but inoculated with the specific microorganism, while the negative control contained only the mixed liquor with 250 ppm V^{5+}. All the inoculated flasks as well as the controls were initially incubated at 30°C±2°C, and aliquots were taken every day for 5 days. The median-lethal concentration (LC_{50}) of the test metal for each of the test microbial isolates was determined as described by previous investigators (Madoni et al. 1996; Malik and Jaiswal 2000; Lyer et al. 2004). The minimum inhibitory concentration (MIC) of the test metal (the smallest concentration necessary to inhibit growth) was determined in accordance with Shirdam and co-workers (2006). After incubation, the microbial isolates were classified as sensitive or tolerant to V^{5+} according to the inhibition of growth cells.

2.3.2 Effect of Temperature and pH on the Tolerance Limits of Test Organisms

To check the effect of temperature and pH on the tolerance limits of bacterial and protozoan species to vanadium, these isolates were separately inoculated in mixed liquor containing the smallest concentration of V^{5+} necessary to inhibit their growth (MIC). The experimental study was conducted at various temperatures (25, 35 and 40°C) with a constant pH of 7 and thereafter at various pHs (pH 4, 6, 8 and 10) with a constant temperature of 30°C in a shaking incubator at a speed of 100 rpm. During each sampling regime, aliquot samples were taken every 24 h for 5 days for microbial estimation.

The specific growth and mortality (die-off) rates were checked every 24 h for 5 days of incubation. The growth/mortality of bacterial species was determined using the spread plate method after dilution (APHA 2001). Briefly, 100 μL of aliquot from each sample was transferred to Mannitol Egg Yolk Polymyxin (MYP) agar, nutrient agar (NA) and *Pseudomonas* Isolation Agar (PIA) for *Bacillus licheniformis*, *Brevibacillus laterosporus* and *P. putida*, respectively. The plates were incubated at 50°C for *Bacillus* (Emptage et al. 2009) and at 30°C for the two other bacterial isolates (Fonseca et al. 2011). The growth/mortality of protozoan species was determined by visual count using an inverted microscope (Axiovert S100, Carl Zeiss) under×100–×400 magnifications. The first-order die-off rate (mortality rate) of microbial

species was calculated using the formula as reported by Peng et al. (2008):

$$Y_t = Y_0 e^{-K_t} \quad (1)$$

where

K	The die-off rate coefficient (dimensionless)
Y_0 and Y_t	Number of microorganisms at time 0 and t, repectively. The die-off rate coefficient was converted in percentage by using the total inhibition/die-off of the colony/cell counts as the 100% die-off rate.

2.3.3 Determination of the COD and DO in Mixed Liquor Inoculated with Test Organisms

The COD and DO were determined as stated earlier in order to identify their effects on the tolerance limits of the isolates to V^{5+} in mixed liquor. The COD concentration of the samples was calculated according to the formula previously used by Kamika and Momba (2011).

2.4 Statistical Analysis

The data were statistically analysed using the Stata computer software. Two-sample *t*-test was used to compare the two groups (bacteria and protozoa). One-way analysis of variances was used to compare isolates within the group. Except for the tolerance limit (MIC), the Wicoxon–Mann–Whitney test was used to compare the two groups of organisms, and the Kruskal–Wallis test was used to compare microbial isolates. The tests for relationships were carried out using the Pearson correlation index, and the interpretation was performed at two-sided 95% confidence limit.

3 Results

3.1 Profile of the Daspoort Activated Sludge Mixed Liquor

Table 1 shows the profile of wastewater samples before inoculation of the test organisms. The DO concentrations in wastewater samples ranged between 5.37 and 6.73 mg/L while the COD and vanadium concentrations were found to be lower than 1 mg/L. Compared to other months, the sample collected during September 2010 was found to have the lowest average concentrations for these three parameters. The average pH of the wastewater mixed liquor ranged between 6.56 and 6.9.

3.2 Tolerance Limit of Test Organisms to V^{5+} in the Modified Wastewater Mixed Liquor

3.2.1 Microbial Responses in the Modified Mixed Liquor

Figure 1a, b summarises the growth responses or the die-off of bacterial and protozoan species to various concentrations of V^{5+} in wastewater mixed liquor. In general, there was a gradual decrease in the number of the test organisms after exposure to gradual increases of V^{5+} concentrations. This resulted in a decrease in the counts of organisms or in a total inhibition/die-off of the microbial population. In contrast, at moderate and less toxic V^{5+} concentrations, bacterial colony/protozoan cells counts increase over time.

When exposed to V^{5+} concentrations up to 50 ppm, *P. putida* showed a positive growth response (from 2 $\log_{10}$ CFU/mL to 7 $\log_{10}$ CFU/mL) which lasted during 5 days of the experimental study. However, a decline on its population occurred when exposed to V^{5+} concentrations greater than 50 ppm (Fig. 1a). For example, an exposure of this organism to 120 or

Table 1 Profile of domestic wastewater mixed liquor prior to the experimental study

Samples[a]	DO (mg/L) Mean ± SD	COD (mg/L) Mean ± SD	V^{5+} (ppm) Mean ± SD	pH Mean ± SD
Sample 1 (3/08/2010)	6.732±0.247	0.037±0.004	0.053±0.001	6.561±0.101
Sample 2 (6/09/2010)	5.370±0.432	0.028±0.001	0.000±0.000	6.901±0.197
Sample 3 (5/10/2010)	6.087±0.761	0.043±0.003	0.027±0.001	6.609±0.431
SA standard	–	75	0.1[b]	5.5–9.5

[a] Each sample was analysed in triplicate

[b] UN-FAO recommended limit (FAO 1985)

