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Synergistic effects of vanadium and nickel on heavy metal-tolerant microbial species in wastewater systems

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ABSTRACT

This study assessed the combined effects of V^{5+} and Ni^{2+} on metal-tolerant microbial isolates (bacteria: *Bacillus licheniformis and Pseudomonas putida* and Protozoa: *Peranema* sp. and *Trachelophyllum* sp). Using the primers for *nccAC1* and *van2* genes, the polymerase chain reaction technique revealed the presence of genes in both *P. putida* and *Peranema* sp., whereas *B. licheniformis* contained only *nccAC1* and *Trachelophyllum* sp. none. *P. putida* appeared to be highly resistant to V^{5+} (growth rate: 0.43 h^{-1}, LC_{10}: 40–50 ppm-V^{5+}) and Ni^{2+} (growth rate: 0.41 h^{-1}, LC_{10}: 50 ppm-Ni^{2+}). The absence of target genes in *Trachelophyllum* sp. was then confirmed by its high sensitivity to V^{5+} (growth rate: 0.08 h^{-1}, LC_{10}: 40-ppm-V^{5+}) and Ni^{2+} (growth rate: 0.04 h^{-1}, LC_{10}: 30–40 ppm-Ni^{2+}) when compared to other test isolates. Although the combination of Ni^{2+} and V^{5+} resulted in a synergistic reaction by increasing their toxicity and impairing the microbial growth survival and their metal-removal ability in the wastewater systems, bacterial isolates were more persistent than protozoan isolates. *P. putida* were able to persist up to 60 h (4 CFU/mL, LC_{50}: 40–50 ppm-V^{5+}–Ni^{2+}) and *Peranema* sp. up to 48 h (~4 Cells/mL, LC_{50}: 30–40 ppm). However, the co-inoculation of bacterial (2×10^2–6.6×10^6 CFU/mL, LC50: 40–50 ppm) and protozoan (2.01×10^2–1.63×10^3 Cells/mL, LC50: 30–40 ppm) isolates decreased the synergistic effect of the test metals. This study suggests that the ability of microbial isolates to resist to the synergistic effect of Ni^{2+}–V^{5+} was due to the presence of metal-resistant genes and the microbial diversity in the media.

Keywords: Vanadium; Nickel; Synergistic effect; Pollution; Wastewater treatment; Bacteria; Protozoa

1. Introduction

Heavy metals are an inherent part of nature and are found deep in the earth's crust [1–3]. Naturally, at certain levels, their presence in the surface of the earth is very limited, but they are found in water, soil and food. It has been pointed out that an increased production of everything, human activities (such as industrialisation and urbanisation) and rapid population growth disrupt the normal biogeochemical activities and this, in turn, results in large quantities of highly concentrated heavy metal waste disposal. Occasionally, a single metal is involved but more often, a mix of metals are present [4]. Although some heavy metals are reported as chemicals that are

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essential to both prokaryotic and eukaryotic organisms, they also present a toxic effect, even at moderate concentrations [5] and their disposal are a global concern [4,6,7].

Nickel (Ni) has been reported as an essential trace element for mammals and Ni^{2+} ion is the prevalent oxidation state under environmental conditions [8,9]. Despite evidence of the importance of Ni^{2+} in several animals and microorganisms, ambiguity still exists regarding its potential role in humans, because the intake from most foodstuffs is sufficiently high [8]. This metal can be considered as a problematic heavy metal due to its ability to inhibit the synthesis of macromolecules such as protein. It also interferes with the role of other essential elements such as Mg^{2+} and Fe^{3+} in the metabolism of carbohydrates and in the removal of pyruvate or potassium ions from cells [10,11]. Nickel is one of the rare chemicals classified by the International Agency of Research on Cancer as falling into the human carcinogens Group 1, while the parameters involved in its carcinogenic activities are not yet clear [12]. However, it is widely known that Ni^{2+} compounds are nephrotoxic, hepatotoxic, clastogenic, immunotoxic and teratogenic [8,13].

On the other hand, vanadium (V) is the 22nd most abundant element in the earth's crust and naturally found in minerals, fossil fuels, coal and living organisms [14]. With a complex chemistry, V exhibits several oxidation states (−1 to +5) and toxicity depends on these states [14,15]. Two of these oxidation states (+4 and +5) are considered predominant in the environment, with the +5 oxidation state being more toxic [16]. Used as a dietary supplement and therapeutic agent, V is regarded as being a more essential rather than a toxic element [4]. Inhalation and ingestion are the main exposure routes. There is some controversy about the V toxicity to humans [14]. A study conducted on V effects revealed that at acute exposure, V could cause local irritation of eyes and the upper respiratory tract [17]. Vanadium pentoxide (V_2O_5), one of the vanadium compounds in air, is of major concern. Acute exposure to V_2O_5 was reported to cause bronchitis and pneumonitis [18]. Despite the lack of evidence in V toxicity and carcinogenicity, this heavy metal still represents a threat to both human and animal health when taken in high concentrations [19,20].

Several studies have also reported the co-occurrence of $V^{4+/5+}$ and Ni^{2+} in the environment; these metals are known to be the most abundant in crude oil and petroleum and thought to exist largely as porphyrin complexes [21–23]. This raises the question of whether $V^{4+/5+}$ and Ni^{2+} can interact with one another by increasing or decreasing their toxicity. Although they can form a complex such as porphyrin, their combined effects to bacterial and protozoan species in the wastewater system remain unknown.

It is well known that microorganisms can acquire resistant mechanisms such as biosorption, bioaccumulation, bioprecipitation and efflux-mediated mechanisms to overcome the toxic effects of heavy metals such as Ni^{2+} and $V^{4+/5+}$ [3,24]. Among these mechanisms, the efflux-mediated mechanism has been well studied [3,25]. This mechanism is basically a plasmid-encoded mechanism involving many operons such as *nccAC1*, *van2*, etcetera in which toxic ions enter the cell via active transport (ATPase pump) or diffusion (chemiosmotic ion or proton pump) [4]. Discovery of these detoxifying mechanisms intensified research into the interaction of heavy metals with microorganisms [26,27]. In micro-fauna, less research has been conducted on protozoa for the tolerance/detoxification of heavy metals when compared to other microorganisms such as bacteria and fungi [28–30]. It is still unclear how protozoan species resist $V^{4+/5+}$ and Ni^{2+} toxicity. Therefore, this study firstly assessed the presence of Ni^{2+}- and V^{5+}-resistant genes (*nccAC1* and *van2 genes* encoding the resistance to Ni^{2+} and V^{5+}, respectively) in two bacterial isolates (*Bacillus licheniformis* and *Pseudomonas putida*) and in two protozoan isolates (*Peranema* sp. and *Trachelophyllum* sp.). Secondly, the study ascertained the combined effects of V^{5+} and Ni^{2+} on microbial isolates in order to mimic the environmental conditions.

2. Materials and methods

2.1. Test organisms

Bacterial species (*B. licheniformis*-ATCC12759 and *P. putida*-ATCC31483) were purchased from Quantum Biotechnologies (Strydompark, Randburg, South Africa). The metal tolerance or removal ability of these bacterial species has been reported elsewhere [30,31]. To obtain a fresh culture, an aliquot of each bacterial species was separately inoculated into a 500 mL Elernmeyer flask containing 100 mL sterile nutrient broth (NB) in aseptic conditions and incubated in a shaking incubator (speed: 100 rpm) at 30˚C, with the exception of *B. licheniformis*, which was incubated at 50˚C overnight [32]. In order to determine the cellular concentration needed for the experiment, the growth of bacterial species was measured every 30 min using the spread plate method [33].

Indigenous protozoan species (*Trachelophyllum* sp. and *Peranema* sp.) previously isolated from wastewater mixed liquors were collected from the aeration tanks of the Daspoort Wastewater Treatment Plant (Pretoria, South Africa). The said protozoan species have been

reported to successfully remove nitrate and phosphorus in modified mixed liquors [34] and to separately tolerate V^{5+} and Ni^{2+} [30,35]. The preparation of these protozoan species was carried out according to Akpor et al. [34]. Briefly, each protozoan isolate was separately transferred from the stock culture to a 500 mL Erlenmeyer Flask containing 100 mL of fresh media of proteose peptone glucose medium (PPG) under aseptic conditions. An antibiotic (streptomycin-50 μg/mL) was added to prevent bacterial contamination, while a heat-killed *Escherichia coli*-WG4 was used as a nutrient source. To obtain the needed protozoan concentrations, the inoculated flasks were incubated at room temperature (25˚C) in the dark, and the number of cells was determined every hour using an inverted microscope (Axiovert S100, Carl Zeiss) at 100–400× magnifications.

Prior to assessing the synergistic effects of V^{5+} and Ni^{2+}, the test organisms were acclimatised to a mixture of V^{5+} and Ni^{2+} (2 ppm) in the NB for bacteria and in PPG for protozoa for 48 h at 30 and 25˚C to enhance their tolerance ability in order to test the heavy metals.

2.2. Screening of metal tolerance-gene in test isolates

2.2.1. Isolation of genomic DNA

The high molecular weight genomic DNA was isolated from the fresh growing cells (6×10^6 Cells/mL for protozoa and 8×10^8 CFU/mL for bacteria) as reported by Ozutsumi et al. [36]. However, the precipitated gDNA was washed with 70% ethanol and then purified using ZR Fungal/Bacterial DNA Kit (Zymo Research, USA).

