Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2020.25843

194 (2020) 172–179
August

Isolation of *Acinetobacter* sp. strain WKDN with capacity for aerobic denitrification and $CaCO_3$ biomineralization and its potential application in dissolved Zn removal

Masataka Aoki[a,*,†], Takuya Noma[a,b,†], Nobuo Araki[c], Takashi Yamaguchi[b,d], Masataka Kusube[e], Kazuyuki Hayashi[a]

[a]*Department of Civil Engineering, National Institute of Technology, Wakayama College, 77 Nada, Noshima, Gobo, Wakayama 644-0023, Japan, Tel. +81-738-29-8445; Fax: +81-738-29-8469; emails: aoki@wakayama-nct.ac.jp (M. Aoki), s195038@stn.nagaokaut.ac.jp (T. Noma), k-hayashi@wakayama-nct.ac.jp (K. Hayashi)*
[b]*Department of Civil and Environmental Engineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan, email: ecoya@vos.nagaokaut.ac.jp (T. Yamaguchi)*
[c]*Department of Civil Engineering, National Institute of Technology, Nagaoka College, 888 Nishikatakai, Nagaoka, Niigata 940-8532, Japan, email: araki@nagaoka-ct.ac.jp (N. Araki)*
[d]*Department of Science of Technology Innovation, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan*
[e]*Department of Applied Chemistry and Biochemistry, National Institute of Technology, Wakayama College, 77 Nada, Noshima, Gobo, Wakayama 644-0023, Japan, email: kusube@wakayama-nct.ac.jp (M. Kusube)*

Received 27 September 2019; Accepted 16 March 2020

ABSTRACT

Geologic and anthropogenic activities have led to increased heavy metal contamination in soils and groundwater. This paper describes a newly isolated aerobic denitrifying bacterial strain, WKDN, with the capacity for calcium carbonate ($CaCO_3$) biomineralization, and evaluates its potential use in dissolved zinc (Zn) removal. Our 16S rRNA gene-based phylogenetic analysis assigned the newly isolated strain to the *Acinetobacter* genus. An *in vitro* experiment indicated that the g acetate–carbon/g nitrate–nitrogen ratio of a biomineralization medium affects WKDN's nitrate–nitrogen removal and $CaCO_3$ biomineralization efficiencies. An X-ray diffraction analysis indicated that calcite-type $CaCO_3$ was the dominant component of precipitates produced *in vitro* by WKDN. Sand columns treated with WKDN and a biomineralization medium effectively removed dissolved Zn from an aqueous zinc chloride solution. The results presented in this study indicate that the bacterial strain WKDN has good application prospects in *in situ* dissolved Zn removal from groundwater.

Keywords: Acinetobacter; Aerobic denitrification; Biomineralization; Calcium carbonate; Zinc removal

1. Introduction

Heavy metal-contaminated soils and groundwater have become a common problem worldwide due to the interaction between geologic and anthropogenic activities [1]. Zinc (Zn), a heavy metal, is an indispensable trace element for living organisms [2]. It is also used for galvanization and in the production of brass, bronze, and zinc-based alloys [3]. However, excessive amounts of Zn have been found to be toxic [4–6]. Therefore, various physical, chemical, and biological methods have been developed for the remediation and treatment of heavy metal-contaminated soils and

* Corresponding author.

† These authors have contributed equally to this work.

groundwater [7]. However, major problems associated with physical and chemical methods include the high cost of complete remediation and the considerable risk of releasing toxic heavy metals to other environments.

In nature, a variety of microbial reactions, including urea hydrolysis, photosynthesis, denitrification, ammonification, sulfate reduction, and methane oxidation, induce calcium carbonate ($CaCO_3$) precipitation [8]. $CaCO_3$ can adsorb heavy metal ions, including Zn, and its potential adsorption mechanisms include ion-exchange and precipitation on the surface of carbonate minerals [9–11]. Therefore, the *in situ* removal of heavy metal ions from groundwater originating from contaminated sites could be made performed using a permeable reactive barrier (i.e., a permeable treatment wall [12]) composed of *in situ* biomineralized $CaCO_3$. Large-scale excavation and transportation of heavy metal-contaminated soils and groundwater would not be required to install biomineralized $CaCO_3$-based permeable reactive barriers, only small-scale excavation is required to create a well for the injection of nutrients and microbes that could precipitate $CaCO_3$. Thus, the risk of releasing toxic heavy metals to other environments is low.

Urea hydrolysis-driven $CaCO_3$ biomineralization has been extensively investigated for soil improvement and the treatment of heavy metal-contaminated wastewater [13,14]. However, urea hydrolysis generates ammonia, which has an unpleasant odor and is detrimental to human health [14]. Denitrification, a microbial process that involves the reduction of nitrate (NO_3^-) to molecular nitrogen (N_2) via intermediates (NO_3^- → nitrite [NO_2^-] → nitric oxide [NO] → nitrous oxide [N_2O] → N_2), occurs under aerobic and anoxic conditions [15,16]. The occurrence of H^+-consuming denitrification increases the pH of the surrounding environment and produces CO_2, which favors carbonate biomineralization [8]. When NO_3^- is efficiently reduced to gaseous nitrogen compounds through denitrification, toxic compounds do not accumulate. Some researchers have studied denitrification-driven $CaCO_3$ biomineralization for soil improvement [17–22]. The installation of a biomineralized $CaCO_3$-based permeable reactive barrier might be a cost-effective method of treating heavy metal-contaminated soils and groundwater when industrial waste or wastewater are used as the substrate for denitrification-driven $CaCO_3$ biomineralization [18].