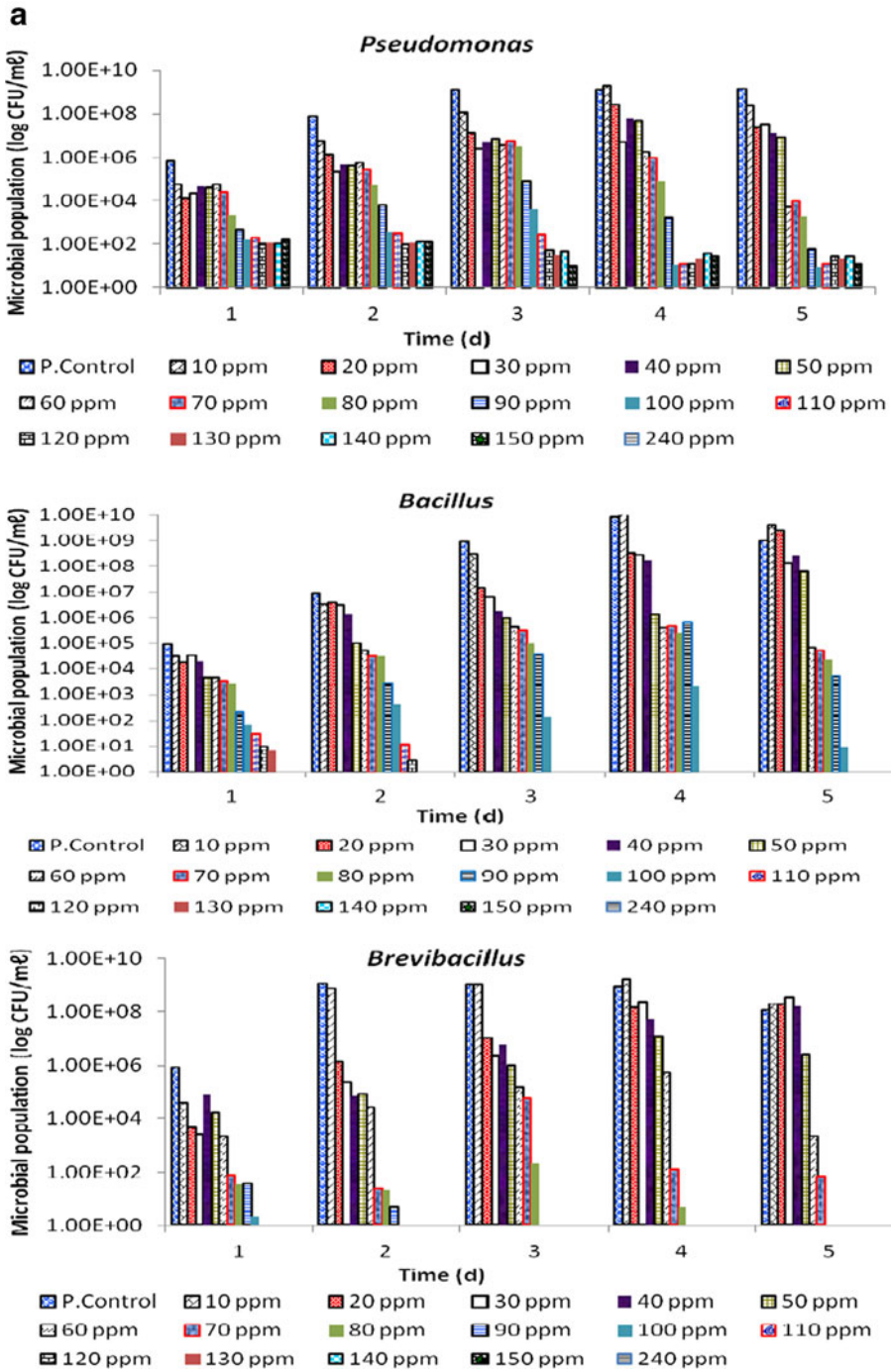
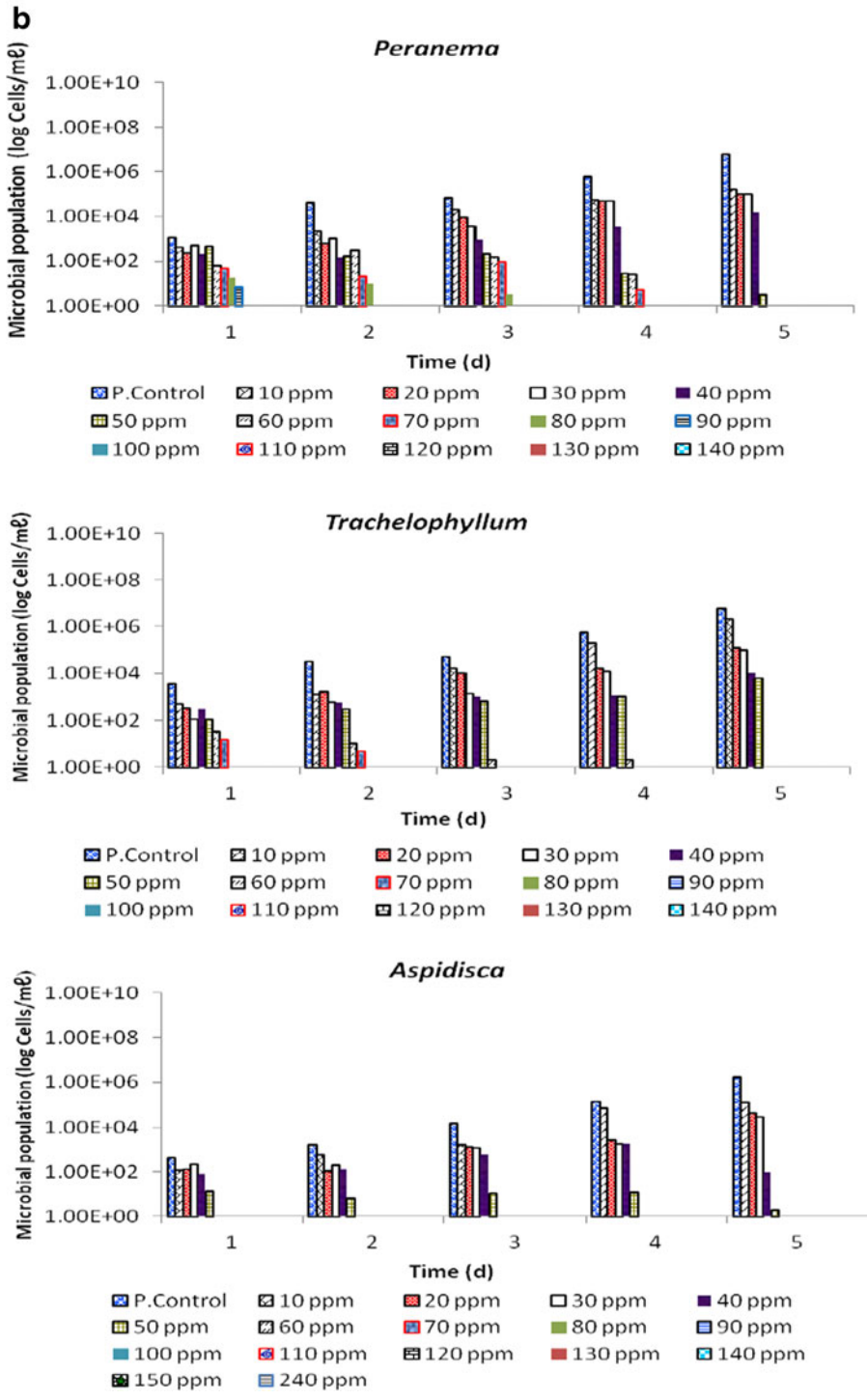