2.2.2. PCR amplication of purified DNA

The molecular characterisation on metal-tolerance ability of the test isolates was done by amplification of the *nccAC1* and *van2* genes that encodes nickel–cobalt–cadmium and vanadium resistance, respectively, using specific primers (Table 1). These primers were synthesised by Inqaba Biotechnological Industries (Pretoria, South Africa). A PCR reaction mixture of a total of 50 μL containing 19 μL Nuclease-free water, 25 μL 2× Dream Taq™ PCR master mix (10× Dream Taq™ buffer, 2 uM dNTP mix and 1.25 U Dream Taq™ polymerase), 2 μL of each PCR primer (10 μM) and 2 μL of genomic DNA (50 ng/μL) was prepared in a 200 μL PCR tube.

The amplification was carried out in a thermal cycler (MJ Mini™ Personal Thermal Cycler, Biorad, SA) consisting of 30 cycles of 1 min at 94˚C of denaturation, 30 s of annealing step of 30 s at 50˚C, 1 min of extension step at 72˚C, followed by the final extension step at 72˚C for 10 min and cooling to 4˚C. The PCR product (10 μL) was analysed using 1% agarose gel (Merck, SA) stained with 5% ethidium bromide (Merck, SA) and electrophoresed to determine and visualise the product size under UV light using an InGenius L Gel documentation system (Syngene).

2.3. Sample collection and preparation of the culture medium

Between November 2010 and February 2011, wastewater samples were collected on a monthly basis from the effluent (before disinfection) of the Daspoort Wastewater Treatment Plant in Pretoria and transported in a cooler box to the laboratory. Prior to usage as culture media, 5 L wastewater samples were allowed to settle for 2 h and filtered using filter paper (Whatman No. 1) to remove biomass and other suspended solids. The filtered samples were screened to determine the concentrations of chemical oxygen demand (COD), dissolved oxygen (DO), and metals (V^{5+} and Ni^{2+}) as well as the pH. The COD concentration was measured using closed reflux methods as described in standard methods (APHA 2001), while the heavy metal concentration was determined using the Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Other parameters, such as the 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), $MgSO_4.7H_2O$ (0.5 g/L) and KNO_3 (0.18 g/L)

Table 1
Primers used for molecular characterisation on metal-tolerance ability of test isolates

Primer name	Sequences	Amplicon size (bp)	Refs.
nccAC1	GCGTGGAAGGCAAGATGTTC ACGTCCACCAACGTTGGC	457	[37]
van2	CAAGTTCGTCGTCAACTT CACTCGAGACAGGTATCA	1,256	[38]

were added to the filtrate to serve as a carbon source and nutrient supplement in the mixed liquor [34,39]. The test metal used in the experimental study was of analytical grade, purchased from Sigma Aldrich (Cape Town, South Africa). Sodium meta-vanadate anhydrous ($NaVO_3$) and Nickel nitrate [$Ni(NO_3)_2$] were used as a source of V^{5+} and Ni^{2+} ions, respectively. The stock solution of V^{5+}, Ni^{2+} and mixture (V^{5+} and Ni^{2+}, 1:1, mol: mol) at a concentration of 1,000 ppm was prepared using the deionised water. The experiment was performed in triplicate for each isolate.

A 150 mL wastewater mixed liquor medium was prepared with either a separate or a mixture of V^{5+} and Ni^{2+} at a concentration of 10 ppm. Since, in the environment, microorganisms are exposed to a consistent variation of heavy metal concentrations, depending on surrounding human activities, metal concentrations in the culture medium were increased on a geometric scale of 10 after each 12 h for 3 days of incubation. To adjust the pH (7.2 ± 0.3) of the medium, 1.0 M HCl and 1.0 M NaOH (Merck, SA) were used. ICP-OES was used to confirm the metal concentrations in the wastewater mixed liquor medium. Before use, the culture medium was autoclaved and cooled down to room temperature. 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.

2.4. Determining single and combined effects of V^{5+} and Ni^{2+}

The experiments were conducted in 250 mL Erlenmeyer flasks containing 150 mL of the modified mixed liquor. The flasks were aseptically inoculated with fresh acclimatised bacterial (100 CFU/mL) or protozoan (100 Cells/mL) isolates. One positive and one negative control were also included in the experimental study. The positive control flask contained the mixed liquor without either V^{5+} or Ni^{2+} or a mixture thereof, which was inoculated with the specific microorganism, while the negative control contained only the mixed liquor with 100 ppm V^{5+}–Ni^{2+}.

To check the effect of V^{5+}–Ni^{2+} on the microbial interaction in mixed liquor media, a parallel experiment using co-inoculation of bacterial isolates and co-inoculation of protozoan isolates (as a group) in the culture media containing the combined test metals was carried out. All inoculated samples as well as the controls were incubated at 30˚C ± 2˚C for three days. The duration of the three-day experiment was selected since the test organisms were reported to reach their exponential phase within this time span [30]. After every 12 h, the sample was homogeneously shaken and an aliquot of 10 mL was taken and analysed for growth/die-off of the microbial isolates and for metal concentrations. The concentration of the metals in all the inoculated flasks as well as in the negative control was subsequently increased at a gradual scale of 10 ppm. The V^{5+}/Ni^{2+} lethal concentration at 10% (LC_{10}) and at 50% (LC_{50}) die-off of each microbial isolate was estimated according to the inhibition concentration approach. Throughout the experimental study, the COD as pollutant and DO as nutrient for microbial growth were determined as mentioned above to identify their effects on the toxicity of the $V^{5+} + Ni^{2+}$ mixture to test organisms in wastewater mixed liquor.

2.5. Effect of temperature, pH and microbial concentration on the toxicity of V^{5+} and Ni^{2+}

To check the effect of temperature and pH on the toxicity of the V^{5+} and Ni^{2+} mixture, these isolates were separately inoculated in mixed liquor containing both V^{5+} and Ni^{2+} at the concentration, which significantly inhibited the test organisms in the modified mixed liquor. The experimental study was conducted at various temperatures (25, 35 and 40˚C) with a constant pH of 7, thereafter at various pH levels (pH 4, 6 and 8) with a constant temperature of 30˚C. Finally, the experimental study was performed with various concentrations of microorganisms (~200, 400; 600; 800 and 1,000 CFU or Cells/mL) at a constant temperature 30˚C and pH 7 in a shaking incubator at a speed of 100 rpm. During each sampling regime, aliquot samples were taken every 12 h over a 60 h period for the determination of the microbial concentration. The growth/mortality of the bacterial and protozoan isolates was determined using the spread plate method and visual counts [30,33]. The specific growth and first order die-off rates of the bacterial and protozoan isolates were calculated using the formula reported by previous investigators [40,41].

2.6. Statistical analysis

The data were statistically analysed using the Stata computer software. To compare effects of both single and combined effects of V^{5+} and Ni^{2+} to the test isolates, one-way analysis of variances was used. The relationship tests were carried out using the Pearson correlation index and the interpretation was performed at a two-sided 95% confidence limit.

3. Results

3.1. Screening of metal tolerance-gene in test isolates

Before assessing the effect of V^{5+} and Ni^{2+} on the isolates, the present study first screened the presence of Ni^{2+}- and V^{5+}-resistant genes in gDNA of the test microbial isolates. The expression levels of specific genes: *nccAC1* (Ni^{2+}, Co^{2+}, Cd^{2+}-resistant) and *van2* ($V^{5+/4+}$-resistant) were measured using the conventional PCR techniques (Fig. 1).

As evident in Fig. 1, an amplified PCR product of approximately 500 bp indicated the presence of *nccAC1* gene in *P. putida* and *Peranema* sp., while this gene appeared to be absent in *B. licheniformis* and *Trachelophyllum* sp. For *van2*, the amplified product of approximately 1256 bp was reproductively detected in bacterial isolates (*P. putida* and *B. licheniformis*) as well as in *Peranema* sp. *Trachelophyllum* sp. was found to be the only microbial isolate lacking both target genes, while *B. licheniformis* showed only the presence of genes encoding the resistance to V^{5+} toxicity.

3.2. Single effects of V^{5+} and Ni^{2+} on test organisms in mixed liquor

Figs. 2 and 3 illustrate the growth response of individual bacterial and indigenous protozoan isolates to a gradual increase of V^{5+} and Ni^{2+} concentrations in a modified wastewater mixed liquor. There was a general growth of the test organisms in the presence of Ni^{2+} concentrations (Fig. 2). With the exception of *Peranema* sp. (die-off rate: 13%), no acclimatised test organisms revealed a die-off after 12 h of incubation. A high bacterial growth response [with bacterial counts ranging from 2.67×10^2 to 1.05×10^{10} CUF/mL (growth rate: 0.23–0.41 h^{-1})] was noted up to 48 h after the incubation period. This was followed by a die-off rate of 1.52 (LC_{10}: 50-ppm-Ni^{2+}) when compared with the highest growth of each isolate. *P. putida*, appeared to have the highest growth response compared with *B. licheniformis*. Its population count reached up to 1.05×10^{10} CFU/mL (growth rate: 0.41 h^{-1}) after 48 h of incubation. As to protozoan isolates, there was a slight increase in the number of cells, which ranged from 7.8×10^1 to 7.02×10^3 Cells/mL (growth rate: 0.02–0.10 h^{-1}) between 0 h and 36 h. The remaining incubation period was characterised by the die-off of both protozoan isolates [die-off rate of 0.80 (LC_{10} between 30 and 40 ppm-Ni^{2+})]. *Peranema* sp. appeared to have the highest cell counts of 7.02×10^3 Cells/mL (Fig. 2) compared with *Trachelophyllum* sp. Although LC_{10} of both bacterial isolates (50-ppm-Ni^{2+}) was close to those of protozoan isolates (between 30 and 40 ppm-Ni^{2+}), the microbial growth among the two groups was found to be significantly different ($p < 0.05$) due to the high bacterial counts.

