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Resilient Denitrifiers Wink at Microbial Self-Healing Concrete

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Abstract— **This study focused on identification of denitrifiers that can be used to achieve microbial self-healing concrete. By using heat treatment and minimal medium, 9 denitrifying strains were isolated from soil. Upon identification of the strains, their capability of handling dehydration stress was investigated. Qualifying 7 strains were further investigated at N:P ratio of 70:1. Finally, 2 strains, *Pseudomonas aeruginosa* and *Diaphorobacter nitroreducens*, were selected and investigated at pH 7, pH 9.5 and pH 13 with and without protection. As a protective carrier diatomaceous earth and expanded clay were used. Significant activity observed at pH 9.5 and with protection both strains could survive pH 13 for 14 days and reduced 20-30 mg/L NO_3^- in 4 days after the pH adjustment to ~10. Overall, the results indicated that *Pseudomonas aeruginosa* and *Diaphorobacter nitroreducens* can resist mild heat, dehydration, starvation and relatively alkali environment, which are the main concerns in use of bacteria for concrete structures.**

***Keywords*—alkali pH, self-healing, Gram-negative, phosphorus limitation**

I. Introduction

Denitrification is known to be an essential part of the N-cycle and an important step in treatment of hazardous nitrogen compounds from waste streams. In the last decade, denitrification mechanism started to be investigated in soil improvement studies to replace ureolysis, since it also leads biological calcium carbonate precipitation in the presence of Ca^{2+} ions [1,2]. Soil improvement studies revealed that significant performance on $CaCO_3$ precipitation could be achieved if necessary conditions, i.e. nutrient availability, enough water and non-inhibitory pH environment, were provided [1,2]. These results bring the idea that, denitrifiers can also be used to achieve microbial self-healing in concrete structures which were defined through ureolysis or oxidation of carbon sources up to now [3-5]. The main concerns for bacteria in concrete environment are; (1) high shear stress during mixing, (2) high temperatures whilst cement hydration, (3) nutrient availability, (4) relatively high pH (pH~13 in concrete and pH~10 in cracks), (5) dehydration stress, (6) small pore sizes (<0.1 μm). Mainly to protect bacteria from high shear stress, high pH and small pore sizes, encapsulation or immobilization of the bacteria is suggested [3]. Several encapsulation techniques, such as porous expanded clay particles, glass capillaries, diatomaceous earth and microcapsules, were investigated and bacterial healing was reported [4,5]. Yet, from our knowledge, no study investigated denitrification in concrete or mortar. If resilient denitrifying strains are identified, they can also be protected with the defined encapsulation methods and further used to investigate denitrification based microbial self-healing in concrete structures. Therefore, this study focused on identification and selection of resilient denitrifying strains and investigation of their performances at pH values similar to the ones found in concrete with and without using protective carriers.

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II. Materials and Methods

A. Isolation and characterization of denitrifiers

The denitrifiers were isolated from soil by inoculating two batch reactors containing sterile M9 media (Table 1) with a specific C-source (either methanol or formate) and exposing to cyclic heat treatment. Anoxic batch reactors having 200 mL liquid volume, 400 mL headspace with rubber stoppers and screw caps were used. The reactors were incubated for 5 cycles with 90% volumetric exchange ratio. Each cycle was 3 days. At the end of each cycle, reactors were exposed to heat treatment (70°C for at least 20 minutes). Afterwards, reactor content was replaced with the sterile fresh medium based on volumetric exchange ratio and the headspace of the reactors were flushed with Argon (Ar) and the next cycle started. The cyclic operation was repeated 5 times in a row and ended up with 10 isolated strains. In order to characterize the isolated strains, DNA extraction, dereplication (BOX-PCR) and 16S-PCR amplification experiments were carried out. Firstly, DNA of all 10 isolates was extracted using the suggested FastPREP DNA extraction protocol [6]. The DNA concentrations were analysed by using Nano-drop ND-100 spectrophotometer. DNA concentrations (data not shown) were diluted to 50 ng/μl for each sample for the further BOX-PCR and 16S-PCR amplification. The BOX-PCR was carried out as described previously [7]. The amplified DNA fragments were separated by electrophoresis on 1.5% agarose gel. The amplified DNA bands after 4.5 h of electrophoresis at 100V were stained in ethidium bromide solution and visualized under UV light. The 16S-PCR amplification was also carried out via universal primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and

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1378R (5'-CGGTGTGTACAAGGCCCGGGAACG-3'), thus the strains were identified.