Aerobic denitrification is a microbial process that reduces NO_3^- into gaseous nitrogen compounds in the presence of dissolved oxygen (DO) [16]. Compared to anaerobic denitrifying microorganisms that require oxygen-limited conditions for effective denitrification, aerobic denitrifying microorganisms may efficiently produce $CaCO_3$ in subsurface environments where molecular oxygen (O_2) is present [23]. However, to our best knowledge, *in situ* heavy metal-contaminated soil-/groundwater-treatment techniques utilizing aerobic denitrification-driven $CaCO_3$ biomineralization have not been reported.

In this paper, we report a newly isolated bacterial strain, WKDN, with the capacity for denitrification and $CaCO_3$ biomineralization under aerobic conditions. Further, we evaluate the dissolved Zn removal potential of a biomineralized $CaCO_3$-based permeable reactive barrier prepared using WKDN and a biomineralization medium.

2. Materials and methods

2.1. Cultivation media used in this study

The composition of the medium used to enrich the denitrifying microbial community was as follows (per L): 3 g of CH_3COONa, 2 g of KNO_3, 0.2 g of $MgSO_4{\cdot}7H_2O$, 1 g of K_2HPO_4, 0.1 g of $CaCl_2{\cdot}2H_2O$, 0.5 g of NH_4Cl, and 1 mL of trace element solution SL-10 [24]. The composition of the bromothymol blue (BTB) agar plate used to isolate strain WKDN was as follows (per L): 3 g of CH_3COONa, 2 g of KNO_3, 0.2 g of $MgSO_4{\cdot}7H_2O$, 1 g of K_2HPO_4, 0.1 g of $CaCl_2{\cdot}2H_2O$, 0.5 g of NH_4Cl, 1 mL of trace element solution SL-10, 50 mg of BTB, and 15 g of agar. The composition of the biomineralization medium was as follows (per L): variable amount of $Ca(CH_3COO)_2$, 1.26 g of $Ca(NO_3)_2{\cdot}4H_2O$, 0.002 g of $MgSO_4{\cdot}7H_2O$, 0.01 g of K_2HPO_4, 0.005 g of NH_4Cl, and 1 mL of trace element solution SL-10. Bacto nutrient broth (3 g/L beef extract, 5 g/L peptone, and 5 g/L NaCl, Becton Dickinson and Company, Sparks, MD, USA) was used to cultivate WKDN following isolation. All medium components were sterilized by autoclaving (121°C, 15 min) or filtration through a 0.22 μm pore size filter. The pH of the media was adjusted to 7.0 using hydrochloric acid or sodium hydroxide solutions prior to sterilization.

2.2. Isolation procedures of WKDN

Soil samples (5 g each) were collected from five distinct locations at the National Institute of Technology at Wakayama College (Gobo, Wakayama, Japan) and mixed for use as an inoculum to enrich a denitrifying microbial soil community. This inoculum was added to 100 mL of the enrichment medium in a 200 mL Erlenmeyer flask. The flask was then incubated for 2 d with constant agitation at 90 rotations per minute (rpm). Subsequently, 1 mL of the enrichment culture was transferred to 99 mL of the freshly prepared enrichment medium in a 200 mL Erlenmeyer flask and incubated for 2 d with constant agitation at 90 rpm, this procedure was repeated twice. The resultant suspension was streaked onto the BTB agar plate, which was then incubated. Based on the relatively rapid color change around the colonies on the BTB agar plate, strain WKDN, which has a high denitrification potential, was selected and isolated by selecting a colony formed on the agar medium. Colony purification was conducted through repeated streaking on a BTB agar plate. All incubation was conducted in the dark under an aerobic atmosphere at 25°C.

2.3. 16S rRNA gene-based phylogenetic analysis

The total genomic deoxyribonucleic acid (DNA) of strain WKDN was extracted following the procedure of Hiraishi [25]. The almost-full length of the bacterial 16S ribosomal ribonucleic acid (16S rRNA) gene of WKDN was amplified by a polymerase chain reaction (PCR) using the extracted genomic DNA, universal bacterial primer set B27F/U1492R (27F: 5′-AGA GTT TGA TCM TGG CTC AG-3′, U1492R: 5′-GGH TAC CTT GTT ACG ACT T-3′), and TaKaRa Ex Taq version 2.0 (TaKaRa Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions. The resulting PCR product was purified using a Micro-Elute DNA Clean/

Extraction Kit (GMbiolab Co., Ltd., Taichung, Taiwan), and the purified product was then sequenced by Macrogen Japan Corp. (Kyoto, Japan). A 16S rRNA gene-based phylogenetic tree was constructed following the neighbor-joining method with the Jukes and Cantor correction, as implemented in ARB [26] version 6.0.4. Bootstrap analyses were conducted for 1,000 replicates to estimate the confidence levels of the tree topologies. The 16S rRNA gene sequence was deposited in the DDBJ/EMBL/GenBank databases under accession number LC466957.

2.4. In vitro denitrification and biomineralization experiments

All WKDN cultures used for *in vitro* denitrification and biomineralization experiments were initially cultivated overnight in bacto nutrient broth at 25°C. Overnight cultures of WKDN (optical density at 600 nm [OD_{600}] = 0.3 = ~2 × 10^7 colony-forming units [CFU]/mL) were inoculated to 80 or 400 mL of the biomineralization medium containing variable amounts of $Ca(CH_3COO)_2$ and 1.26 g of $Ca(NO_3)_2{\cdot}4H_2O$ (inoculum concentration: 1% [vol./vol.]). The flasks were then incubated in the dark under an aerobic atmosphere at 25°C with constant agitation at 90 rpm. Moderately aerated conditions were created with a low flask-to-medium ratio of 5:4 [27,28]. An abiotic control experiment (using the biomineralization medium without WKDN inoculation) was also conducted to assess whether the occurrence of denitrification and calcium ion (Ca^{2+}) precipitation were attributable to the strain. The nitrate–nitrogen (NO_3^-–N) removal efficiency (%) was calculated as the percent of NO_3^-–N removed in relation to the starting concentration. The precipitated Ca^{2+} concentration reported in this study was calculated based on changes in the Ca^{2+} concentration in the biomineralization medium.