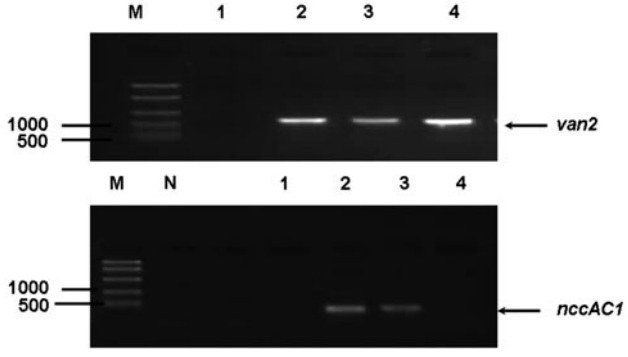

Fig. 1. Agarose gel electrophoresis of PCR products of total genomic DNAs with primer pair *nccAC1*-fwd and *nccAC1*-rev, primer pair *van2*-fwd and *van2*-rev. Lanes: M: High range DNA ladder, N: Negative, 1–4, amplified PCR product of: *B. licheniformis* (4), *P. putida* (3), *Peranema* sp. (2) and *Trachelophyllum* sp. (1).

In examining the response of acclimatised test organisms to the gradual increase in V^{5+} during 60 h of the incubation period at 30 °C (Fig. 3), a general positive growth response was recorded throughout the experimental study. The highest microbial counts were revealed in the media inoculated with bacterial isolates (from 2.04×10^2 to 9.65×10^8 CFU/mL) compared to protozoan isolates. After 36 h of the exposure to V^{5+}, *P. putida* had the highest colony counts of 9.65×10^8 CFU/mL (growth rate: 0.43 h^{-1}), followed by *B. licheniformis* [4.75×10^8 CFU/mL after 48 h (growth rate: 0.34 h^{-1})], *Peranema* sp. [1.47×10^4 Cells/mL (growth rate: 0.11 h^{-1})] and *Trachelophyllum* sp. [2.10×10^3 Cells/mL (growth rate: 0.08 h^{-1})]. No microbial isolates revealed a die-off after a 12 h exposure to V^{5+}. When compared to the highest microbial counts, microbial isolates had a similar LC_{10}, ranging between 40 and 50 ppm-V^{5+}, while displaying a die-off rate of 5.96 and 5.69 for *P. putida* and *B. licheniformis*, respectively. Protozoan isolates however, exhibited a die-off rate of 2.64 for *Peranema* sp. and 3.27 (LC_{50}: 50 ppm-V^{5+}) for *Trachelophyllum* sp. Although LC_{10} of all the test isolates appeared to range between 40 and 50 ppm-V^{5+}, significant differences in growth response were observed between the isolates ($p < 0.05$) and also between the two groups of isolates ($p < 0.05$).

In terms of microbial counts and growth response, V^{5+} appeared to be more toxic to bacterial isolates than Ni^{2+}, but less toxic to protozoan isolates than Ni^{2+}. In the presence of V^{5+}, bacterial counts reached up to 9.65×10^8 CFU/mL; while in Ni^{2+} presence, the bacterial counts were up to 1.05×10^{10} CFU/mL. Protozoan isolates were found to be more sensitive to

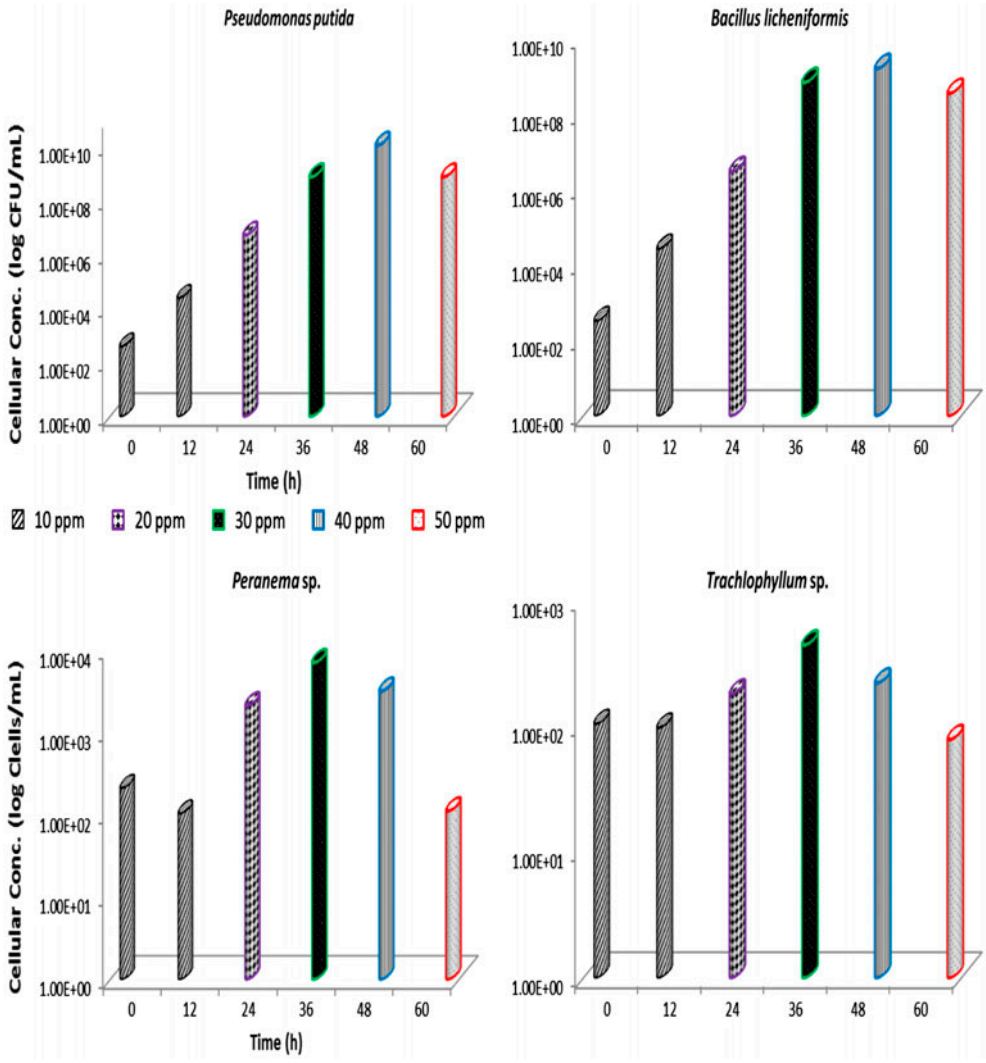

Fig. 2. Ni^{2+} effects on microbial isolates incubated at 30°C, pH 7 for 60 h in wastewater systems.

Ni^{2+} than to V^{5+}, with the highest cell counts up to 7.03×10^3 Cells/mL and 1.47×10^4 Cells/mL, respectively. Statistical evidence indicates significant differences ($p < 0.05$) when comparing the effects of Ni^{2+} between bacterial counts of the target isolates to those of V^{5+} effects. The toxicity effects of these metals were not found to be significant ($p > 0.05$) between protozoan cell counts.

3.3. Combined effects of V^{5+} and Ni^{2+} on test organisms in mixed liquor

Fig. 4 summarises the combined effects of V^{5+} and Ni^{2+} to individual acclimatised test organisms and to concomitant inoculation of acclimatised bacterial isolates (group 1) as well as protozoan isolates (group 2). Throughout the experimental study, a general decrease in colony/cells counts for all the test organisms was observed. Bacterial isolates revealed an insignificant increase in counts (10^3 CFU/mL) and the highest growth rate of 0.08 h^{-1} that was reached after 48 h for *P. putida*, and 0.037 h^{-1} after 36 h for *B. licheniformis*. *P. putida* persisted with the combined effect of V^{5+} and Ni^{2+} until up to 60 h (4 CFU/mL) with a die-off rate of 98.84%, while *Bacillus licheniformis* could reach only 48 h (7.2×10^1 CFU/mL) with a die-off rate of 3.91 (73.84%) and 5.60 (100%) at 48 and 60 h, respectively. The lethal concentration of V^{5+} and Ni^{2+} combined was observed between 30 and 40 ppm-V^{5+}–Ni^{2+} (LC_{10}) and between 40 and 50 ppm-V^{5+}–Ni^{2+} (LC_{50}) for both *B. licheniformis* and *P. putida*, respectively. Unlike bacterial isolates, protozoan cells gradually decreased throughout the experimental study. *Peranema* sp. could persist up to 48 h (~4 Cells/mL) while *Trachelophyllum* sp. persisted