Fig. 1 **a** Bacterial concentrations in modified wastewater mixed liquor containing various concentration of V^{5+}. **b** Protozoan concentrations in modified wastewater mixed liquor containing various concentration of V^{5+}

230 ppm V^{5+} resulted in die-off rates of 18% and 100%, respectively, from the onset of the experimental study. Similar observations were also noted with *Bacillus licheniformis* (7 $\log_{10}$ CFU/mL) which showed a growth response up to day 5 when exposed to V^{5+} concentrations up to 50 ppm, but when exposed to V^{5+} concentrations ranging between 60 and 100 ppm, there was a decline on microbial population (i.e. 1 $\log_{10}$ CFU/mL at 100 ppm on day 5). This was followed by a further inhibition of bacterial number and a total inhibition/die-off at 140 ppm V^{5+} (die-off rate: 100%) starting from the first day of exposure. *Brevibacillus*

Fig. 1 (continued)



laterosporus (8 $\log_{10}$ CFU/mL) was able to grow for 5 days when exposed to V^{5+} at concentrations up to 40 ppm V^{5+} but showed a decline on microbial population and a total die-off at 110 ppm V^{5+} (die-off rate: 100% of bacterial population).

Compared to bacterial species, protozoan isolates (Fig. 1b) showed very slow positive growth responses when exposed to V^{5+} concentrations although an increase in cell densities occurred over time. While there was no positive growth response for *Aspidisca* sp (2

$\log_{10}$ cells/mL) during the first day of exposure to vanadium, slight increases in cell densities were observed for *Peranema* sp. (3 $\log_{10}$ cells/mL) and for *Trachelophyllum* sp. (3 $\log_{10}$ cells/mL) when exposed to V^{5+} concentrations up to 50 and 40 ppm, respectively. A gradual increase in *Peranema* sp. cell densities up to 4 $\log_{10}$ cells/mL was reached at the end of its 5-day exposure to 40 ppm V^{5+}. However, its exposure to high vanadium concentrations such as 100 ppm V^{5+} resulted in a decrease in cell densities and total inhibition/die-off (die-off rate: 100%). For *Trachelophyllum* sp. and *Aspidisca* sp. an increase in their cell densities was noted when these organisms were exposed to V^{5+} concentrations up to 50 ppm (4 $\log_{10}$ cells/mL) and 30 ppm V^{5+} (4 $\log_{10}$ cells/mL), respectively. However, a decline in their cell densities and total die-off occurred from the first day (die-off rate: 100%) of exposure at 80 and 60 ppm V^{5+}, respectively.

3.2.2 Determination of 24-h LC_{50} and MIC of Vanadium to the Microbial Isolates

Results on tolerance to V^{5+} are summarised in Table 2 and revealed that all isolates were able to grow in the presence of V^{5+} with bacterial species being the most tolerant organisms. Among bacterial isolates, *P. putida* had the highest tolerance limit to V^{5+} (MIC: 230 ppm) with a 24-h LC_{50} that ranged from 200 to 210 ppm of V^{5+}. *Bacillus licheniformis* (MIC: 140 ppm) and *Brevibacillus laterosporus* (MIC: 110 ppm) had a 24-h LC_{50} at values ranging from 110 to 120 ppm and 90 to 100 ppm V^{5+}, respectively. Of the protozoan isolates, *Peranema* sp. was found to be the most tolerant isolate (MIC: 100 ppm) with 24-h LC_{50} values ranging between 70 and 80 ppm. When compared to all the microbial isolates, *P. putida* appeared to be the most tolerant isolate to V^{5+} and *Aspidisca* sp. the most sensitive organism, with a MIC value of 60 ppm V^{5+} and a 24-h LC_{50} value of 50 ppm V^{5+}. The order of tolerance of isolates to V^{5+}, in terms of MIC, was as follows: *P. putida*>*Bacillus licheniformis*>*Brevibacillus laterosporus*>*Peranema* sp.>*Trachelophyllum* sp.>*Aspidisca* sp. There were significant differences between the MIC values of all the six isolates ($p<0.005$) and also between MIC mean values of microbial isolates when grouped as bacterial species and protozoan species ($p<0.001$).

Table 2 Twenty-four-hour LC_{50} and MIC of V^{5+} to the isolates in mixed liquor after 24 hours at 30°C

	Microorganisms	24-h LC_{50} (ppm)	MIC (ppm)
Bacteria isolates	Pseudomonas putida	160–200	230
	Bacillus licheniformis	100–120	140
	Brevibacillus laterosporus	70–90	110
Protozoan isolates	Peranema sp.	60–80	100
	Aspidisca sp.	40–50	60
	Trachelophyllum sp.	60–70	80

3.2.3 Effect of pH and Temperature on the Tolerance Limit of Microbial Isolates to Vanadium Concentrations

Table 3 illustrates the effects of pH on the tolerance limit of the isolates when inoculated in mixed liquor containing the smallest concentration of V^{5+} necessary to inhibit their growth (MIC) at 30°C (MIC: 230 ppm V^{5+} for *P. putida*, 140 ppm V^{5+} for *Bacillus licheniformis*, 110 ppm V^{5+} for *Brevibacillus laterosporus*, 100 ppm V^{5+} for *Peranema* sp., 80 ppm V^{5+} for *Trachelophyllum* sp. and 60 ppm V^{5+} for *Aspidisca* sp.). In spite of a decrease in bacterial counts and protozoan cell densities that occurred over time, all test organisms were able to survive at pH 8 but not at pH 4. This gave an indication of pH effects on the toxicity of vanadium. Of the bacterial species, at pH 8, *P. putida* could persist up to day 2 with a percentage die-off rate of 57% and 72% for day 1 and 2, respectively. Moreover, *Bacillus licheniformis* and *Brecibacillus laterosporus* were able to persist up to day 3 with a percentage die-off rate ranging from 56% to 78% and from 34 to 86%, respectively.

Among protozoan species, *Peranema* sp. and *Aspidisca* sp. showed slow die-off rates (55% and 79%, respectively) on the first day and could survive up to day 2 (65% and 87%, respectively), while *Trachelophyllum* sp. had a fast die-off rate of 69% on the first day and could only resist on day 1 at pH 8. However, *Bacillus licheniformis* (die-off rate: 75% at day 2) and *Trachelophyllum* sp. (die-off rate: 73% at day 1) were the only isolates able to survive at pH 10 and 6, respectively, in the presence of V^{5+} at the concentrations able to inhibit their growth.