TABLE I. MEDIUM COMPOSITIONS IN DIFFERENT EXPERIMENTS

Compounds		Isolation	Dehydration, Re-activation
Buffer (g/L)	$Na_2HPO_4.7H_2O$	8.5	-
	KH_2PO_4	3	-
M9 Salt Solution (g/L)	NaCl	0.5	0.5
	$MgSO_4$	0.24	0.24
	$CaCl_2$	0.011	0.011
C source (g/L)	NaCOOH	6	6
	CH_3OH	4	4
NO_3^- source (g/L)	KNO_3	0.72	1.011
P source (g/L)	Na_3PO_4	-	0.105 0.0105*

*During the test of PO_4-P limitation

B. *Strain responses to dehydration and limited PO_4-P*

All the isolated strains were grown in non-buffered M9 medium (Table 1). Afterwards, each strain was plated on an agar plate having identical composition with the liquid medium and specific C-source (formate or methanol specific to strains), and incubated at 28 °C for 3 days. Upon bacterial growth, they were incubated at 37°C for 3 weeks. After that, dry agars were submerged back in 50 mL of liquid medium and investigated under anoxic conditions. NO_3^- reduction was monitored for 2 weeks. The isolates showing no or negligible activity were discarded and 7 qualifying strains were investigated at N:P ratio of 70:1 to determine the NO_3^- reduction performances at N:P ratio of 70:1 (Table 1).

C. *Strain performances at different pH values with and without protection*

Pseudomonas aeruginosa and *Diaphorobacter nitroreducens* (top performing strains at N:P ratio 70:1) were investigated at pH 7, pH 9.5 and pH 13 with and without protection. The composition of the buffered media are given in Table 2. As protective carriers diatomaceous earth (DE) and ARGEX® expanded clay (0-2) were used. The diatomaceous earth used in this study had a particle size of 4-20 µm with large amount of pores (0.1µm to 0.5µm).

The NO_3^- reduction performances of the unprotected strains were investigated at pH 7, pH 9.5 and pH 13. In addition to that, from each strain two batches of bacterial suspension were prepared. First batch was mixed with sterile diatomaceous earth (60 w/v) and put on 120 rpm shaker for 1 hour to facilitate bacterial attachment to the DE surface. Second batch was incorporated into expanded clay particles by using vacuum saturation. Bacterial solution were added to the bottles containing sterile clay particles (60w/v) under vacuum (-0.7 bar) and over pressurized (1.2 bar) to improve bacterial impregnation into pores. Initial bacterial concentration for all cases was set to 10^9 cells/mL.

TABLE II. COMPOSITION OF DIFFERENT PH ENVIRONMENTS

Compounds	pH 7 (mM)	pH 9.5 (mM)	pH 13 (mM)
Na_2HPO_4	75	96	-
NaOH	-	3	-
HCl	24	-	-
Cement*	-	-	20*
NaCl	8.6	8.6	8.6
$MgSO_4$	2	2	2
$CaCl_2$	0.1	0.1	0.1
NaCOOH	88	88	88
$NaNO_3$	10	10	10
Na_3PO_4	-	-	0.65

*Concentration is given as g/L

III. RESULTS AND DISCUSSION

The isolation process ended up with 10 strains (5 from Formate-fed, 5 from MeOH-fed batch). Molecular analysis revealed that 9 out of 10 strains were unique and all the strains were Gram-negative denitrifying strains (Fig. 1, Table 3).

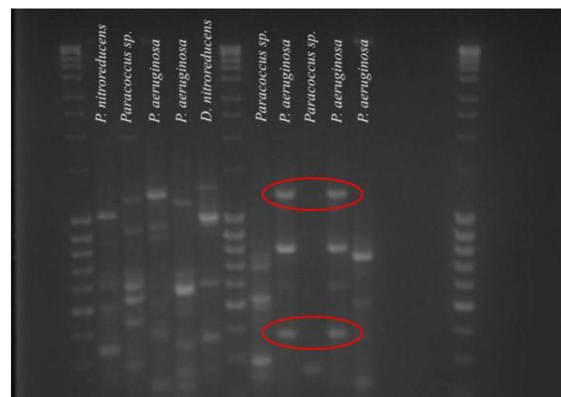

Figure 1. Dereplication-separation of DNA fragments via electrophoresis on 1.5% agarose gel (left to right the strains: first group m1-m5, second group f1-f5)