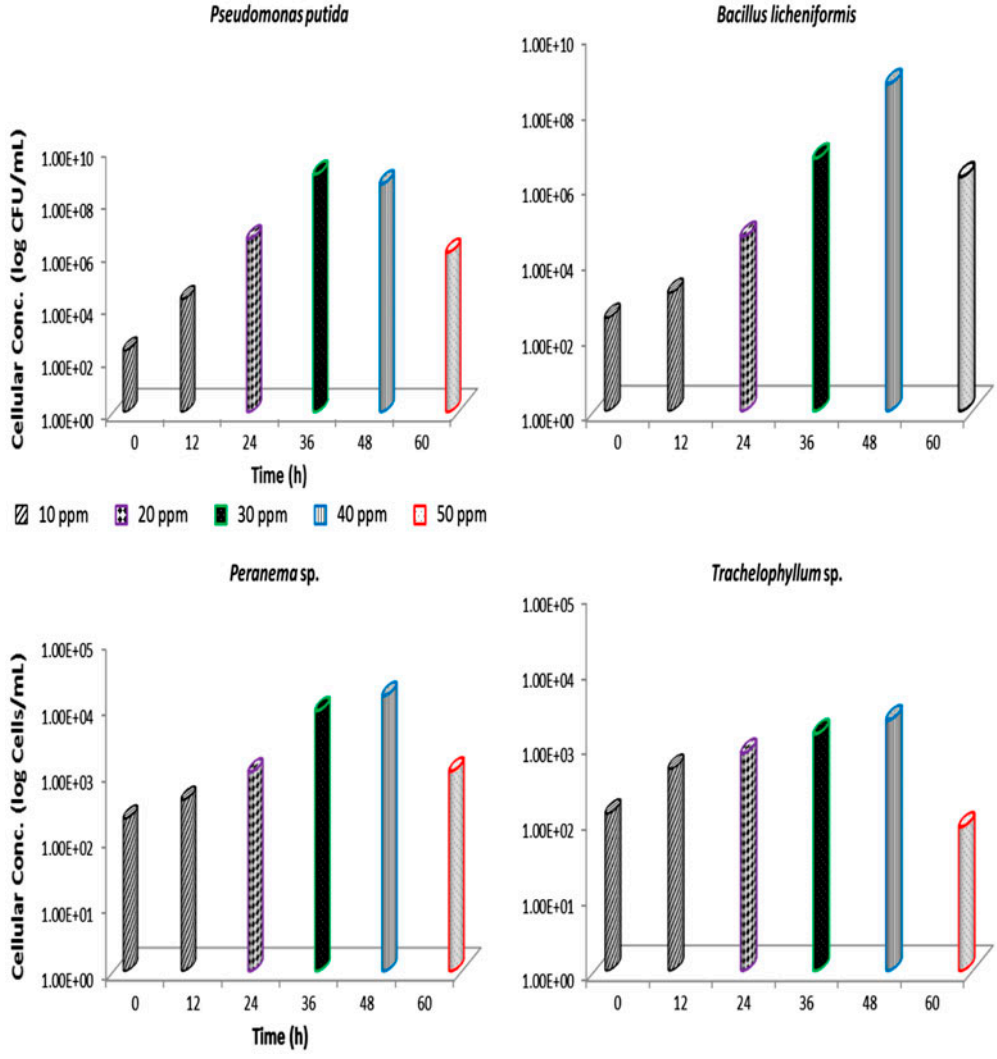

Fig. 3. V^{5+} effects on microbial isolates incubated at 30°C, pH 7 for 60 h in wastewater systems.

up to 36 h (~3 Cells/mL), followed by a total die-off (100%) at 60 and 48 h, respectively. The lethal concentrations of combined V^{5+} and Ni^{2+} on protozoan isolates were observed between 30 and 40 ppm-V^{5+}–Ni^{2+} (LC_{50}) for *Peranema* sp. and below 10 ppm-V^{5+}–Ni^{2+} (LC_{50}) for *Trachelophyllum* sp. with a die-off rate of 100% was observed after 60 h of incubation. Although the two bacterial isolates appeared to be more resistant to the combined effects of V^{5+}–Ni^{2+} than protozoan isolates, statistical evidence revealed no significant difference between these two groups ($p > 0.05$).

This study also evaluated the combined effects of V^{5+} and Ni^{2+} to concomitant inoculation of acclimatised bacterial isolates (group 1) as well as protozoan isolates (group 2) (Fig. 4). In general, in the presence of both V^{5+} and Ni^{2+}, a gradual increase in microbial number was observed in the culture media inoculated by acclimatised bacteria as well as by acclimatised protozoa. Bacteria (6.6×10^6 CFU/mL, growth rate: $0.45\ h^{-1}$) and protozoa (1.63×10^3 Cells/mL, growth rate: $0.068\ h^{-1}$) could grow for as long as 48 h in 40 ppm-V^{5+}–Ni^{2+} followed by a slight decrease in the number of colonies/cells at 60 h [die-off rate of 1.52 (LC_{10}: 50 pm-V^{5+}–Ni^{2+}) and 2.32 (LC_{10}: 40–50 pm-V^{5+}–Ni^{2+}), respectively].

3.4. Effect of temperature, pH and microbial concentration to the toxicity of V^{5+} and Ni^{2+}

Table 2 illustrates the effect of pH and temperature on the synergistic actions of V^{5+} and Ni^{2+} (50 ppm) to the acclimatised test organisms incubated in a modified wastewater mixed liquor. The variations of pH (4, 6, 8) of the mixed liquor media and tempera-

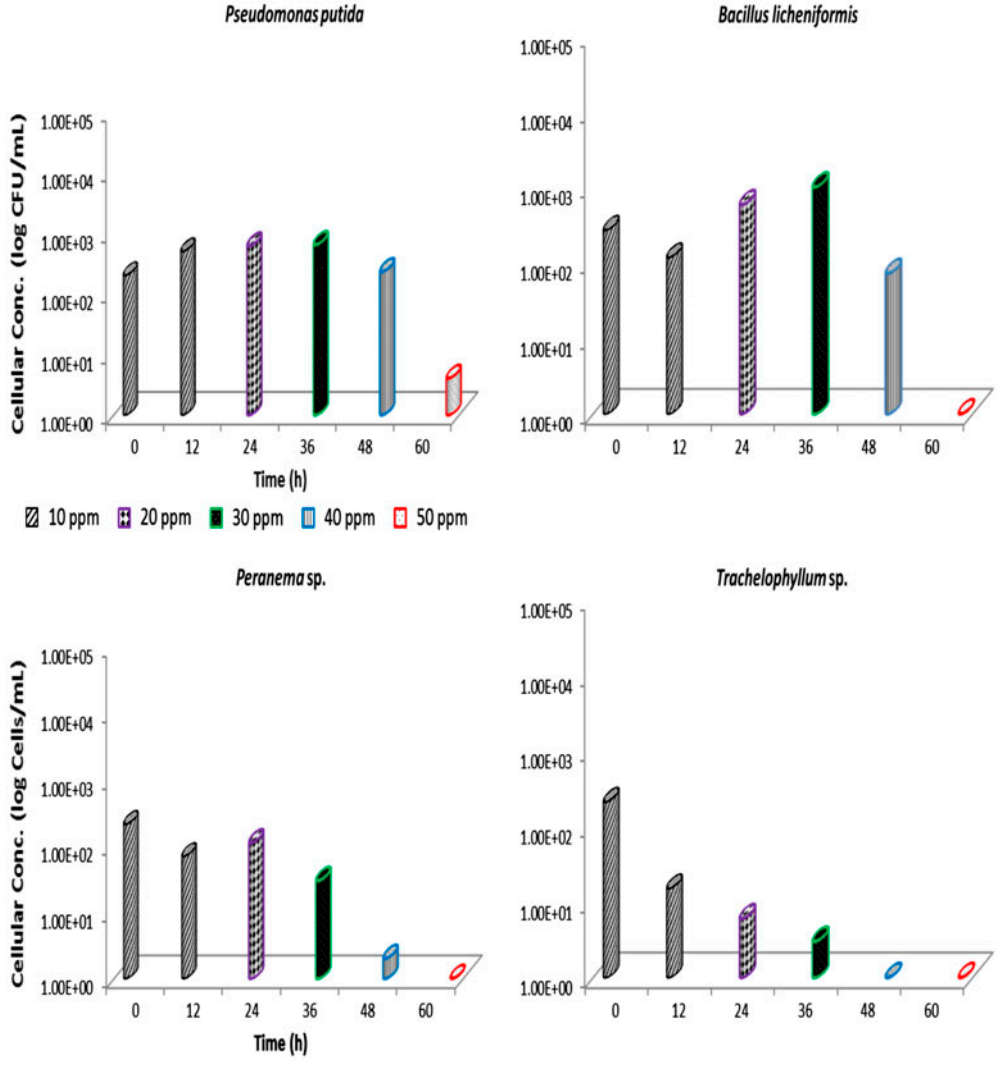

Fig. 4. Synergistic effects of V^{5+} and Ni^{2+} on individual acclimatised test organisms at 30°C and pH 7 during 60 h.

ture (25, 35, 40°C) during the incubation period did not have a major effect on the synergistic action of Ni^{2+} and V^{5+} to the test organisms. Although the combined effect of V^{5+} and Ni^{2+} at 50 ppm appeared to be more toxic to all the isolates, there was an increase in bacterial counts when varying the pH of the media and the temperature of incubation. After 12 h of exposure to V^{5+} and Ni^{2+}, a drastic die-off of bacterial isolates occurred. Subsequently, a slight gradual growth of *P. putida* in the media was noted between the periods within 24 h and 94 h which had pH values of 6 (94 CFU/mL) and 8 (152 CFU/mL), respectively. For *B. licheniformis*, this observation could be noted only at pH 6 (64 CFU/mL) between a 24 and 36 h incubation period. In terms of temperature variation, a gradual growth of *P. putida* and *B. licheniformis* were recorded, up to 68 CFU/mL after 48 h at 25°C and up to 124 CFU/mL after 36 h at 40°C, respectively. No increase in cell count was observed in the culture media inoculated with protozoan isolates. However, *Peranema* sp. could resist the combined effect of V^{5+} and Ni^{2+} (50 ppm) up to 36 h of incubation when the temperature was adjusted to 25°C and pH at 8.