Table 4 summarises the effects of temperature on the tolerance limits of test organisms in the modified mixed liquor containing the smallest concentrations of

Table 3 pH effects vs V^{5+} toxicity to the tolerance limit of test organisms in modified mixed liquor incubated at 30°C (C_1: Mixed liquor with vanadium only at specific MIC, C_2: Mixed liquor with vanadium at specific MIC and inoculated with a specific isolate)

Bacterial isolates					Protozoan isolates			
Pseudomonas putida					*Peranema* sp.			
Time (day)	pH 4	pH 6	pH 8	pH 10	pH 4	pH 6	pH 8	pH 10
1	100	100	57	100	100	100	55	100
2	100	100	72	100	100	100	65	100
3	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
Bacillus licheniformis					*Aspidisca* sp.			
1	100	100	56	38	100	100	79	100
2	100	100	55	75	100	100	87	100
3	100	100	78	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
Brevibacillus laterosporus					*Trachelophyllum* sp.			
1	100	100	34	100	100	73	69	100
2	100	100	50	100	100	100	100	100
3	100	100	86	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100

V^{5+} necessary to inhibit their growth (MIC) at 30°C and pH 7 (MIC: 230 ppm V^{5+} for *P. putida*, 140 ppm V^{5+} for *Bacillus licheniformis*, 110 ppm V^{5+} for *Brevibacillus laterosporus*, 100 ppm V^{5+} for *Peranema* sp., 80 ppm V^{5+} for *Trachelophyllum* sp. and 60 ppm V^{5+} for *Aspidisca* sp.). While the controls were incubated at 30°C, inoculated mixed liquor samples were exposed at various temperatures (25, 35 and 40°C). In spite of a decrease in bacterial counts and protozoan cell densities that occurred over time, all the isolates, with exception of *Bacillus licheniformis*, persisted at 25°C. *P. putida*, *Peranema* sp. and *Trachelophyllum* sp. could survive up to day 3 with die-off rates of 75%, 88% and 87%, respectively, while *Brevibacillus laterosporus* (die-off rate: 80%) and *Aspidisca* sp. (die-off rates: 85%) could only survive up to day 2. However, the presence of *Brevibacillus laterosporus* (die-off rates: 76%), *Bacillus licheniformis* (die-off rate: 80%), and *Peranema* sp. (die-off rate: 81%) was apparent at 35°C up to day 3, 2 and 1, respectively. With the exception of *Bacillus licheniformis*, none of the isolates could be grown at 40°C.

3.3 Profile of DO and COD in Mixed Liquor during the Study Period

Figure 2 shows the DO removal by the test organisms in mixed liquors containing various concentrations of vanadium during an incubation period of 5 days at 30°C and pH 7. In spite of fluctuations that occurred during the DO removal by test organisms, an increase in V^{5+} concentration resulted in a gradual decrease in DO levels. In general, all test organisms were able to take up all the DO present in the modified mixed liquor, with *Trachelophylum* sp. and *Aspidisca* sp. showing an early decrease of the total uptake when exposed to 90 ppm V^{5+}. Compared to other organisms, an earlier decrease of the total uptake of DO by *P. putida* occurred when exposed to 200 ppm V^{5+}. In addition, a negative percentage removal of DO was observed in the inoculated mixed liquor, starting from 90 ppm V^{5+} for *Aspidisca* sp. Significant differences were found between the mean DO values of the three bacterial species ($p<0.001$) and also between the mean DO values of the three protozoan species ($p=0.0025$).

Table 4 Temperature effects vs V^{5+} toxicity to the tolerance limit of test organisms in modified mixed liquor incubated at pH 7

Bacteria					Protozoa			
Pseudomonas putida					*Peranema* sp.			
Time (day)	25°C	35°C	40°C	C1	25°C	35°C	40°C	C1
1	55	100	100	100	55	81	100	100
2	64	100	100	100	58	100	100	100
3	75	100	100	100	88	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
Bacillus licheniformis					*Trachelophyllum* sp.			
1	100	65	56	100	54	100	100	100
2	100	80	71	100	62	100	100	100
3	100	100	63	100	88	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
Brevibacillus laterosporus					*Aspisdisca* sp.			
1	71	55	100	100	56	100	100	100
2	80	57	100	100	85	100	100	100
3	100	76	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100

Further statistical analysis showed strong significant differences between bacteria and protozoa ($p<0.001$) as groups of organisms.

As can be seen from Table 5, an increase in COD in the inoculated mixed liquors was observed throughout the study period. For bacterial species, more than 100% of COD increase was noted in mixed liquor containing V^{5+}, and the highest COD concentration was recorded in the mixed liquor containing 10 ppm V^{5+} and inoculated with *P. putida* (237.11%). No COD increase of more than 100% was observed in mixed liquor inoculated with protozoan species. The highest COD increase in this medium was found in the mixed liquor containing 20 ppm V^{5+} and inoculated with *Trachelophyllum* sp. (99.58%). A gradual decrease in COD occurred when the V^{5+} concentrations gradually increased, and this decrease in COD was observed for both groups of test organisms. There were strong significant differences between the mean COD values of the three bacterial species ($p<0.001$) while no significant differences were

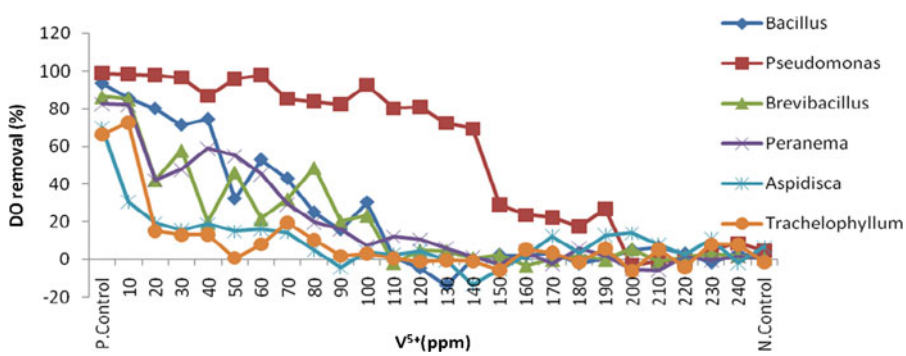


Fig. 2 DO removal by the isolates in the presence of various concentrations of V^{5+} in mixed liquor at 30°C, pH 7

Table 5 Average percentage of the increase of COD in modified mixed liquor inoculated with test organisms throughout the experimental period