The precipitates subjected to X-ray diffraction (XRD) analysis were prepared using 400 mL of the biomineralization medium with WKDN (OD_{600} = 0.3, inoculum size: 1% [vol./vol.]). After 14 d of *in vitro* incubation in the dark under an aerobic atmosphere at 25°C with constant agitation at 90 rpm, the precipitates produced in the biomineralization medium were collected by filtration using Whatman filter paper No. 1 (GE Healthcare UK Ltd., Buckinghamshire, UK) and washed with distilled-deionized water. The filter with the precipitates were then dried at 105°C for 24 h for XRD analysis.

2.5. Sand column experiment

The sand columns (diameter: 3.6 cm, height: 7.2 cm) used to evaluate the dissolved Zn removal potential of the biomineralized $CaCO_3$-based permeable reactive barrier were prepared using Toyoura sand (soil particle density: 2.64 g/cm^3, maximum void ratio [void ratio in the loosest state]: 0.978, minimum void ratio [void ratio in the densest state]: 0.605) in 100 mL plastic syringes (inside diameter: 3.6 cm) with a porous plastic filter (diameter: 3.6 cm, thickness: 2.0 mm, pore size: 30 μm; WINTEC, Niigata, Japan) (Fig. 1). The sand columns were prepared following the water pluviation method [29]. The initial relative density [30] and void volume of the sand columns were 70% and 31 mL, respectively. The sand columns were sterilized under a germicidal

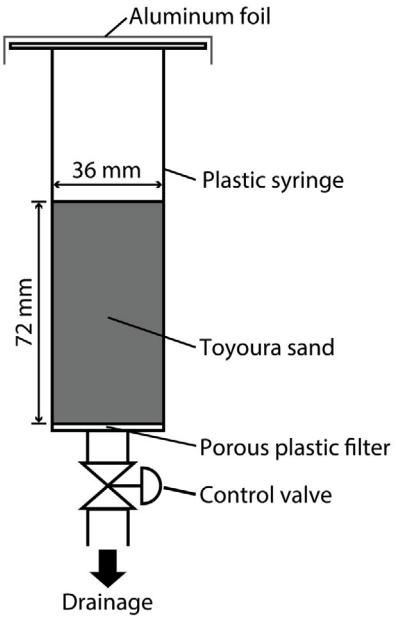

Fig. 1. Schematic of the sand column used in this study.

ultraviolet light lamp (wavelength: 254 nm) in a biosafety cabinet for 2 h. Following sterilization, 46 mL of the live or autoclave (121°C, 15 min)-killed WKDN cells (OD_{600} = 0.3, prepared by overnight cultivation in bacto nutrient broth) was supplied to the sand columns, which were then incubated for 6 h for biofilm formation. Subsequently, 31 mL of the biomineralization medium (g acetate–carbon [acetate–C]/g NO_3^-–N ratio: 6) was supplied to the sand columns, and they were incubated for another 7 d for biomineralization. Following treatment with the biomineralization medium, 310 mL of distilled-deionized water was supplied to the sand columns to wash away any remaining biomineralization medium. To evaluate the dissolved Zn removal potential of the sand columns, 31 mL of aqueous zinc chloride ($ZnCl_2$) solution (298 mg dissolved Zn/L, pH 6.8) was supplied to the sand columns. After 24 h of incubation, 31 mL of the aqueous $ZnCl_2$ solution was again supplied to the sand columns, and the drained liquid was collected to measure the dissolved Zn and Ca^{2+} concentrations. All incubation was conducted in the dark under an aerobic atmosphere at 25°C. In all procedures, the solutions were supplied from the top of the sand columns, and the drainage from the lower part of the sand columns was immediately stopped by a control valve after the complete permeation of the solutions into the sand columns. To prevent microbial contamination from the air, the sand columns were covered with aluminum foil during incubation. The dissolved Zn removal efficiency (%) was calculated using Eq. (1):

$$\text{Removal efficiency}(\%) = \frac{C_{in} - C_{out}}{C_{in}} \times 100(\%) \quad (1)$$

where C_{in} is the dissolved Zn concentration in the aqueous $ZnCl_2$ solution supplied to the sand columns, and C_{out} is the dissolved Zn concentration of the drainage obtained

after the second addition of the aqueous $ZnCl_2$ solution (mg dissolved Zn/L).

2.6. Analytical methods

All samples used in quantifying the NO_3^-–N, nitrite–nitrogen (NO_2^-–N), Ca^{2+}, and dissolved Zn concentrations were filtered through a 0.22 μm pore size filter prior to quantification.