As illustrated in Table 3, there were variations in toxic-synergistic effects of V^{5+} and Ni^{2+} (50 ppm) on different microbial concentrations in modified mixed liquors. In general, approximately 10% of the microbial population inoculated into the media survived the combined effects of Ni^{2+} and V^{5+} after 12 h of exposure, although the die-off rates depended on the initial concentrations of the organisms. Moreover, the growth response of all the isolates, except for *Tachelophyllum* sp, was affected by an increase in microbial concentrations. Bacterial isolates revealed the highest increase in

Table 2
Temperature and pH effects to the synergistic effects of V^{5+} and Ni^{2+}

	Time (h)	pH/Isolate counts			Temperature/isolate counts		
		4	6	8	25	35	40
Pseudomonas putida	0	125	140	120	140	146	144
	12	24	49	64	39	59	84
	24	10	68	94	44	21	9
	36	1	94	152	46	7	1
	48	1	21	95	68	1	1
	60	1	2	38	12	1	1
Bacillus licheniformis	0	167	164	180	150	140	162
	12	35	12	86	12	75	60
	24	21	38	31	3	39	80
	36	1	64	9	1	28	124
	48	1	32	1	1	2	90
	60	1	6	1	1	1	68
Peranema sp.	0	160	180	153	150	138	130
	12	1	27	94	94	3	1
	24	1	5	27	36	1	1
	36	1	1	9	2	1	1
	48	1	1	1	1	1	1
	60	1	1	1	1	1	1
Trachelophyllym sp.	0	157	135	162	160	130	130
	12	1	1	21	9	1	1
	24	1	1	1	2	1	1
	36	1	1	1	1	1	1
	48	1	1	1	1	1	1
	60	1	1	1	1	1	1

numbers compared with protozoan isolates. *P. putida* and *B. licheniformis* could grow for up to 48 h and 36 h when the microbial concentration was adjusted from 400 to 1,000 CFU/mL and from 800 to 1,000 CFU/mL, respectively. In addition, *B. licheniformis* also showed a slight growth after 24 h in the media inoculated with 600 CFU/mL. For protozoan isolates, *Peranema* sp. was the only isolate that showed growth up to 60 h in the media inoculated with 800 and 1,000 Cells/mL.

In the media with combined Ni^{2+}–V^{5+} and inoculated with acclimatised isolates, the variations of DO and COD were also assessed (Fig. 5). In general, the variations of COD and DO in the inoculated media appeared to be inversely proportional. At the end of the experiment, the DO and COD concentrations in the media inoculated with *P. putida* were observed to be lower at 74.8% (1.68 mg/L) and higher at 239.9% (765 mg/L) compared with their initial concentrations, respectively. For the media inoculated with *B. licheniformis*, it was observed that the DO and COD concentrations were 95.9% (0.27 mg/L) lower and at 231.52% (712 mg/L) higher than their respective initial concentrations. Moreover, for protozoan isolates, the media inoculated with *Peranema* sp. were found to be the only media with significant variations in DO (69.13%) and COD (71.40%) concentrations. The media inoculated with *Trachelophyllum* sp. exhibited the lowest variations in DO (9.12%) and COD (15.96%) concentrations.

Statistical evidence indicated a significant negative correlation between COD and DO in the mixed liquor inoculated with bacterial isolates ($r = -0.935$, $p < 0.05$) and a weak negative correlation in the mixed liquor inoculated with protozoan isolates ($r = -0.351$, $p < 0.05$) except for *Peranema* sp., which revealed a significant negative correlation ($r = -0.734$, $p < 0.05$).

3.5. Bioremoval of V^{5+}+Ni^{2+} in the modified mixed liquor

As it can be seen in Table 4, microbial isolates were highly able to remove both V^{5+} and Ni^{2+} at single or mixture form when in low concentration (10 ppm) and this decreased with a gradual increase of the test metals in the mixed liquor. A significance difference ($p < 0.05$) between the percentage removal of 10 ppm and 20 ppm was remarkably observed in

Table 3
Effect of microbial concentration disparity on the toxic effects of V^{5+} and Ni^{2+}

		Microbial concentration				
	Time (h)	200	400	600	800	1,000
Pseudomonas putida	12	15	36	59	80	160
	24	25	96	124	196	281
	36	16	291	163	239	369
	48	7	364	230	350	394
	60	1	237	139	128	234
Bacillus licheniformis	12	25	68	89	120	134
	24	36	37	124	168	213
	36	21	13	75	197	259
	48	6	5	26	120	150
	60	1	1	4	68	89
Peranema sp.	12	31	38	57	64	110
	24	9	17	18	97	267
	36	1	5	25	120	219
	48	1	1	29	135	298
	60	1	1	7	157	320
Trachelophyllum sp.	12	15	60	87	95	120
	24	3	21	38	35	45
	36	1	1	6	7	21
	48	1	1	1	1	4
	60	1	1	1	1	1

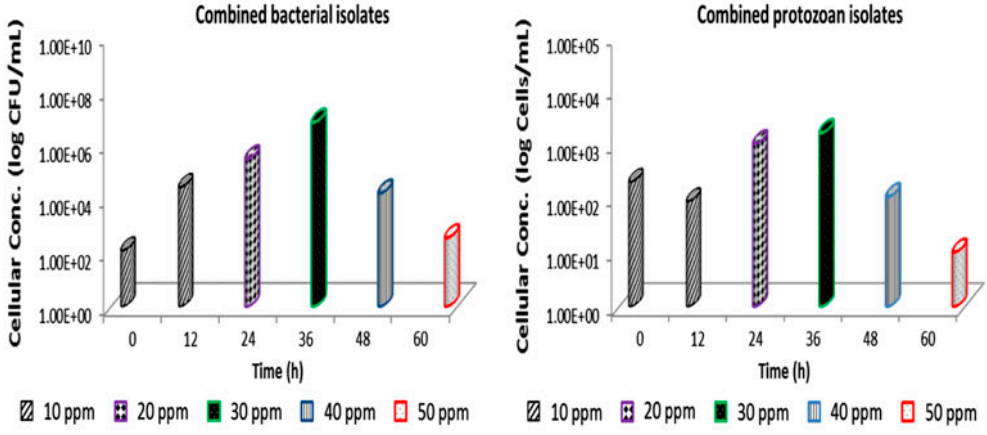

Fig. 5. Effects of V^{5+} and Ni^{2+} on test organisms concomitantly inoculated as a group (bacterial isolates or protozoan isolates) and incubated at 30°C, pH 7 during a 60 h period.

the media containing of Ni^{2+} and this could have been due to the shock-load effect of Ni^{2+} to the microbial isolates which disturbed their removal ability. Similar observation ($p < 0.05$) occurred with the addition of the combined V^{5+} and Ni^{2+}.

However, no significant ($p > 0.05$) shock-load effect was observed with V^{5+} when compared to those of Ni^{2+}. In general, *P. putida* appeared to be the isolates with the highest removal of both Ni^{2+} (99.67%) and V^{5+} (99.56%) in a single form as well as when combined (39.29%-Ni^{2+}, 21.36%-V^{5+}). *Trachelophyllum* sp. was, however, the isolates with the lowest percentage removal for single metal effect (69.68%-Ni^{2+} and 52.24%-V^{5+}) and combined metal effect (21%-Ni^{2+}, 11.65%-V^{5+}). Although the combined effects of the test metals negatively affected the removal ability of the microbial isolates, the results indicate a slight increase of percentage co-removal of Ni^{2+}–V^{5+} occurred in the

Table 4
Bioremoval of V^{5+} and Ni^{2+} in a single and combined form from the mixed liquor by test isolates

	10 ppm		20 ppm		30 ppm		40 ppm		50 ppm	
Ni^{2+}										
Pseudomonas putida	99.67 ± 1.29		58.65 ± 9.29		94.32 ± 7.51		85.64 ± 2.92		71.29 ± 13.43	
Bacillus licheniformis	98.65 ± 0.72		52.69 ± 5.65		86.95 ± 12.56		62.98 ± 16.07		47.01 ± 8.19	
Peranema sp.	88.36 ± 1.15		45.32 ± 3.94		56.84 ± 0.65		26.96 ± 12.02		10.98 ± 22.31	
Trachelophyllum sp.	69.68 ± 12.93		39.65 ± 0.12		36.44 ± 5.74		16.69 ± 1.09		6.28 ± 2.93	
V^{5+}										
Pseudomonas putida	99.56 ± 0.83		98.97 ± 11.21		95.32 ± 1.83		89.69 ± 9.94		86.67 ± 0.94	
Bacillus licheniformis	78.69 ± 2.82		87.69 ± 15.49		72.36 ± 0.84		59.36 ± 15.03		42.65 ± 7.01	
Peranema sp.	87.95 ± 5.71		67.95 ± 7.54		45.12 ± 2.48		37.36 ± 6.21		17.29 ± 9.41	
Trachelophylum sp.	52.24 ± 9.94		56.98 ± 2.19		44.28 ± 9.86		25.64 ± 4.75		12.27 ± 3.85	
V^{5+}+Ni^{2+}	$^{*}Ni^{2+}$	$^{*}V^{5+}$	$^{*}Ni^{2+}$	$^{*}V^{5+}$	$^{*}Ni^{2+}$	$^{*}V^{5+}$	$^{*}Ni^{2+}$	$^{*}V^{5+}$	$^{*}Ni^{2+}$	$^{*}V^{5+}$
Pseudomonas putida	39.29 ± 9.54	21.36 ± 8.08	17.28 ± 2.58	11.38 ± 3.69	24.28 ± 2.84	19.67 ± 1.28	21.28 ± 0.93	7.07 ± 1.36	4.00 ± 0.83	4.01 ± 3.54
Bacillus licheniformis	27.71 ± 7.21	17.39 ± 5.27	15.86 ± 6.89	9.67 ± 5.28	12.67 ± 2.95	18.36 ± 2.49	10.20 ± 0.83	7.28 ± 1.28	3.25 ± 2.39	2.69 ± 0.36
Peranema sp.	21.40 ± 9.64	13.27 ± 2.97	12.20 ± 2.39	8.04 ± 1.25	14.83 ± 2.03	14.25 ± 0.64	10.42 ± 5.52	5.38 ± 3.34	2.10 ± 9.84	2.09 ± 1.25
Trachelophylum sp.	21.00 ± 1.65	11.65 ± 0.38	10.60 ± 2.46	7.36 ± 4.94	10.70 ± 0.76	9.68 ± 0.04	10.00 ± 3.93	9.63 ± 10.32	4.00 ± 6.76	1.36 ± 0.37
Combined bacteria	57.95 ± 9.85	32.64 ± 4.94	32.90 ± 5.78	17.98 ± 0.58	46.60 ± 7.94	23.28 ± 1.28	25.50 ± 4.01	17.03 ± 5.31	13.40 ± 2.31	6.68 ± 0.64
Combined protozoa	52.01 ± 2.64	27.08 ± 7.75	29.20 ± 9.87	15.67 ± 7.18	26.30 ± 2.95	20.39 ± 6.25	1.07 ± 2.27	12.69 ± 1.97	10.00 ± 1.94	4.67 ± 1.29