TABLE III. ISOLATED STRAINS AND CLOSEST TAXONOMY IDENTIFICATION

Label*	Species	Similarity
m1	*Pseudomonas nitroreducens*	98%
m2	*Paracoccus sp. Ol 18*	90%
m3	*Pseudomonas aeruginosa* (*Pseudomonas citronellolis*)	98%
m4	*Pseudomonas aeruginosa* or *Pseudomonas sp WW9*	96% - 98%
m5	*Diaphorobacter nitroreducens*	99%
f1	*Paracoccus sp. R-24650*	100%
f2	*Pseudomonas aeruginosa*	98%
f3	*Paracoccus sp. R-24650*	99%
f4	*Pseudomonas aeruginosa*	99%
f5	*Pseudomonas aeruginosa* (*Pseud. citronellolis DSM 50332T*)	98%

*m: The species cultured by using methanol as carbon source
f: The species cultured by using formate as carbon source

The existing information about the heat resistance of the strains isolated in this study (*Paracoccus* sp., *Pseudomonas*

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sp., *Diaphorobacter* sp.) is limited. Viable cells of *P. aeruginosa* after exposure to 63°C for 30 minutes were reported [8], yet the resilience was attributed to the NaCl concentration which was 40 to 80 times higher than the concentration (0.05 w/v) in our study. To our knowledge, there is no information about heat resilient *Paracoccus* sp and *Diaphorobacter* sp. which explains the majority (5 out of 9 specific isolates) of the *Pseudomonas* sp. in isolated strains.

A. *Selection of the top performing strains*

The unique strains were further exposed to 3 weeks of dehydration and starvation to assure their resilience. All of the strains belonging to the MeOH-fed batch were active, while only two of the strains (f2 and f5) belonging to the Formate batch were active in terms of NO_3^- reduction to NO_2^- and N_2 gas (Fig. 2).

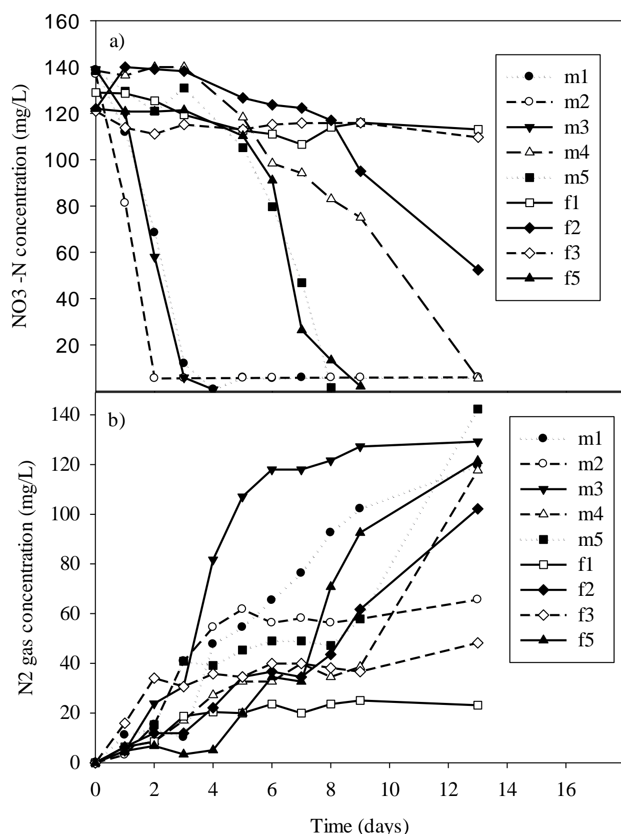

Figure 2. Nitrate reduction performances of the 9 unique strains after 3 weeks of dehydration, m: strains belong to MeOH-fed batch f: strains belong to Formate-fed batch

Although it is not common, Gram-negative bacteria can resist dehydration stress by producing EPS or osmoprotectants, e.g. *Pseudomonas* sp. could change their microenvironment by producing extracellular polymeric substances (EPS), to enhance their survival in case of intracellular water loss [9]. An outdoor air strain which could survive in absence of water and has 97% similarity to *Diaphorobacter nitroreducens* was also reported [10]. Moreover, *Paracoccus denitrificans* was reported to utilize L-and D-Pro-B as well as tHyp-B as osmoprotectans under high osmotic stress [11].