V^{5+} (ppm)	Bacterial species			Protozoan species		
	Pseudomonas putida COD (%)	*Bacillus licheniformis* COD (%)	*Brevibacillus laterosporus* COD (%)	*Peranema* sp. COD (%)	*Trachelophyllum* sp. COD (%)	*Aspidisca* sp. COD (%)
P.control	251.54	263.09	175.88	154.11	110.14	132.04
10	237.11	186.95	153.16	80.24	78.78	70.07
20	161.72	152.23	128.78	60.16	99.58	77.53
30	188.79	87.02	128.43	69.02	58.94	62.65
40	177.75	141.29	105.12	40.85	73.04	64.84
50	181.92	108.25	98.75	53.99	65.24	28.17
60	201.69	105.59	144.36	40.69	12.08	6.07
70	196.32	70.77	63.46	35.04	−1.33	9.77
80	230.27	92.85	51.42	2.28	0.27	4.74
90	187.62	78.72	29.86	7.91	−1.58	5.15
100	88.66	103.83	8.64	−0.40	1.41	0.19
110	59.93	20.51	−0.90	0.80	2.60	−0.95
120	76.27	17.90	6.24	−1.33	−2.49	−0.05
130	42.04	13.82	−2.30	3.13	−0.11	0.30
140	59.66	−11.74	3.60	−3.08	0.81	−0.67
150	29.86	−17.75	7.64	2.26	−3.19	−0.59
160	22.58	2.82	−2.38	−8.61	−9.60	−5.96
170	2.66	6.81	3.68	−7.39	−9.75	−3.41
180	0.86	1.56	−6.85	2.32	−8.10	4.70
190	0.61	1.58	−1.34	−6.44	−2.59	1.01
200	13.98	2.12	−2.59	11.66	3.11	0.59
210	22.28	−4.05	−0.39	−2.86	−3.80	−0.60
220	−6.51	6.60	−1.97	0.93	5.69	0.68
230	−9.49	−3.42	−2.74	−0.08	−2.60	−0.76
240	2.39	−2.96	5.35	1.98	−2.45	3.18
N.control	−4.48	−0.79	1.20	0.33	0.08	1.51

found between protozoan species ($p<0.05$). The comparison of isolates in terms of group of species showed a strong statistical significance between bacteria and protozoa ($p<0.001$).

To establish the relationship between the growths/die-off of isolates and the physicochemical parameters (COD and DO), the Pearson correlation index was carried out. Statistical evidence revealed positive correlations between microbial number and COD removal ($r=0.8488$ for bacterial species and $r=0.8437$ for protozoan species), and also between microbial number and DO removal ($r=0.7629$ for bacterial and $r=0.6700$ for protozoan species).

4 Discussion

Extensive industrialisation and the rapid growth of the human population have resulted in the pollution of water resources. Increasing concern has been expressed about the discharge of heavy metals into the environment. Bacteria and protozoa are members of the dynamic population that plays an important role in the treatment of wastewater. Consequently, their presence in wastewater systems is desperately needed for the effectiveness of biological processes and the control and management of water resource pollution. Although certain heavy metals such as vanadium are regarded as

essential elements and are used as electron donors by certain microorganisms (Antipov et al. 2000; Rehder 2008) at high concentrations, they can interfere with the operation of biological sewage treatment processes (Madoni 2011).

At high concentrations, certain metals have shown to be toxic to both aerobic and anaerobic sewage organisms and have caused deflocculation of biological sludge and reduced treatment efficiencies (Neufeld 1976; Mytelka et al. 1973). The release of these metals without proper treatment poses a significant threat to not only the environment but also to public health because of their persistence, biomagnifications and accumulation in the food chain (Alluri et al. 2007). Due to the negative impact of heavy metals on public health and the environment, many countries and international agencies have established regulations for the use and discharge of wastewater in the receiving water bodies (ATSDR 2009).

During the study period, wastewater effluent collected from the Daspoort Wastewater Treatment Plant was screened for COD, DO and pH and the presence of vanadium. The results (Table 1) revealed that COD and pH values were within the South African permissible limits of 75 mg/L and 5.5 to 9.55, respectively (National Water Act. 1998). The vanadium concentration in the effluent was within the permissible limit of 0.1 mg/L as recommended by the United Nations Food and Agriculture Organisation Standard (FAO 1985). In terms of these variables, the Daspoort Wastewater Treatment Plant complied with the regulations, and the discharge of the effluent into the receiving water body could not impact negatively on the environment.

According to Burton et al. (1987), the survival of microorganisms in wastewater is affected by several interacting factors including antibiotics, organic matters, dissolved nutrients, algal toxicants, heavy metals, temperature and the physicochemical nature of the aquatic environment. In his study, Nilsson (1981) reported that heavy metals can affect the survival of microbial isolates in many ways, such as reduction of food uptake, inhibition of growth and reduction in the rate of endocytosis. In the present study, the growth inhibition was used as an indication of V^{5+} toxicity in the modified mixed liquor and the MIC values used as the tolerance level of isolates against V^{5+}. The results revealed a decrease in the number of the test organisms after exposure to gradual increases of V^{5+} concentrations which resulted in an inhibition and total die-off of the microbial population. Moreover, the factor time played a major role, in certain concentration of V^{5+}. Microbial population increases with a gradual increase in exposure time (Fig. 1a, b). Compared to bacterial isolates, protozoan isolates had a long lag phase to acclimatise to the new environmental conditions and slow growth. This was followed by significant decrease in their cell densities starting from 50 ppm V^{5+} (Fig. 1b). In general, all the bacterial species were more tolerant to vanadium compared to the tolerance of protozoan species (Table 2). Statistical evidence also confirmed significant differences between the MIC values of bacterial and protozoan species ($p<0.001$). *P. putida* and *Peranema* sp. were classified as the organisms with the highest tolerance to V^{5+} when considered in terms of group of species (bacteria and protozoa). Furthermore, *P. putida* was revealed to be the most tolerant organism by far compared to other organisms, and *Aspidisca* sp. the most sensitive to V^{5+} in the modified wastewater mixed liquor ($p<0.05$).

A large number of studies have also reported the tolerance of bacterial species to heavy metals. Shirdam et al. (2006) found that bacterial species such as *P. putida*, *Bacillus cereus* and *P. peudoalkaligenes* were able to tolerate cadmium, nickel and even vanadium, and could accumulate approximately 40–50% Cd, 5–6% Ni and 10–12% V, respectively. Chen et al. (2006) showed that *P. putida* was able to tolerate copper and zinc at MIC values of 3 and 5 mmol/L, respectively. A study conducted by Canovas et al. (2003) revealed that *P. putida*'s genome encodes an unexpected capacity to tolerate heavy metals. In a study by Clausen (2000), it was reported that *Bacillus licheniformis* was able to tolerate copper, chromium and arsenic, with the ability to remove 93% of copper and 48% of arsenic from the media. Hernandez et al. (1998) reported that *Escherichia hermannii* and *E. cloacae* were able to grow in a very high concentration of vanadyl sulphate. Briand et al. (1996) found that *Thiobacillus thiooxidans* was able to grow in the presence of vanadium and reduced vanadium (V) to vanadium (IV) in cultures. However, a study conducted on the interaction between *Streptococcus pneumonia* and vanadium salts revealed that *S. pneumonia* was very sensitive to vanadium (Fukuda and Yamase 1997).