Note: * Concentration of V^{5+} or Ni^{2+} from the combined system.

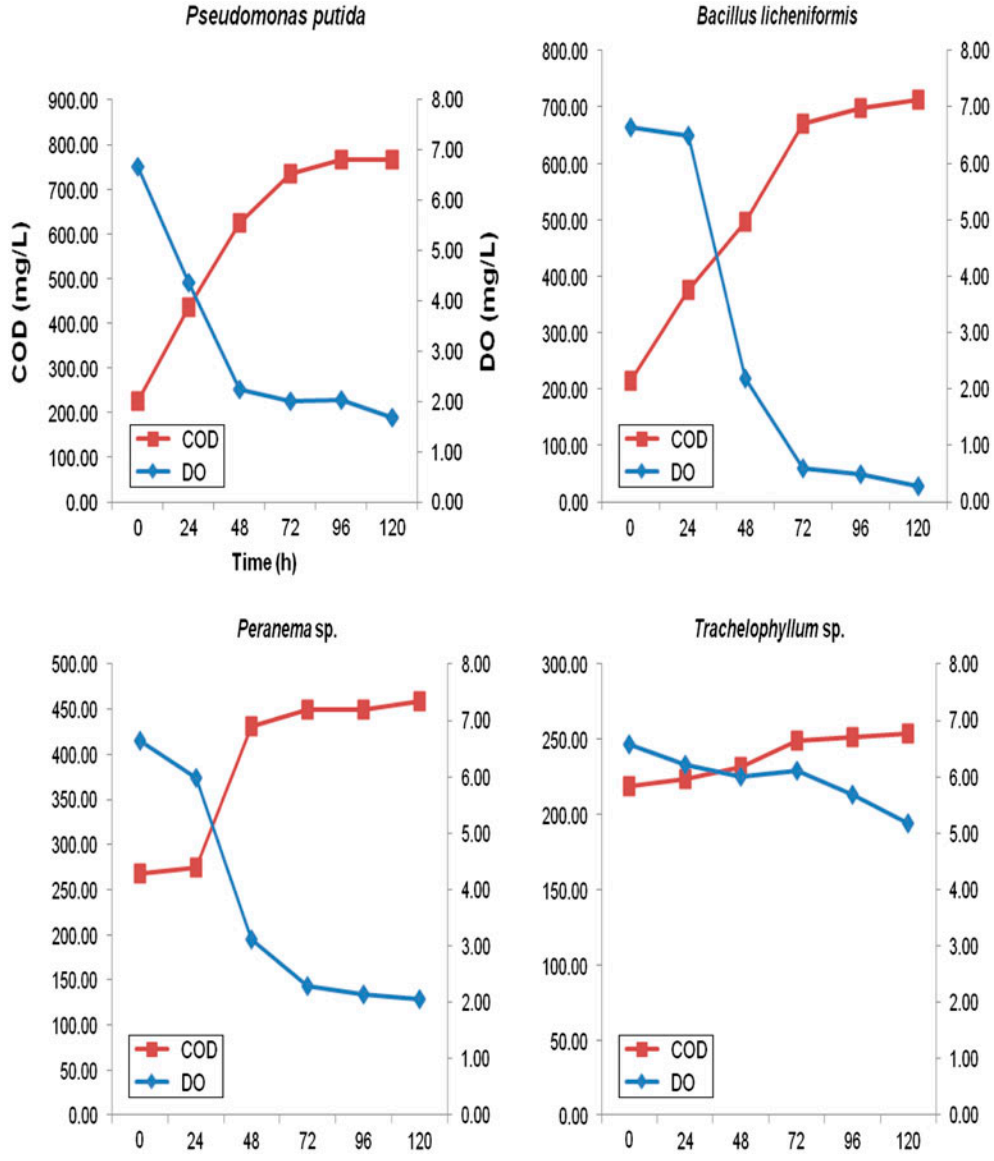

Fig. 6. Variation of DO and COD in the media inoculated with test organisms.

modified mixed liquor co-inoculated with all bacterial isolates (57.95%-Ni^{2+}, 32.64-V^{5+}) and also in those with all protozoan isolates (52.01%-Ni^{2+}, 27.08%-V^{5+}).

4. Discussion

In recent years, increasing concern has been expressed regarding the disposal of heavy metals into the environment, especially in water sources [42]. This is an alarming situation in most of the third-world countries [28,43]. In the environment, heavy metals co-occur mostly in mixture form; this phenomenon might have synergistic or antagonistic reactions to their toxicity, which render their toxicity unpredictable. Due to the co-occurrence of V^{5+} and Ni^{2+} in nature, their ability to complex and the uncertainty in V^{5+} toxicity, this study investigated the combined effects of V^{5+} and/or Ni^{2+} on selected bacterial and protozoan isolates in the wastewater system. Since it has been reported that heavy metal-resistant genes can reduce or eliminate the toxicity of heavy metals [44], prior to assessing the combined effects of Ni^{2+} and V^{5+}, the present study firstly screened the presence/absence of both *nccAC1* and *van2* genes encoding the resistance of Ni^{2+} and V^{5+}, respectively. Secondly, the single and combined effects of V^{5+} and Ni^{2+} on these four metal-resistant isolates were also assessed.

The present study revealed that microbial isolates, bacterial as well as protozoan, except *Trachelophyllum* sp., appeared to be tolerant of both Ni^{2+} and V^{5+}, separately (Figs. 2 and 3) though Ni^{2+} revealed to be more toxic than V^{5+} and also had more shock-load effect than the later test metal. Furthermore, the microbial isolates also showed high percentage removal of both test metals (Table 4). The findings of this study also revealed that the high resistance and metal removal ability observed from *P. putida* and *Peranema* sp. to Ni^{2+} and V^{5+}, separately, could be explained by the presence of *nccAC1* and *van2* genes (Fig. 1) encoding the resistance to both Ni^{2+} and V^{5+} toxicity, respectively. However, the high growth response observed with *B. licheniformis* when exposed to both Ni^{2+} and V^{5+}, separately, and the absence of the *van2* gene in its gDNA, indicated that this isolate has an alternative resistance mechanism for V^{5+}. In addition, the sensitivity of *Trachelophyllum* sp. to both Ni^{2+} and $V^{5+,}$ when compared to other test isolates, was revealed to be due to the absence of *van2* and *nccAC1* genes. This could also be due to the absence of cell wall (which is present in bacterial cell) during the vegetative stage as reported by Martin-Gonzalez and co workers [25]. Compared with their growth curves in a free-metal media, as reported by Kamika and Momba [30], the single effects of Ni^{2+}/V^{5+} to acclimatised protozoan isolates as well as bacterial isolates were found not to be significant. Furthermore, the study revealed that the toxic effects of the two metals depend on the species/group of organisms. This is in agreement with the findings of Draggan and Giddings [45] who reported that the chemical effects can be acute or chronic and observed in individual organisms, populations of organisms, or in total ecosystems. As an operon, which contains a cluster of genes controlling the resistance of Ni^{2+}, Co^{2+} and Cd^{2+}, *nccAC1* has been previously reported as being present in several bacteria such as *Bacillus subtilis, Bacillus cereus, staphylococcus aureus, Ralstonia metallidurans* CH34, *Microbacterium arabinogalactanolyticum*, etcetera [26,46]. However, the *van2* gene was firstly reported to be present in eukaryotic cells, *saccharomyces cerevisiae* [38]. In addition, reports revealed that *P. putida* contains several heavy metal-resistant mechanisms [28,47,48]. Among the six possibly well-known, metal-resistant mechanisms developed by microorganisms, at least three, namely bioaccumulation, active export and bio-sorption, have been reported to be found in protozoan isolates [25]. However, no literature to date has reported the presence of *nccAC1* and *van2* genes in *Peranema* sp. and *Trachelophyllum* sp.