The NO_3^-–N concentration was determined following the brucine method [31] with slight modification. In brief, 1 mL aliquots of the samples were mixed with 0.2 mL of a sodium chloride solution (300 g/L), 1 mL of a sulfuric acid solution (4:1 mixture of concentrated sulfuric acid and distilled-deionized water), and 0.05 mL of a brucine-sulfanilic acid solution (10 g/L brucine sulfate heptahydrate, 1 g/L sulfanilic acid, and 30 mL/L concentrated hydrochloric acid) and heated at 100°C for 20 min. After cooling, the absorbance at 410 nm was determined using a DR6000 spectrophotometer (HACH Company, Loveland, CO, USA). The NO_2^-–N concentration was determined based on the Griess reaction [32]. In brief, 2 mL of a sample was mixed with 0.08 mL of the Griess reagent (1 g/L *N*-1-naphthylethylenediamine dihydrochloride, 10 g/L sulfanilamide, and 100 mL/L concentrated phosphoric acid) and incubated at room temperature (~25°C) for 10 min. After incubation, the absorbance at 540 nm was determined using a DR6000 spectrophotometer (HACH Company). The Ca^{2+} concentration was measured using a Ca^{2+} meter (LAQUAtwin B-751; HORIBA, Kyoto, Japan). The dissolved Zn concentration was measured following the Zincon method (HACH method 8009; HACH Company). The DO concentration was measured using a DO meter (PDO-520; FUSO Co., Ltd., Tokyo, Japan).

The pH was measured using either a pH meter (HM-25R; DKK-TOA Corporation, Tokyo, Japan) or a potable pH meter (LAQUAtwin B-712; HORIBA, Tokyo, Japan).

The OD_{600} was measured using a DR6000 spectrophotometer (HACH Company).

XRD analysis of the precipitates produced by strain WKDN was performed using a Smart Lab (Rigaku Corp., Tokyo, Japan) with Cu Kα radiation (λ = 1.541862 Å) operated at 40 kV and 30 mA. The precipitates were powdered with a mortar and pestle prior to XRD analysis. The XRD analysis was conducted by the Industrial Technology Center of Wakayama Prefecture (Wakayama, Japan).

2.7. Statistical analysis

Statistical significance was assessed with a one-way analysis of variance (ANOVA) with *post-hoc* Tukey's honestly significant difference (HSD) test or two-tailed Student's *t*-test. A *p*-value < 0.05 was considered statistically significant. All statistical analyses were conducted using statistical software R version 3.3.2 (http://www.r-project.org/).

3. Results and discussion

3.1. Identification of WKDN by 16S rRNA gene-based phylogenetic analysis

Microbial denitrification is one of most suitable methods to induce $CaCO_3$ biomineralization in soils, based on not only the type and amount of by- or side-products, but also the substrate solubility, $CaCO_3$ precipitate yield, and reaction rate [19]. Therefore, in this study, a non-motile, rod-shaped denitrifying bacterial strain, WKDN, was isolated from soil collected from the grounds of the National Institute of Technology at Wakayama College using the BTB agar plate technique developed for isolating aerobic denitrifiers [16,33]. A neighbor-joining phylogenetic tree was constructed based on the 16S rRNA gene sequence of WKDN and other phylogenetically-related gammaproteobacterial strains (Fig. 2). Based on the phylogenetic tree, we concluded that WKDN is a species of *Acinetobacter*. Members of this genus are frequently found in diverse environments, including soils, foods, water, and sewage [34]. Several isolates of *Acinetobacter* have been identified as bacterial denitrifiers [16]. For safe use of this strain in real groundwater treatments, further phenotypic characterization of WKDN is required.

3.2. In vitro aerobic denitrification and biomineralization capacity of WKDN

The simultaneous denitrification and biomineralization capacity of strain WKDN under an aerobic atmosphere was examined *in vitro*. As the carbon to nitrate (C/N) ratio of the biomineralization medium affects denitrification performance [16], the influence of the g acetate–C/g NO_3^-–N ratio on WKDN's denitrification and biomineralization was evaluated. The effect of the g acetate–C/g NO_3^-–N ratio on the removal efficiency of NO_3^-–N (%) and precipitated Ca^{2+} concentrations (mg/L) in the WKDN-inoculated biomineralization medium (initial medium volume: 80 mL) after 7 d of incubation is shown in Fig. 3. Among the tested medium compositions, NO_3^-–N removal and Ca^{2+} precipitation occurred most efficiently at a g acetate–C/g NO_3^-–N ratio of 6, with efficiency and concentration of 87.4% ± 2.0% and 250 ± 20 mg/L (average ± standard deviation [SD], n = 3), respectively.

We further examined temporal changes in the concentrations of NO_3^-–N, NO_2^-–N, precipitated Ca^{2+}, and DO, OD_{600}, and pH in the WKDN-inoculated biomineralization medium (initial medium volume: 400 mL) at a g acetate–C/g NO_3^-–N ratio of 6 (Fig. 4). Over the 14 d of this experiment, the DO concentration in the biomineralization medium was maintained at approximately 4–5 mg/L. The OD_{600} value of the biomineralization medium increased significantly until day 4 due to the growth of strain WKDN and Ca^{2+} precipitation in the biomineralization medium. The OD_{600} value was then maintained at approximately 0.2–0.3 until *in vitro* incubation ceased. The pH of the biomineralization medium increased slightly from 7.0 to 7.3–7.4 after *in vitro* incubation. The slight increase in pH during the progression of aerobic denitrification was likely due to the pH-buffering effect of carbonate precipitation [22]. The precipitated Ca^{2+} concentration in the biomineralization medium increased with the decrease in the NO_3^-–N concentration, while the precipitated Ca^{2+} concentration plateaued after 10 d of *in vitro* incubation, which was most likely due to the depletion of the NO_3^-–N in the biomineralization medium. The observed Ca^{2+} precipitate yield (g) per g NO_3^-–N consumed was 1.8 g Ca^{2+} precipitate/g NO_3^-–N. This precipitate yield (equivalent to 4.5 g $CaCO_3$ precipitate/g NO_3^-–N) is similar to or lower than those obtained by using anaerobic denitrifying bacterial strains