While the tolerance of protozoan species to a number of heavy metals has been reported elsewhere (Madoni et al. 1996; Abraham et al. 1997; Leborans

et al. 1998; Rehman et al. 2005, 2010; Nicolau et al. 2005; Kamika and Momba, 2011), as far as we know, no specific studies have pointed out the tolerance of *Peranema* sp., *Trachelophyllum* sp. and *Aspidisca* sp. to vanadium. A recent experimental study by Kamika and Momba (2011) revealed that these protozoan isolates could tolerate nickel at 52, 34 and 32 ppm, respectively. A study by Schlenk and Moore (1994) revealed that the ciliated protozoan *Tetrahymena thermophila* was resistant to copper sulphate toxicity and this resistance ability was attributed to an intracellular mechanism. Madoni and co-workers (1996) investigated the toxic effect of heavy metals on the activated sludge protozoan community and found that ciliated species such as *Chilodonella uncinata* and *Trochilia minuta* showed the highest sensitivity to Cd, Cu, Pb, Zn and Cu than *Opercularia coarctata* and *O. minima*. Abraham et al. (1997) found that ciliated protozoan species such as *Aspidisca cicada*, *C. uncinata* and *Vorticella convallaria* were resistant to Fe (> 2000 ppb), Zn (> 500 ppb), Cu (> 60 ppb) and Cr (> 100 ppb) in activated sludge systems. The authors pointed out that the ciliate species and metal content correlated negatively. Nicolau et al. (2005) reported that the sessile *Opercularia* sp. was exceptionally tolerant to copper and was related to the low quality of the activated sludge. Bitton (1999) pointed out that *A. costata* food intake decreases when in the presence of cadmium. This author observed that protozoan species transform themselves into inactive, nonmotile, environmentally resistant cysts or shells in adverse environmental conditions. According to Reynolds and Pepper (2000), in that state, protozoa are incapable of immediate growth or reproduction, but are able to survive in harsh ecological niches. This situation could clearly explain the long lag phase periods that occurred on the first day of *Peranema* sp., *Trachelophyllum* sp. and *Aspidisca* sp., which was followed by a slow increase in cell densities over time (Fig. 1b). Compared to bacterial isolates, the sensitivity of protozoan isolates to vanadium on the first day could also be due to the absence of a cell wall at the trophic stage (Martin-Gonzalez et al. 2006).

The results of this study show that temperature (25, 35 and 40°C) and pH (pH 4, 6, 8 and 10) changes could affect the tolerance limits of test organisms to V^{5+} in mixed liquor. This confirmed findings by Kamika and Momba (2011) who also reported the effect of these two parameters on the ability of similar test organisms to have certain resistance to nickel. In this study, test organisms were able to persist at pH 8 but not in acidic mixed liquors (pH 4, 6 and 10), except for *Bacillus licheniformis* (10) and *Trachelophyllum* sp. (pH 6). This was an indication of the toxic V^{5+} and pH effects on these isolates. These results corroborated the findings of Roane and Peper (2000), who reported that at high pH, the solubility of some metals decreases, while at low pH, those metals are found as free ionic species in aqueous solutions and are capable to express their toxicities. In their study, Van Nostrand and co-authors (2005), when assessing the effect of pH and the toxicity of nickel and other divalent metals, reported growth inhibition of *Burkholderia cepacia* PR1 with increasing pH. According to Bell et al. (2004), the pH effects play a major role in the toxicity of vanadium salts in the nutrient broth. Gikas (2008) reported that a number of environmental factors, such as pH, biomedium and biomass concentration, can effect the microbial toxicity of metals such as Ni^{2+}. Moreover, in their study, Stevik et al. (2004) recorded that the survival of bacterial counts decreases with the increase of temperature.

An increase of COD in the modified mixed liquor was noticed throughout the experimental study when this medium was inoculated with test organisms. These results confirmed the findings by Akpor et al. (2008), who also reported an increase of COD in mixed liquor incubated with *Peranema* sp., *Aspidisca* sp. and *Trachelophyllum* sp. at 30°C while the authors investigated the ability of these three protozoan species to remove nitrate and phosphorus in wastewater mixed liquor. However, both these studies agree with the findings of Arican and Yetis (2003), who reported a significant increase of the COD removal rate in activated sludge stressed with an increased level of heavy metals such as Ni^{2+}.

In this study, a drastic DO uptake was observed in mixed liquor inoculated with test organisms throughout the experimental period. A similar decrease was reported by Sedlak (1991). Since DO is a very important factor and a limiting nutrient for the growth of aerobic microorganisms, its uptake by microbial isolates in mixed liquor containing vanadium explains an excessive growth of microbial isolates (Herman and Maier 2000). Given the fact that the level of DO in water or wastewater is strongly related to the temperature (Herman and Maier 2000) and also due to the inactivity of the test organisms at certain concentrations, the negative percentage removal of DO observed in this study, especially with *Bacillus licheniformis* at 130 ppm V^{5+}

(−13.78%) and *Aspidisca* sp. at 140 ppm V^{5+} (−13.46%), could be influenced by the ambient temperature, which increased DO in the mixed liquor samples when compared to the DO level in the appropriate media at day zero of the study period.

Strong correlations were found between test organisms' growth response and COD (r=0.8488 for bacterial, r=0.8437 for protozoan species), and between growth response and DO removal (r=0.7629 for bacterial and r=0.6700 for protozoan species). These results support Akpor et al. (2008), who also found a positive correlation between COD concentrations and growth rates of the same protozoan species (r=0.806, p<0.01) in wastewater mixed liquor, but disagree with the results of Pala and Sponza (1995), who illustrated a removal of COD in the activated sludge system inoculated with *Pseudomonas* sp.

5 Conclusion

This study reveals that both bacterial and protozoan species tolerate vanadium, with bacterial species being the most tolerant isolates (p<0.001). *P. putida*-ATCC31483 was by far the most tolerant isolate (MIC: 230 ppm V^{5+}) with *Aspidisca* sp. the most sensitive (MIC: 60 ppm V5+) (p<0.05). Bacterial isolates generally showed an increase in their counts from the first day, which gradually increase over time, depending on the V^{5+}concentrations they were exposed to. The growth responses of *Peranema* sp., *Trachelophyllum* sp. and *Aspidisca* sp. in the presence of V^{5+} concentrations were characterised by a long lag phase and slow growth over time and depending on the V^{5+}concentrations. However, at excessive V^{5+} concentrations, die-off was noted which resulted to a total inhibition/die-off rate with an increase of V^{5+} concentrations for both bacterial and protozoan species. This study also found that pH and temperature alteration affects the tolerance limits of test isolates to V^{5+}. Furthermore, positive correlations were found between the growth responses of all isolates and COD increase and also DO removal in mixed liquor. The findings of this study indicate that both the bacterial species and the protozoan species used in this investigation have potential to tolerate V^{5+}. Tolerant protozoan species can also be used for the bioremediation of V^{5+} from domestic and industrial wastewater under the control of pH and temperature.