When assessing the combined effects of the two metals on the test isolates as an individual or a group (bacteria or protozoa), the results indicated an increase in toxicity (more than the sum of the single effects of V^{5+} and Ni^{2+}), which might be related to the synergistic actions of Ni^{2+} to V^{5+} or V^{5+} to Ni^{2+} (Figs. 4 and 5). Which was also observed in the metal-removal ability of the test isolates (Table 4). However, the synergistic effects of these metals decreased when all bacterial isolates were co-inoculated in the mixed liquor culture media (Fig. 5). The same result was observed for protozoan isolates when co-inoculated in the same culture media. This could be explained by the microbial diversity that might be facilitating the resistant isolates to fastly grow and overcome the toxic effects of V^{5+} and Ni^{2+}. Although no litterature was found on the synergistic effects of V^{5+} and Ni^{2+} to microbial species in wastewater systems, this study corroborates an epidemiological study of Campen et al. [49] that assessed the potential interaction between nickel and vanadium sulfate. The said authors pointed out that there is a possible synergistic relationship between inhaled Ni^{2+} and V^{5+} in male Sprague-Dawley rats. Gadd and Griffiths [50] reported that the toxicity of a metal might increase when other ions are present. This was confirmed by Cross et al. [51] who, when working on the effects of nickel and cobalt chlorides on some foetal rat cells, found that the mixtures of $NiCl_2$ and $CoCl_2$ induced a synergistic toxic response in cell culture. Herkovits et al. [52] reported that the effect of nickel–zinc interactions in amphibian embryos (*Bufo arenarum*) were dependent on the zinc (Zn) concentration, which ranged from no effect, on Ni^{2+} toxicity to synergistic effects. These authors also pointed out that when mixing Ni^{2+} with high concentrations of Zn (30 mg/L), instead of being toxic, the results revealed a beneficial effect on amphibian embryos. Khangarot and Ray [53] also observed that chromium and Ni^{2+} mixtures had synergistic effects on *Poecilia reticulata*.

It has been reported that heavy metal toxicity in activated sludge depends mainly on speciation, chemical properties and concentrations. However, other factors such as pH, microbial concentration and temperature have been reported to also affect the toxicity of metals [54]. According to Roane and Pepper [4], pH has an appreciable effect on metal solubility and hence on metal bioavailability in the solution. These authors also reported that, at a high pH, some metals are predominantly found in the form of insoluble mineral phosphates and carbonates. To the contrary, at a low pH, they are present in ionic forms and are free to exert their toxicity on microbial cells. Abel [55] revealed that changes in pH could have an adverse effect on the toxicity of the metals and on the ability of microorganisms to develop resistance

mechanisms. The present results revealed that pH, temperature and microbial concentrations had significant adverse effects on the synergistic toxicity of V^{5+} and Ni^{2+} to the test isolates (Tables 2 and 3). In addition, the results indicated that the effect of pH variations and metal toxicity varied between bacterial and protozoan species. An increase in pH resulted in a decrease in the synergistic effect of the test metals on all test isolates, except *B. licheniformis*. This results support the findings by Kamika and Momba [30] who reported a decrease in Ni^{2+} toxicity on bacteria isolates by an increase in pH. Babich and Stotzky [56], when investigating the toxicity of Ni^{2+} to microorganisms in soil, reported that the toxicity of Ni^{2+} was raised in acidic rather than in alkaline medium. Sandrin and Maier [57] stated that increasing the pH reduces the toxicity of Ni^{2+} in bacteria. However, these results disagreed with those of van Nostrand et al. [58] who found that by increasing the pH, the growth of *Burkholderia cepacia* PR1 was inhibited. Similar behaviour was observed with an increase and decrease in temperature regarding the synergistic effects of the two metals during the present study and concurred with Kamika and Momba [30,35]. In contrast, Khan et al. [59] reported a threefold increase in the inhibitory effect of copper on juvenile crayfish Orconectes immunis (Hagen) when the temperature (20˚C) increased by 3–4˚C.

The results of this study showed an increase in COD and a decrease in DO concentrations in mixed liquors inoculated with test isolates. Statistically, a significant negative correlation was recorded between COD and DO in mixed liquors inoculated with bacterial isolates ($r = -0.935$, $p < 0.05$) and a weak negative correlation in the mixed liquor inoculated with protozoan isolates ($r = -0.351$, $p < 0.05$), except for *Peranema* sp., which revealed a significant negative correlation ($r = -0.734$, $p < 0.05$). Kelly et al. [60] reported that an oxygen uptake rate of activated sludge decreases when the wastewater contains toxicants. Akpor et al. [34] also reported a strong negative correlation ($r = -0.602$, $p < 0.05$) between COD and DO in the mixed liquor inoculated with protozoan species. Recently, Kamika and Momba [30,35] recorded a negative correlation between COD and DO in activated sludge mixed liquor.

5. Conclusion

Pollution caused by heavy metals is a global issue. Assessing their toxicity in an aquatic environment is an additional concern due to the co-occurrence in the form of a mixture. In this study, the combined effects of V^{5+} and Ni^{2+} on bacterial and protozoan isolates in wastewater mixed liquors were investigated. This study was able to demonstrate the resistance ability of *P. putida*, *B. licheniformis* and *Peranema* sp. due to the presence of both V^{5+} and Ni^{2+} resistance genes found in their DNA, except for *B. licheniformis*, which did not have the *van2* gene. This was confirmed by their ability to overcome Ni^{2+} and V^{5+} toxicity and also by their removal ability when exposed to the test metals separately. In addition, the significant shock-load effects on microbial isolates observed by the continuous increase in Ni^{2+} concentration revealed that the consistent variation of heavy metal concentrations is a major concern for the effectiveness of wastewater treatment process. The sensitivity of *Trachelophyllum* sp. to the test metals was demonstrated to be due to the lack of both genes (*van2* and *nccAC1*). Beside the presence of V^{5+} and Ni^{2+} resistance genes, when microbial isolates were exposed to combined V^{5+} and Ni^{2+}, the study revealed that these metals can synergistically react by increasing their toxic effect to more than the sum of the single effects of V^{5+} and Ni^{2+}. Unlike the acclimatisation, the microbial species' diversity and concentration in the culture media were also found to enhance their ability to resist the toxic and synergistic effects of V^{5+} and Ni^{2+} and also remove the test metals in wastewater. In addition, the synergistic effect also demonstarted that it was a function of pH and temperature. This study suggests that, in order to mimic the environmental conditions and evaluate the real toxicity of metals in the aquatic environment, further research on heavy metals in mixture forms is needed. Such an approach would provide an efficient and accurate assessment of the toxic effect of heavy metals in the aquatic environments.

References

[1] K.G. Malle, Zink in der Umwelt [Zinc in the environment], Acta Hydrochim. Hydrobiol. 20 (1992) 196–204.
[2] T.M. Mighall, J.P. Grattan, S. Timberlake, J.A. Lees, S Forsyth, An atmospheric pollution history for lead-zinc mining from the Ystwyth Valley, Dyfed, Mid-Wales, UK, as recorded by an upland peat, Environment 2 (2002) 175–184.
[3] D.H. Nies, Microbial heavy-metal resistance, Appl. Microbiol. Biotechnol. 51 (1999) 730–750.
[4] T.M. Roane, I.L. Peper, Microorganisms and metal pollutants, in: R.M. Maier, I.L. Pepper, C.P. Gerba (Eds.), Environmental Microbiology, Academic Press, San Diego, 2000, pp. 403–423.
[5] P. Gikas, 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 (2008) 187–203.
[6] J.H. Duffus, Heavy metals—A meaningless term? International Union of Pure Applied Chemistry 74 (2002) 793–807.
[7] R.M. Maier, I.L. Pepper, C.P. Gerba, Environmental Microbiology, second ed., Academic Press, New York, NY, 2008.
[8] ATSDR (Agency for Toxic Substances and Disease Registry), Draft of toxicological profile for nickel. US Department of Health and Human Services Public Health Service, Atlanta, GA, 2005.