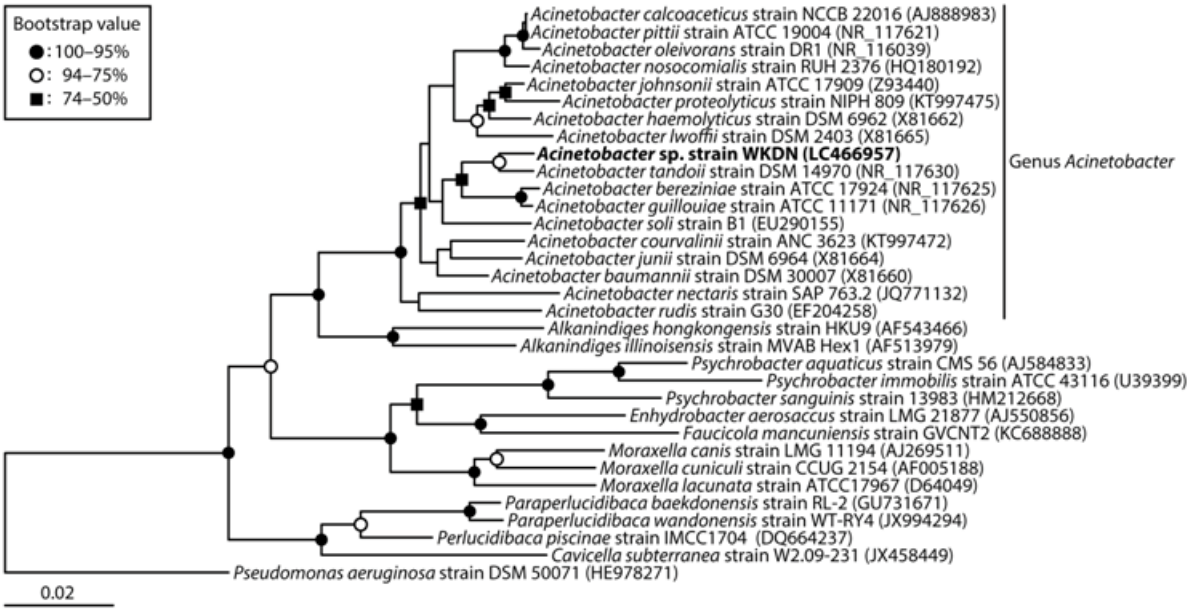

Fig. 2. Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences showing the phylogenetic position of strain WKDN. The scale bar represents the estimated number of nucleotide changes per sequence position. The symbols at the nodes indicate the bootstrap values (only those ≥50% are indicated) obtained after 1,000 resamplings. DDBJ/EMBL/GenBank accession numbers are shown in parentheses.

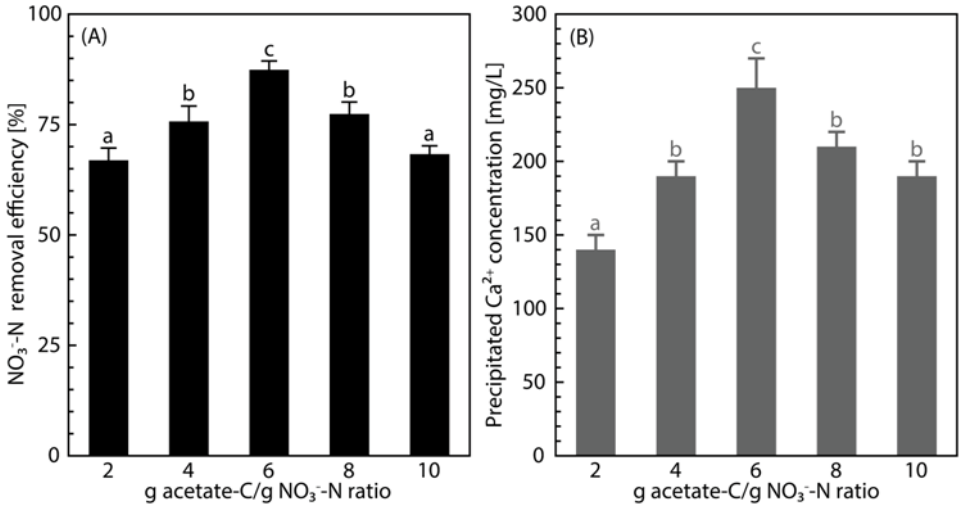

Fig. 3. Influence of the g acetate–C/g NO_3^-–N ratio of the biomineralization medium on the removal efficiency (%) of 150 mg NO_3^-–N/L and precipitated Ca^{2+} concentration (mg/L) by strain WKDN. Mean bars with different letters are statistically different (p-value < 0.05) according to a one-way analysis of variance with *post-hoc* Tukey's honestly significant difference test. The error bars represent the standard deviation (n = 3).

of *Pseudomonas denitrificans* (1.3–10.6 g $CaCO_3$ precipitate/g NO_3^-–N [17]), *Diaphorobacter nitroreducens* (14.1 g $CaCO_3$ precipitate/g NO_3^-–N [20]), and *Pseudomonas aeruginosa* (18.9 g $CaCO_3$ precipitate/g NO_3^-–N [20]). It should be noted that the presence of O_2 inhibits and represses anaerobic denitrification activity [15,16], which produces the alkalinity required for effective $CaCO_3$ precipitation [19]. Therefore, for use in the installation of a biomineralized $CaCO_3$-based permeable reactive barrier in subsurface environments where O_2 is present, WKDN might be a useful denitrifying bacterial strain capable of $CaCO_3$ biomineralization.