After discarding the inactive two strains (f1, f3), belong to Formate-fed batch, nitrate reduction performances of the qualifying 7 strains were compared at N:P ratio of 70:1. It was found that 4 of the strains (m1, m3, m4, f5) were more sensitive to PO_4-P deficiency (Fig. 3).

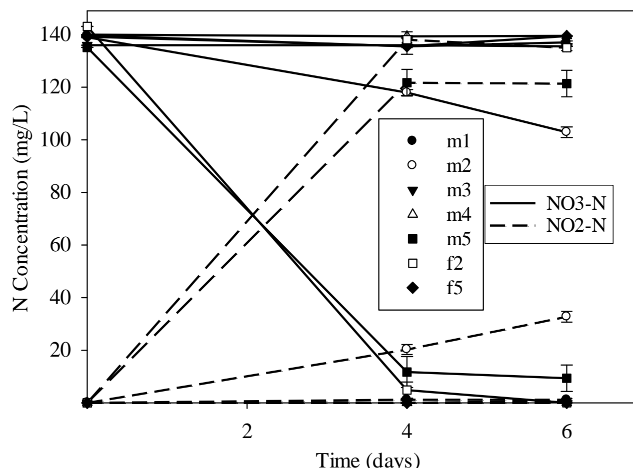

Figure 3. Nitrate reduction performances at N:P ratio of 70:1 m: strains belong to MeOH-fed batch f: strains belong to Formate-fed batch

On the other hand, three of the strains (m2-*Paracoccus sp*., m5-*Diaphorobacter nitroreducens* and f2-*Pseudomonas aeruginosa*) showed activity at N:P ratio of 70:1. There was a little difference between the performances of *Diaphorobacter nitroreducens* (m5) and *Pseudomonas aeruginosa* (f2), while their performance was significantly better than *Paracoccus* sp. Ol.18 (m2) (p=0.05). Phosphate limitation was reported to interrupt denitrification partially and cause NO_2^- accumulation; however, a N:P ratio of 100 or less is enough for complete denitrification [12]. However, as seen in our results nitrate reduction activity of some strains can be completely inhibited under PO_4-P limited conditions, since it is an essential microbial nutrient particularly contributing to the cell growth and being used in cellular polymer (nucleic acids, phospholipids, proteins) and adenosine triphosphate (ATP) synthesis.

Based on the performances of the strains whilst or after exposure to certain stress, *Diaphorobacter nitroreducens* and *Pseudomonas aeruginosa* were chosen for further investigation of performances at pH values found in concrete cracks and concrete itself.

B. *Strain performances at different pH values with and without protection*

After determination of the most useful strains among all the isolated strains, they were investigated under different pH conditions with and without protective carriers. Although both strains showed significant nitrate reduction activity at pH 7 and pH 9.5 (p=0.05) as expected, their performances were

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enhanced with either DE protection or expanded clay protection (Fig. 4a,b, Fig. 5a,b).