[9] M. Cempel, G. Nikel, Nickel: A review of its sources and environmental toxicology, Polish J. Environ. Stud. 15 (2006) 375–382.
[10] I.C. Farcasanu, C. Paraschivescu, L. Ruta, E. Oprea, S. Avramescu, Manipulation of Ni^{2+} tolerance of *Sacchromyces cerevisiae* cells: A primary step to bioremediation by removal and recovery of Ni^{2+} from contaminated waters, Rev. Roum. Chim. 53 (2008) 647–651.
[11] A.F. Rehman, R. Shakoori, A.R. Shakoori, Resistance and uptake of heavy metals by *Vorticella microstoma* and its potential use in industrial wastewater treatment, Environ. Prog. Sustain. Energy 29 (2010) 481–486.
[12] IARC (International Agency for Cancer Research), Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Chromium, Nickel and Welding, 49, IARC Press, Lyon, 1990.
[13] A. Hartwig, T. Schwerdtle, Interactions by carcinogenic metal compounds with DNA repair processes: Toxicological implications, Toxicol. Lett. 127 (2002) 47–54.
[14] ATSDR (Agency for Toxic Substances and Disease Registry), Draft of toxicological profile for vanadium and compounds. US Department of Health and Human Services, Public Health Service, Atlanta, GA, 2009.
[15] I. Mannazzu, Vanadium detoxification and resistance in yeast: A mini-review, Ann. Microbiol. 51 (2001) 1–9.
[16] M. Hu, P.P. Coetzee, Vanadium speciation by chromatographic separation of V(IV) and V(V) in acidic solution followed by ICP-OES determination, Water SA. 33 (2007) 291–296.
[17] T.I. Fortoul, A. Quan-Torres, I. Sanchez, I.E. Lopez, P. Bizarro, M.L. Mendoza, L.S. Osorio, G. Espejel-Maya, C. Avila-Casado Mdel, M.R. Avila-Costa, L. Colin-Barenque, D.N. Villanueva, G. Olaiz-Fernandez, Vanadium in ambient air: Concentrations in lung tissue from autopsies of Mexico City residents in the, and 1990s, Arch. Environ. Health 57(2002) (1960) 446–449.
[18] B.V. Venkataraman, S. Sudha, Vanadium toxicity, Asian J. Exp. Sci. 19 (2005) 127–134.
[19] M.A. Amezcua-Allieri, L. Salazar-Coria, Nickel and vanadium concentrations and its relation with sediment acute toxicity, Bull. Environ. Contam. Toxicol. 80 (2008) 555–560.
[20] C.D. Klaassen, Casarett & Doull's Toxicology: The Basic Science of Poison, seventh ed., McGraw Hill Medical, New York, NY, 2008.
[21] M.D. Lewan, Factors controlling the proportionality of vanadium to nickel in crude oils, Geochim. Cosmochim. Acta 48 (1984) 2231–2238.
[22] A.J.G. Barwise, Role of nickel and vanadium in petroleum classification, Energy Fuels 4 (1990) 647–652.
[23] G. Caumette, C.-P. Lienemann, I. Merdrignac, B. Bouyssiere, R. Lobinski, Element speciation analysis of petroleum and related materials, J. Anal. At. Spectrom. 24 (2009) 263–276.
[24] D.H. Nies, Efflux mediated heavy metal resistance in prokaryotes, FEMS Microbiol. Rev. 27 (2003) 313–339.
[25] J.C. Gutiérrez, F. Amaro, A. Martin-Gonzalez, From heavy metal-binders to biosensors: Ciliate metallothioneins discussed, BioEssays 31 (2009) 805–816.
[26] L.M. Abdelatey, W.K.B. Khalil, T.H. Ali, K.F. Mahrous, Heavy metal resistance and gene expression analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Egyptian soils, J. Appl. Sci. Environ. Sanitation 6 (2011) 201–211.
[27] S. Murthy, G. Bali, S.K. Sarangi, Effect of lead on metallothionein concentration in lead-resistant bacteria *Bacillus cereus* isolated from industrial effluent, Afr. J. Biotechnol. 10 (2011) 15966–15972.
[28] I. Kamika, M.N.B. Momba, Assessing the resistance and bioremediation ability of selected bacterial and protozoan species to heavy metals in metal-rich industrial wastewater, BMC Microbiol. 13 (2013). 28doi: 10.1186/1471-2180-13-28.
[29] B. Muneer, A. Rehman, F.R. Shakoori, A.R. Shakoori, Evaluation of consortion of microorganisms for efficient removal of hexavalent chromium from industrial wastewater, Bull. Environ. Contam. Toxicol. 82 (2009) 597–600.
[30] I. Kamika, M.N.B. Momba, Comparing the tolerance limits of selected bacterial and protozoan species to nickel in wastewater systems, Sci. Total Environ. 410 (2011) 172–181.
[31] C. Clausen, Isolating metal-tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood, Waste Manage. Res. 18 (2000) 264–268.
[32] C.D. Emptage, R.J. Knox, M.J. Danson, D.W. Hough, Nitroreductase from *Bacillus licheniformis*: A stable enzyme for prodrug activation, Biochem. Pharmacol. 77 (2009) 21–29.
[33] APHA, Standard Methods for the Examination of Water and Wastewater, twentieth ed., American Public Health Association (APHA), Washington DC, 2001.
[34] O.B. Akpor, M.N.B. Momba, J.O. Okonkwo, The effects of pH and temperature on phosphate and nitrate uptake by wastewater protozoa, Afr. J. Biotechnol. 7 (2008) 2221–2226.
[35] I. Kamika, M.N.B. Momba, Comparing the tolerance limits of selected bacterial and protozoan species to vanadium in wastewater systems, Water Air Soil Pollut. 223 (2012) 2525–2539.
[36] Y. Ozutsumi, K. Tajima, A. Takenaka, H. Itabashi, The effect of protozoa on the composition of rumen bacteria in cattle using 16S rRNA gene clone libraries, Biosci. Biotechnol. Biochem. 69 (2005) 499–506.
[37] E. Shoeb, Genetic basis of heavy metal tolerance in bacteria [PhD diss.] University of Karachi; 2006.
[38] C. Kanik-Ennulat, E. Montalvo, N. Neff, Sodium orthovanadate-resistant mutants of *Saccharomyces cerevisiae* show defects in Golgi-mediated protein glycosylation, sporulation and detergent resistance, Genetics 140 (1995) 933–943.
[39] M.N.B. Momba, T.E. Cloete, Biomass relationship to growth and phosphate uptake of *Pseudomonas fluorescens*, *Escherichia coli* and Acinetobacter radioresistens in mixed liquor medium, J. Ind. Microbiol. 16 (1996) 364–396.
[40] C. Farrier-Pagès, F.N. Rassoulzadegan, Mineralization in planktonic protozoa, Limnol. Oceanogr. 39 (1994) 411–419.
[41] X. Peng, T. Murphy, N.M. Holden, Evaluation of the effect of temperature on the die-off rate for *Cryptosporidium parvum* oocycts in water, soils, and feces, Appl. Environ. Microbiol. 74 (2008) 7101–7107.
[42] C.P. Gerba, Domestic waste and waste treatment, in: R.M. Maier, I.L. Pepper, C.P. Gerba (Eds.), Environmental Microbiology, Academic Press, San Diego, CA, 2000, pp. 505–534.
[43] M.A. Senzia, D.A. Mashauri, A.W. Mayo, Suitability of constructed wetlands and waste stabilization ponds in wastewater treatment: Nitrogen transformation and removal, Phys. Chem. Earth 28 (2003) 20–27.
[44] G. Wei, L. Fan, W. Zhu, Y. Fu, J. Yu, M. Tang, Isolation and characterization of the heavy metal resistant bacteria CCNWRS33-2 isolated from root nodules of *Lespedeza cuneata* in gold mine tailings in China, J. Hazard. Mater. 162 (2009) 50–56.
[45] S. Draggan, J.M. Giddings, Testing toxic substances for protection of the environment, Sci. Total Environ. 9 (1978) 63–74.
[46] R.A.I. Abou-Shanab, P. van Berkum, J.S. Angle, Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*, Chemosphere 68 (2007) 360–367.
[47] D. Canovas, I. Casses, V. de Lorenzo, Heavy metal tolerance and metal homeotasis in *Pseudomonas putida* revealed by complete genome analysis, Environ. Microbiol. 5 (2003) 1242–1256.
[48] N. Hu, B. Zhao, Key genes involved in heavy-metal resistance in *Pseudomonas putida* CD2, FEMS Microbiol. Lett. 267 (2006) 17–22.
[49] M.J. Campen, J.P. Nolan, M.C.J. Schladweiler, U.P. Kodavanti, P.A. Evansky, D.L. Costa, W.P. Watkinson, Cardiovascular and thermoregulatory effects of inhaled PM-associated transition metals: A potential interaction between nickel and vanadium sulfate, Toxicol. Sci. 64 (2001) 243–252.
[50] G.M. Gadd, A.J. Griffiths, Microorganisms and heavy metal toxicity, Microb. Ecol. 4 (1978) 303–317.

[51] D.P. Cross, G. Ramachandran, E.V. Wattenberg, Mixtures of nickel and cobalts chlorides induce synergistic cytotoxic effects: Implications for inhalation exposure modelling, Ann. Occup. Hyg. 45 (2001) 409–418.
[52] J. Herkovits, C.S. Perez-Coll, F.D. Herkovits, Evaluation of nickel-zinc interactions by means of bioassays with amphibian embryos, Ecotoxicol. Environ. Saf. 45 (2000) 266–273.
[53] B.S. Khangarot, P.K. Ray, Acute toxicity and toxic interaction of chromium and nickel to common guppy *Poecilia reticulata* (Peters), Bull. Environ. Contam. Toxicol. 44 (1990) 832–839.
[54] M. Sa'idi, Experimental studies on effect of heavy metals presence in industrial wastewater on biological treatment, Int. J. Environ. Sci. 1(2010) 666–676.
[55] P.B. Abel, Water Pollution Biology, second ed., Taylor & Francis, London, 1996.
[56] H. Babich, G. Stotzky, Toxicity of nickel to microorganisms in soil: Influence of some physicochemical characteristics, Environ. Pollut. Ser. A. 29 (1982) 303–315.
[57] T.R. Sandrin, R.M. Mair, Impact of metals on the biodegradation of organic pollutants, Environ. Health Perspect. 111 (2003) 1093–1101.
[58] J.D. van Nostrand, A.G. Sowder, P.M. Bertsch, P.J. Morris, Effect of pH on the toxicity of nickel and other divalent metals to *Burkholderia cepacia* $PR1_{310}$, Environ. Toxicol. Chem. 24 (2005) 2742–2750.
[59] M.A. Khan, S.A. Ahmed, B. Catalin, A. Khodadoust, O. Ajayi, M. Vaughn, Effect of temperature on heavy metal toxicity to juvenile crayfish, *Orconectes immunis* (Hagen), Envviron. Toxicol. 21 (2006) 513–520.
[60] C.J. Kelly, N. Tumsaroj, C.A. Lajoie, Assessing wastewater metal toxicity with bacterial bioluminescence in a bench-scale wastewater treatment system, Water Res. 38 (2004) 423–431.