The accumulation of NO_2^-–N, a denitrification intermediate, should be avoided as it is highly toxic to living organisms [35–37]. In this study, NO_2^-–N was detected in the biomineralization medium during incubation, but its concentration was below 0.1 mg NO_2^-–N/L. This indicates that WKDN

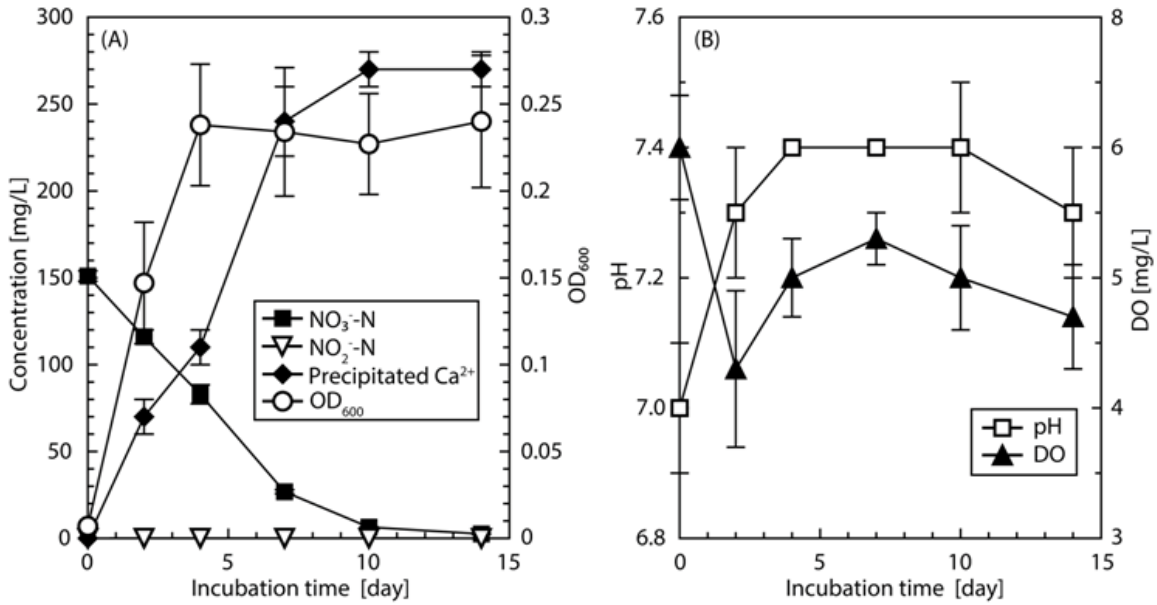

Fig. 4. Temporal changes in the concentrations of (a) NO_3^-–N, NO_2^-–N, and precipitated Ca^{2+}, and OD_{600}; (b) pH and DO concentration in the WKDN-inoculated biomineralization medium (g acetate–C/g NO_3^-–N ratio of 6). The error bars represent the standard deviation (n = 3).

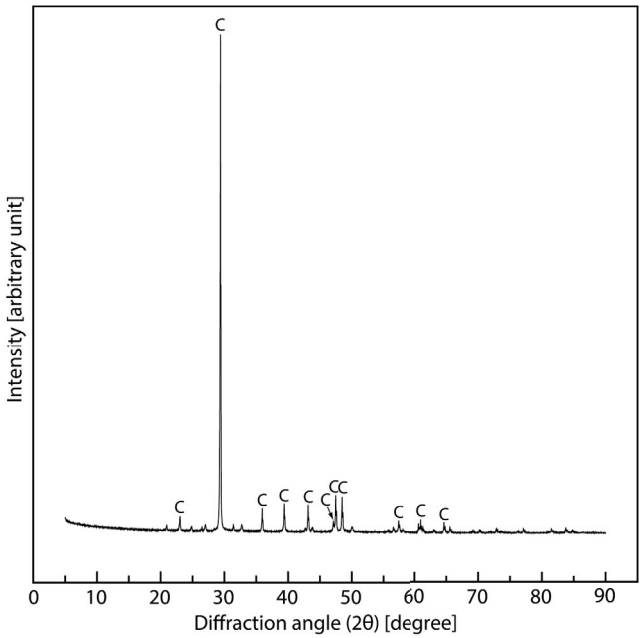

Fig. 5. X-ray diffraction patterns of the precipitates produced *in vitro* by strain WKDN. The major peaks associated with calcite are marked as "C."

can reduce NO_3^-–N into gaseous nitrogen compounds without significant accumulation of NO_2^-–N, making WKDN promising for use in biotechnological applications.

XRD analysis was conducted to elucidate the mineralogy of the precipitates produced by WKDN (Fig. 5). The XRD patterns indicated that calcite-type $CaCO_3$ was the dominant component of the precipitates produced by WKDN in the biomineralization medium.

3.3. Dissolved Zn removal potential of the biomineralized $CaCO_3$-based permeable reactive barrier

The dissolved Zn removal potential of the biomineralized $CaCO_3$-based permeable reactive barrier prepared using strain WKDN and the biomineralization medium was evaluated by passing an aqueous $ZnCl_2$ solution (298 mg dissolved Zn/L, pH 6.8) through sand columns treated with the biomineralization medium and live or deceased WKDN cells. The dissolved Zn concentration reflected the range of the Zn concentrations in landfill leachates (0.03–1,000 mg/L) summarized by Christensen et al. [38]. The sand column experiment showed that dissolved Zn could be almost completely removed from the $ZnCl_2$ aqueous solution by passing through the sand columns treated with live WKDN cells (removal efficiency: 98.5% ± 0.7% [average ± SD], n = 3) (Fig. 6). This removal efficiency was significantly higher (p-value < 0.05 by the two-tailed student's t-test) than that achieved in the sand columns treated with autoclave-killed WKDN cells (1.1% ± 1.0% [average ± SD], n = 3). The results indicated that live WKDN cells with the capacity for $CaCO_3$ biomineralization are required for the efficient removal of dissolved Zn. Notably, 190 ± 20 mg Ca^{2+}/L (average ± SD, n = 3) was detected in the drainage from the sand columns treated with live WKDN cells, while the Ca^{2+} concentration in the drainage from the sand columns treated with autoclave-killed cells was below the quantification limit of the Ca^{2+} meter used in this study (i.e., <40 mg Ca^{2+}/L). The average molar ratio of the removed dissolved Zn concentration to the produced Ca^{2+} concentration in the $ZnCl_2$ aqueous solution that passed through the sand columns treated with