Acknowledgments The authors are grateful to the National Research Foundation (NRF) for the funding of this project. We are also grateful to Mathapelo Seopela, a technician at the Department of Chemistry, for her technical assistance.

References

Abraham, J. V., Butler, R. D., & Sigee, D. C. (1997). Ciliate populations and metals in an activated-sludge plant. *Water Res., 31*(5), 1103–1111.

Akpor, O. B., Momba, M. N. B., & Okonkwo, J. O. (2008). The effects of pH and temperature on phosphate and nitrate uptake by wastewater protozoa. *AJB, 7*(13), 2221–2226.

Alluri, H. K., Ronda, S. R., Settalluri, V. S., Bondili, J. S., Suryanarayana, V., & Venkateshwar, P. (2007). Biosorption: an eco-friendly alternative for heavy metal removal. *AJB, 6*(25), 2924–2931.

Antipov, A. N., Lyalikova, N. N., & L'vov, N. P. (2000). Vanadium-binding protein excreted by vanadate-reducing bacteria. *IUBMB Life, 49*, 137–141.

APHA (2001). *Standard Methods for examination of water and wastewater* (20th ed.) Washington D.C.: American Public Health Association.

Arican, B., & Yetis, U. (2003). Nickel sorption by acclimatized activated sludge culture. *Water Res., 37*(14), 3508–3516.

ATSDR, 2009. Toxicological profile for vanadium and compounds. Agency for Toxic Substances and Disease Registry, Atlanta, GA. US Department of Health and Human Services, Public Health Service.

Avery, S. V. (2001). Metal toxicity in yeast and the role of axidative stress. *Adv Appl Microbiol, 49*, 111–142.

Bell, J. M. L., Philp, J. C., Kuyukina, M. S., Ivshina, I. B., Dundar, S. A., Cunningham, C. J., & Anderson, P. (2004). Methods evaluating vanadium tolerance in bacteria isolates from crude oil contaminated land. *J Microbiol Methods, 58*, 87–100.

Bitton, G. (1999). *Wastewater microbiology* (2nd ed.). New York: Wiley-Liss.

Briand, L., Thomas, H., & Donati, E. (1996). Vanadium (V) reduction in *Thiobacillus thiooxidans* cultures on elemental sulfur. *Biotechnol. lett., 18*(5), 505–508.

Burton, G. A., Gunnison, D., & Lanza, G. R. (1987). Survival of pathogenic bacteria in various fresh water sediments. *Appl. Environ. Microbiol., 53*(4), 633–638.

Canovas, D., Cases, I., & de Lorenzo, V. (2003). Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. *Environ Microbiol, 5*(12), 1242–1256.

Chandy, C. P. (1999). Heavy metal tolerance in chromogenic and non-chromogenic marine bacteria from Arabian gulf. *Environ Monit Assess, 59*(3), 321–330.

Chen, X. C., Shi, J. Y., Chen, Y. X., Xu, X. H., Xu, S. Y., & Wang, Y. P. (2006). Tolerance and biosorption of copper and zinc by *Pseudomonas putida* CZ1 isolated from metal-polluted soil. *Can. J. Microbiol*., 52(4), 308–316.

Cheremisinoff, P. N. (1995). *Handbook of water and wastewater treatment technology.* New York: Marcel Dekker.

Choopan, A., Nakbud, K., Dawveerakul, K., Chawawisit, K., & Lertcanawanichakul, M. (2008). Anti-methicillin resistant

Staphylococcus aureus activity of *Brevibacillus laterosporus* strain SA14. *Walailak J. Sci Tech., 5*(1), 47–56.

Clausen, C. A. (2000). Isolating metal-tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood. *Waste Manag Res, 18*(3), 264–268.

Evangelou, A. M. (2002). Vanadium in cancer treatment. *Crit Rev Oncol Hematol, 42*, 249–265.

Emptage, C. D., Knox, R. J., Danson, M. J., & Hough, D. W. (2009). Nitroreductase from bacillus licheniformis: a stable enzyme for prodrug activation. *Biochem. Pharmacol., 77*, 21–29.

Ezzouhri, L., Castro, E., Moya, M., Espinola, F., & Lairini, K. (2009). Heavy metal tolerance of filamentous fungi isolated from polluted sites in Tangier, Morocco. *AJMR, 3* (2), 035–048.

Fonseca, P., Moreno, R., & Rojo, F. (2011). Growth of *Pseudomonas putida* at low temperature: global transcriptomic and proteomic analyses. *Environ Microbiol Rep, 3*(3), 329–339.

United Nations-Food and Agriculture Organisation (UN-FAO), 1985. *Water quality for agriculture*. Ayers ORS, Westcot DW. FAO Irrigation and Drainage Paper 29 (rev 1), FAO, Rome, p. 174.

Fukuda, N., & Yamase, T. (1997). In vitro antibacterial activity of vanadate and vanadyl compounds against *Streptococcus pneumoniae*. *Biol Pharm Bull, 20*, 927–930.

Gikas, P. (2008). Single and combined effects of nickel (Ni(II)) and cobalt (Co(II)) ions on activated sludge and on other aerobic microorganisms: a review. *J. Hazard. Mater., 159* (2–3), 187–203.

Gosh, S., Sadhukhan, P. C., Gosh, D. K., Chaudhuri, J., & Mandal, A. (1997). Elimination of mercury bynitrogen-fixing bacteria. *Bull Environ Contam Toxicol, 58*(6), 993–998.

Herman, D. C., & Maier, R. M. (2000). Physiological methods. In R. M. Maier, I. L. Pepper, & C. P. Gerba (Eds.), *Environmental microbiology* (pp. 235–265). New York: Academic.

Hernandez, A., Mellado, R. P., & Martınez, J. L. (1998). Metal accumulation and vanadium-induced multidrug resistance by environmental isolates of *Escherichia hermannii* and *Enterobacter cloacae*. *Appl. Environ. Microbiol., 64*, 4317–4320.

Kadiiska, M. B., Mason, R. P., Dreher, K. L., Costa, D. L., & Ghio, A. J. (1997). In vivo evidence of free radical formation in the rat lung after exposure to an emission source air pollution particle. *Chem. Res. Toxicol., 10*, 1104–1108.