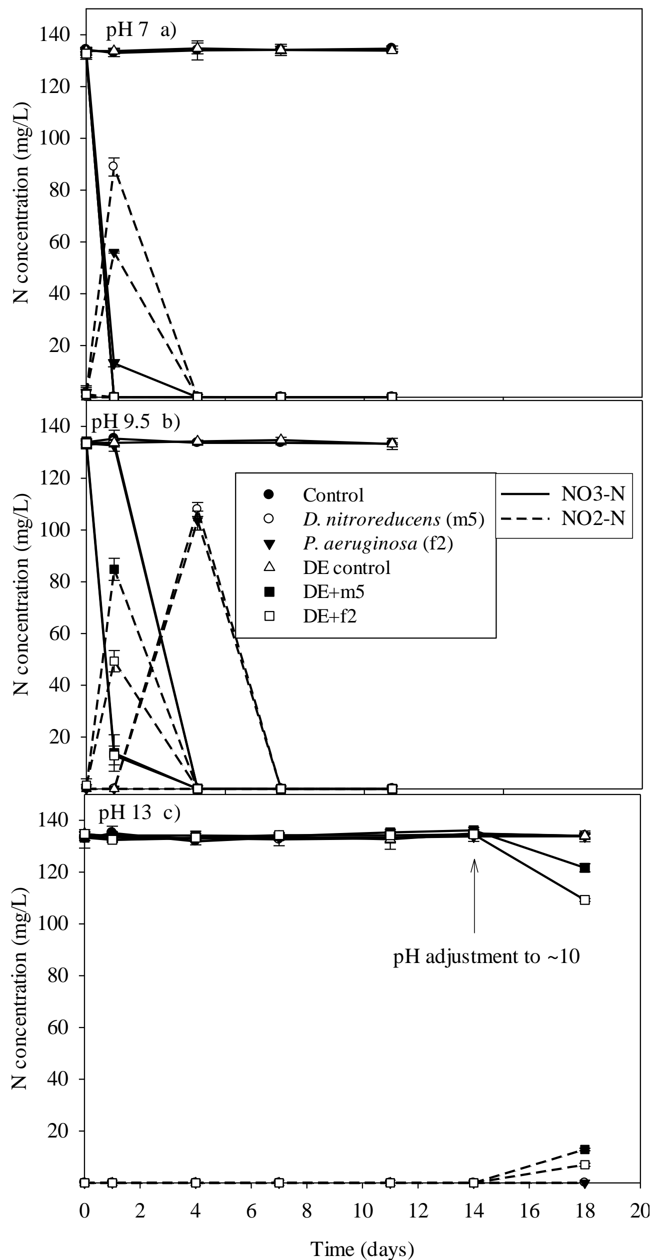

Figure 4. Nitrate reduction activities of strains with and without diatomaceous earth (DE) protection a) at pH 7 b) at pH 9.5 c) at pH 13

At pH 7, both protected and unprotected strains reduced all the NO_3^- in 24 hours. The main difference was observed in NO_2^- accumulation (partial denitrification) in the system. There might be three reasons for NO_2^- accumulation; (1) strain preference on NO_3^- over NO_2^- (2) total denitrification rate (3) free nitrous acid (FNA) inhibition. It was known from the previous experiments (data not shown) that *D. nitroreducens* was reducing NO_3^- to NO_2^- first and further in the absence of NO_3^- it was using NO_2^- and completing the total denitrification (of 140 mg/L NO_3-N) in 2 days. On the other hand, *P. aeruginosa* was using NO_3^- and NO_2^- together but still completing the total denitrification in 2 days. The performance of the unprotected strains in this experiment was consistent with the earlier results and total denitrification achieved at pH 7 which indicated that possibility (1) and (3) were not valid. Achieving total denitrification in 24 hours (instead of minimum 48 hours) with DE protection or expanded clay protection indicated that immobilization was beneficial for strain performances.