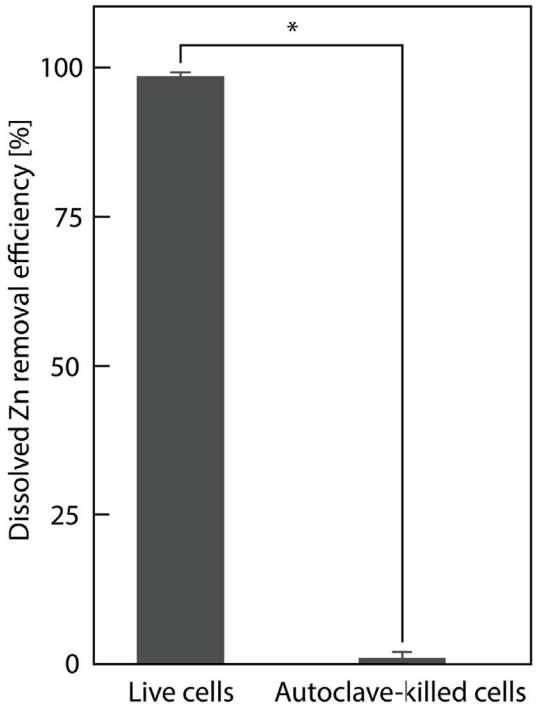

Fig. 6. Dissolved Zn removal efficiency [%] of the sand columns treated with live or autoclave-killed strain WKDN cells. The dissolved Zn concentration of the aqueous $ZnCl_2$ solution supplied to the sand columns was 298 mg/L. The error bars represent the standard deviation (n = 3). The asterisk indicates statistical significance (p-value < 0.05) according to the two-tailed Student's t-test.

live WKDN cells was 0.94 ± 0.06 (average ± SD, n = 3). The data suggested that the ion-exchange capability of $CaCO_3$ significantly contributed to the efficient removal of dissolved Zn from the aqueous $ZnCl_2$ solution. Further large-scale experiments are essential to clarify the applicability of the biomineralized $CaCO_3$-based permeable reactive barrier for the removal of dissolved Zn from real groundwater.

4. Conclusion

Overall, the results obtained in this study showed that bacterial strain WKDN with the capacity for aerobic denitrification and $CaCO_3$ biomineralization has good application prospects in the *in situ* removal of dissolved Zn from groundwater. However, owing to the limitations of these results, further investigation is required to clarify the applicability of the biomineralized $CaCO_3$-based permeable reactive barrier for the removal of dissolved Zn from real groundwater.

Acknowledgments

We thank Takuma Okada for his technical assistance in the *in vitro* experiments.