Klaassen, C. D. (2008). *Casarett & Doull's toxicology: the basic science of poison* (7th ed.). New York: Mc.Graw-Hill.

Kamika, I., & Momba, M. N. B. (2011). Comparing the tolerance limits of selected bacterial and protozoan species to nickel in wastewater systems. *Sci. Total Environ., 410*, 172–181.

Leborans, G. F., Herrero, O. Y., & Novillo, A. (1998). Toxicity and bioaccumulation of lead in marine protozoa communities. *Ecotoxicol. Environ. Saf., 39*, 172–178.

Lyer, A., Mody, K., & Jha, B. (2004). Biosorption of heavy metals by a marine bacterium. *Mar. Pollut. Bull., 50*, 340–343.

Madoni, P. (2011). Protozoa in wastewater treatment processes: a minireview. *Ital J Zool, 78*(1), 3–11.

Madoni, P. (2000). The acute toxicity of nickel to freshwater ciliates. *Environ. Pollut., 109*(1), 53–59.

Madoni, P., Davoli, D., Gorbi, G., & Vescovi, L. (1996). Toxic effect of heavy metals on the activated sludge protozoan community. *Water Res., 30*(1), 135–141.

Malik, A., & Jaiswal, R. (2000). Metal resistance in *Pseudomonas* strains isolated from soil treated with industrial wastewater. *World J Microbiol Biotechno, 16*, 177–182.

Mannazzu, I. (2001). Vanadium detoxification and resistance in yeast: a minireview. *Ann. Microbiol., 51*, 1–9.

Martin-Gonzalez, A., Diaz, S., Borniquel, S., Gallego, A., & Gutiérrez, J. C. (2006). Cytotoxicity and bioaccumulation of heavy metals by ciliated protozoa isolated from urban wastewater treatment plants. *Res Microbiol, 157*(2), 108–118.

Migliore, L., Bocciardi, R., Macri, C., & Lo Jacono, F. (1993). Cytogenetic damage induced in human lymphocytes by four vanadium compounds and micronucleus analysis by fluorescence in situ hybridization with a centromeric probe. *Mutat. Res., 319*, 205–213.

Momba, M. N. B., & Cloete, T. E. (1996). The relationship of biomass to phosphate uptake by *Acinetobacter junii* activated sludge mixed liquor. *Water Res., 30*(2), 364–370.

Mukherjee, B., Patra, B., Mahapatra, S., Banerjee, P., Tiwari, A., & Chatterjee, M. (2004). Vanadium—an element of atypical biological significance. *Toxicol. Lett., 15*, 135–143.

Mytelka, A. I., Czachor, J. S., Guggino, W. B., & Golub, H. (1973). Heavy metals in wastewater and treatment plant effluents. *J Water Pollut Control Fed, 45*, 1859–1864.

National Water Act. (1998). *Act No 36 of 1998*. Pretoria: Department of Water Affairs and Forestry.

Neufeld, R. D. (1976). Heavy metals-induced deflocculation of activated sludge. *J. Water Pollut. Control Fed., 48*(8), 1940–1947.

Nicolau, A., Martins, M. J., Mota, M., & Lima, N. (2005). Effect of copper in the protistan community of activated sludge. *Chemosphere, 58*, 605–614.

Nilsson, J. R. (1981). Effect of copper on phagocytosis in etrahymena. *Protoplasma, 109*, 359–370.

Owusu-Yaw, J., Cohen, M. D., Fernando, S. Y., & Wei, C. I. (1990). An assessment of the genotoxicity of vanadium. *Toxicol. Lett., 50*, 327–336.

Pala, A. I., & Sponza, D. T. (1995). Biological treatment of petrochemical wastewaters by *Pseudomonas* sp. added activated sludge culture. *Environ. Technol., 17*(7), 673–685.

Peng, X., Murphy, T., & Holden, N. M. (2008). Evaluation of the effect of temperature on the die-off rate for *Cryptosporidium parvum* oocycts in water, soils, and feces. *Appl. Environ. Microbiol., 74*(23), 7101–7107.

Rajbanshi, A. (2008). Study on heavy metal resistant bacteria in Guheswori Sewage Treatment Plant. *Our Nature, 6*(1), 52–57.

Rehder, D. (2008). Is vanadium a more versatile target in the activity of primordial life forms than hitherto anticipated? *Org. Biomol. Chem., 6*, 957–964.

Rehman, A., Shakoori, F. R., & Shakoori, A. R. (2010). Resistance and uptake of heavy metals by *Vorticella microstoma* and its potential use in industrial wastewater treatment. *Environ. Prog. Sustainable Energy, 29*(4), 481–486.

Rehman, A., Ashraf, S., Qazi, J. I., & Shakoori, A. R. (2005). Uptake of lead by a ciliate, *Stylonychia mytilus*, isolated from industrial effluents: potential use in bioremediation of wastewater. *Bull. Environ. Contam Toxicol., 75*, 290–296.

Reynolds, K. A., & Pepper, I. L. (2000). Microorganisms in the environment. In R. M. Maier, I. L. Pepper, & C. P. Gerba (Eds.), *Environmental microbiology* (pp. 7–41). San Diego: Academic.

Roane, T. M., & Peper, I. L. (2000). Microorganisms and metal pollutants. In R. M. Maier, I. L. Pepper, & C. P. Gerba (Eds.), *Environmental microbiology* (pp. 403–423). San Diego: Academic.

Schlenk, D., & Moore, C. T. (1994). The effect of pH on the toxicity of copper sulphate to the ciliate protozoan (*Tetrahymena thermophila*). *Bull. Environ. Contam Toxicol, 53*, 800–804.

Sedlak, R. (1991). *Phosphorus and nitrogen removal from municipal wastewater: principles and practice* (2nd ed.). New York: Lewis.

Shirdam, R., Khanafari, A., & Tabatabaee, A. (2006). Cadmium, nickel and vanadium accumulation by three strains of marine bacteria. *Iran. J. Biotechnol., 4*(3), 180–187.

Silva, E. S., Pinto, P. C. A. G., Lima, J. L. F. C., & Saraiva, M. L. M. F. S. (2009). Sequential injection spectrophotometric determination of V (V) in environmental polluted waters. *Water SA, 35*(3), 283–287.

Stevik, T. K., Aa, K., Austand, G. & Hanssen, J. F. (2004). Retention and removal of pathogenic bacteria in wastewater percolating through porous media: A review. *Water Res*., 38, 1355–1367.

Van Nostrand, J. D., Sowder, A. G., Bertsch, P. M., & Morris, P. J. (2005). Effect of pH on the toxicity of nickel and other divalent metals to *Burkholderia cepacia* $PR1_{310}$. *Environ. Toxicol. Chem., 24*(11), 2742–2750.