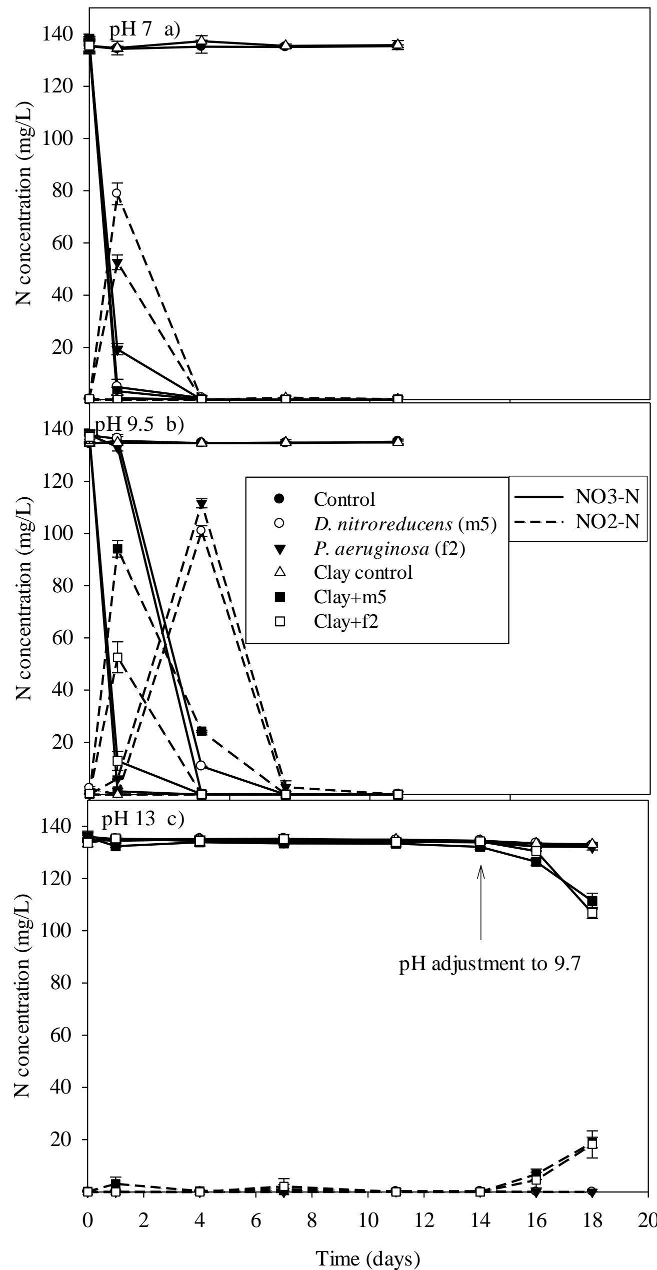

Figure 5. Nitrate reduction activities of strains with and without expanded clay protection a) at pH 7 b) at pH 9.5 c) at pH 13

The performance increase was also observed at pH 9.5. Unprotected strains performed with a delay, most probably due to the inhibitory pH level, while protected strains showed almost the same reduction activity as unprotected strains at pH

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7 (Fig. 4a,b, Fig. 5a,b). Consistent positive effect of DE on bacterial performances were also reported on treatment of chlorinated compounds, oxidation of ferrous ions and in situ remediation of contaminated soil via incorporating with bacteria [13-15]. Therefore, it can be said that, the negative charge of DE and porous structure of the expanded clay provided safe space for the bacteria, hence exposure to inhibitory pH levels was not as severe as it was for the unprotected ones. Small decrease in performances of the clay protected strains were observed at pH 9.5 compared to their performance with DE protection. This might be due to the leaking of bacterial solution from the clay particles whilst incubation which led more exposure of the bacteria to the inhibitory conditions compared to DE protection. Using small (0.5-1 mm) clay particles might improve the protection performance, since it can provide higher surface area for bacterial attachment.

The results at pH 13 revealed that both protected and unprotected strains were inactive/dead. After 2 weeks, pH of the solution was adjusted to ~10 to check re-activation. It was observed that, only the protected strains could reduce NO_3^- and produced NO_2^- which was indication for survival of the strains at pH 13 only if they were protected with DE or expanded clay (Fig. 4c, Fig. 5c).

Although studies reported efficient protection of spores (*Bacillus* sp.) via diatomaceous earth and expanded clay [4-7], our findings indicated that vegetative cells can also be protected from the detrimental pH environments via similar encapsulation methods. Vegetative cells are known to be disadvantageous in certain aspects compared to spores. However, the strains *Diaphorobacter nitroreducens* and *Pseudomonas aeruginosa* investigated in this study could survive alkali pH when necessary protection is provided. Therefore, their advantage over spores, immediate growth and activation when pH drops regardless of nutrient conditions, came into consideration. This kind of rapid response can be beneficial for the performance of microbial self-healing concrete and decrease the time required for crack closure.

IV. CONCLUSION

- Vegetative Gram-negative denitrifying strains *Pseudomonas aeruginosa* and *Diaphorobacter nitroreducens* can resist heat (70°C >20 min), dehydration stress and starvation stress (3 weeks).
- Both strains showed significant nitrate reduction activity at N:P ratio of 70.
- Both strains can survive pH 13 if protected with either diatomaceous earth or expanded clay, and further reduce nitrate at pH ~10, which makes them proper candidates for concrete application.
- Diatomaceous earth and expanded clay have significant positive effect on denitrification performances of the strains.
- Strains can also be used for enhanced soil improvement and wastewater treatment.

Acknowledgment

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Pseudomonas aeruginosa and *Diaphorobacter nitroreducens* are resilient enough to survive concrete environment.

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