References

[1] G.U. Chibuike, S.C. Obiora, Heavy metal polluted soils: effect on plants and bioremediation methods, Appl. Environ. Soil Sci., 2014 (2014) 752708.
[2] S. Frassinetti, G. Bronzetti, L. Caltavuturo, M. Cini, C.D. Croce, The role of zinc in life: a review, J. Environ. Pathol. Toxicol. Oncol., 25 (2006) 597–610.
[3] U.S. Geological Survey, Zinc Statistics and Information. Available at: http://minerals.usgs.gov/minerals/pubs/commodity/zinc/ (accessed: September 27, 2019).
[4] J.F. Skidmore, Toxicity of zinc compounds to aquatic animals with special reference to fish, Q. Rev. Biol., 39 (1964) 227–248.
[5] G.R. Rout, P. Das, Effect of metal toxicity on plant growth and metabolism: I. Zinc, Agronomie, 23 (2003) 3–11.
[6] L.M. Plum, L. Rink, H. Haase, The essential toxin: impact of zinc on human health, Int. J. Environ. Res. Public Health, 7 (2010) 1342–1365.
[7] S. Khalid, M. Shahid, N.K. Niazi, B. Murtaza, I. Bibi, C. Dumat, A comparison of technologies for remediation of heavy metal contaminated soils, J. Geochem. Explor., 182 (2017) 247–268.
[8] T. Zhu, M. Dittrich, Carbonate precipitation through microbial activities in natural environment, and their potential in biotechnology: a review, Front. Bioeng. Biotechnol., 4 (2016) 4.
[9] Y. Kitano, N. Kanamori, S. Yoshioka, Adsorption of zinc and copper ions on calcite and aragonite and its influence on the transformation of aragonite to calcite, Geochem. J., 10 (1976) 175–179.
[10] P. Papadopoulos, D.L. Rowell, The reactions of copper and zinc with calcium carbonate surfaces, Eur. J. Soil Sci., 40 (1989) 39–48.
[11] X. Ma, L. Li, L. Yang, C. Su, K. Wang, S. Yuan, J. Zhou, Adsorption of heavy metal ions using hierarchical $CaCO_3$-maltose meso/macroporous hybrid materials: adsorption isotherms and kinetic studies, J. Hazard. Mater., 209–210 (2012) 467–477.
[12] C.N. Mulligan, R.N. Yong, B.F. Gibbs, Remediation technologies for metal-contaminated soils and groundwater: an evaluation, Eng. Geol., 60 (2001) 193–207.
[13] Á.E. Torres-Aravena, C. Duarte-Nass, L. Azócar, R. Mella-Herrera, M. Rivas, D. Jeison, Can microbially induced calcite precipitation (MICP) through a ureolytic pathway be successfully applied for removing heavy metals from wastewaters?, Crystals, 8 (2018) 438.
[14] D. Mujah, M.A. Shahin, L. Cheng, State-of-the-art review of biocementation by microbiallyinduced calcite precipitation (MICP) for soil stabilization, Geomicrobiol. J., 34 (2017) 524–537.
[15] W.G. Zumft, Cell biology and molecular basis of denitrification, Microbiol. Mol. Biol. Rev., 61 (1997) 533–616.
[16] B. Ji, K. Yang, L. Zhu, Y. Jiang, H. Wang, J. Zhou, H. Zhang, Aerobic denitrification: a review of important advances of the last 30 years, Biotechnol. Bioprocess Eng., 20 (2015) 643–651.
[17] I. Karatas, Microbiological Improvement of the Physical Properties of Soil, Ph.D. Thesis, Arizona State University, 2008.
[18] W.R.L. van der Star, E. Taher, M.P. Harkes, M. Blauw, M.C.M. van Loosdrecht, L.A. van Paassen, Use of Waste Stream and Microbes for *in situ* Transformation of Sand into Sandstone, C.F. Leung, J. Chu, R.F. Shen, Eds., Ground Improvement Technologies and Case Histories, Research Publishing Services, Singapore, 2009, pp. 177–182.
[19] L.A. van Paassen, C.M. Daza, M. Staal, D.Y. Sorokin, W. van der Zon, M.C.M. van Loosdrecht, Potential soil reinforcement by biological denitrification, Ecol. Eng., 36 (2010) 168–175.
[20] Y.Ç. Erşan, N. de Belie, N. Boon, Microbially induced $CaCO_3$ precipitation through denitrification: an optimization study in minimal nutrient environment, Biochem. Eng. J., 101 (2015) 108–118.
[21] N. Hamdan, E. Kavazanjian Jr., B.E. Rittmann, I. Karatas, Carbonate mineral precipitation for soil improvement through microbial denitrification, Geomicrobiol. J., 34 (2016) 139–146.
[22] V.P. Pham, A. Nakano, W.R.L. van der Star, T.J. Heimovaara, L.A. van Paassen, Applying MICP by denitrification in soils: a process analysis, Environ. Geotech., 5 (2018) 79–93.
[23] I.J. Winograd, F.N. Robertson, Deep oxygenated ground water: anomaly or common occurrence?, Science, 216 (1982) 1227–1230.
[24] F. Widdel, G.-W. Kohring, F. Mayer, Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov. and *Desulfonema magnum* sp. nov., Arch. Microbiol., 134 (1983) 286–294.

[25] A. Hiraishi, Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification, Lett. Appl. Microbiol., 15 (1992) 210–213.
[26] W. Ludwig, O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A.W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, K.-H. Schleifer, ARB: a software environment for sequence data, Nucleic Acids Res., 32 (2004) 1363–1371.
[27] G.A. Somerville, R.A. Proctor, Cultivation conditions and the diffusion of oxygen into culture media: the rationale for the flask-to-medium ratio in microbiology, BMC Microbiol., 13 (2013) 9.
[28] E. Stevens, M. Laabei, S. Gardner, G.A. Somerville, R.C. Massey, Cytolytic toxin production by *Staphylococcus aureus* is dependent upon the activity of the protoheme IX farnesyltransferase, Sci. Rep., 7 (2017) 13744.
[29] R. Kuerbis, Y.P. Vaid, Sand sample preparation – the slurry deposition method, Soils Found., 28 (1988) 107–118.
[30] K. Terzaghi, R.B. Peck, Soil Mechanics in Engineering Practices, John Wiley & Sons Inc., New York, NY, 1948.
[31] D. Jenkins, L.L. Medsker, Brucine method for the determination of nitrate in ocean, estuarine, and fresh waters, Anal. Chem., 36 (1964) 610–612.
[32] D. Tsikas, Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric oxide area of research, J. Chromatogr. B, 851 (2007) 51–70.
[33] N. Takaya, M.A.B. Catalan-Sakairi, Y. Sakaguchi, I. Kato, Z. Zhou, H. Shoun, Aerobic denitrifying bacteria that produce low levels of nitrous oxide, Appl. Environ. Microbiol., 69 (2003) 3152–3157.
[34] K. Towner, The genus *Acinetobacter*, Prokaryotes, 6 (2006) 746–758.
[35] W.M. Lewis Jr., D.P. Morris, Toxicity of nitrite to fish: a review, Trans. Am. Fish. Soc., 115 (1986) 183–195.
[36] A.M. Fan, V.E. Steinberg, Health implications of nitrate and nitrite in drinking water: an update on methemoglobinemia occurrence and reproductive and developmental toxicity, Regul. Toxicol. Pharm., 23 (1996) 35–43.
[37] H. Kroupova, J. Machova, Z. Svobodova, Nitrite influence on fish: a review, Vet. Med., 50 (2005) 461–471.
[38] T.H. Christensen, P. Kjeldsen, H.-J. Albrechtsen, G. Heron, P.H. Nielsen, P.L. Bjerg, P.E. Holm, Attenuation of landfill leachate pollutants in aquifers, Crit. Rev. Environ. Sci. Technol., 24 (1994) 119–202.