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## Interaction between palladium-doped zerovalent iron nanoparticles and biofilm in granular porous media: characterization, transport and viability†

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Palladium-doped nanoscale zerovalent iron (Pd-NZVI) can induce rapid transformations of a variety of pollutants during *in situ* remediation of contaminated aquifers. Pd-NZVI stabilized with polymeric surface modifiers has shown substantially improved stability and transport compared to bare Pd-NZVI in model subsurface granular media such as clean quartz sand. The natural subsurface environment is, however, much more complex. For example, biofilms may coat aquifer grain surfaces and thus alter the transport of these reactive nanoparticles. Herein, we compare the transport behavior of Pd-NZVI coated with carboxymethylcellulose or rhamnolipid as surface modifiers, in clean and biofilm-coated sand packed columns. Transport studies suggest that, for both types of nanoparticles tested, the particle attachment efficiency to the collector surface generally increases (up to 6 fold in NaCl and 26 fold in CaCl<sub>2</sub>) in the presence of biofilm. This result indicates the potential for reduced Pd-NZVI transport in a natural groundwater system. The retentive behavior of biofilm-coated media increases with particle–particle aggregation (e.g.,  $C/C_0 \sim 0.15$  for a markedly aggregated CMC-coated Pd-NZVI in divalent salt), implicating physical straining as an important retention mechanism. Retained Pd-NZVI on biofilm-laden matrices was characterized using enhanced darkfield hyperspectral imaging. Assessments of biofilm viability imply that the retained surface-modified Pd-NZVI is non-toxic to the cells within the biofilm matrices (viability >95%). Moreover, the coating molecules do not negatively impact the viability of the biofilm bacteria.

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### Nano impact

The success of Pd-NZVI based site remediation depends on the mobility of injected Pd-NZVI to reach the target contaminants. The mobility of Pd-NZVI is shown to be reduced in the presence of biofilm on the surface of aquifer grains. The retained surface-modified Pd-NZVI is shown to be generally non-toxic to the biofilm cells and enhanced darkfield hyperspectral imaging is used for identification of Pd-NZVI retention on the biofilm-coated sand. Overall, this study emphasizes that the influence of biofilm should be taken into account when predicting the fate and transport of reactive nanoparticles in the aquatic environment. Thus, our results have implications for the successful implementation of nanotechnology enabled soil and groundwater remediation.

## Introduction

Palladium-doped nanoscale zerovalent iron (Pd-NZVI) has been shown to remediate a wide range of contaminants.<sup>1–3</sup> Direct injection has been suggested as a viable method to deliver these reactive nanoparticles to contaminated subsurface zones.<sup>2,4</sup> A major challenge in implementing this technology is that bare Pd-NZVI rapidly aggregates and thus exhibits negligible transport whereas the success of nanotechnology enabled remediation largely depends on the mobility of injected Pd-NZVI to reach the targeted contaminants. Several studies have demonstrated that surface modification of the nanoparticles reduces aggregation and concurrently enhances transport.<sup>5–8</sup> However, these studies were based on mobility experiments conducted in a clean sand-

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† Electronic supplementary information (ESI) available: Biomass distribution along the column, Pd-NZVI characterizations (EPM and sizing), column breakthrough curves, a summary table for column experiments ( $C/C_0$  values), a summary table for measured influent and effluent DLS sizes, hyperspectral imaging for Pd-NZVI suspension, and Live/Dead microscopy images. See DOI: 10.1039/c5en00109a





### Biofilm characterization by confocal microscopy

The biofilm-coated sand surface was characterized using confocal laser scanning microscopy (CLSM, Zeiss LSM 510 META). Two sets of confocal images were acquired: (i) after 24 h biofilm growth using sand grains collected from the top and bottom of the batch in the glass bottle used for growth, and (ii) from two segments (top and bottom) of the column packed with biofilm-coated sand after ~10 pore volumes (PVs) equilibration with either 10 mM NaCl or 10 mM CaCl<sub>2</sub>. The biofilm staining protocol was adapted from our previous work.<sup>14</sup> Briefly, prior to imaging, the collected sand grains were placed on a microscope slide and stained with (i) Film Tracer FM 1-43 green biofilm cell stain (Life Technologies), and (ii) Film Tracer SYPRO Ruby Biofilm Matrix Stain (Invitrogen) for 15 min in the dark. After rinsing with DI water, the grains were then transferred into a bead of glycerol on a glass cover slip, and the confocal imaging (slicing with z-stack) was immediately started. The average biofilm thickness on the sand grains was determined by analyzing the images using Zen software as described previously.<sup>14</sup>

### Biomass along the column

The distribution of biofilm along the sand-packed column was determined by gravimetric analysis as described elsewhere.<sup>16</sup> Briefly, the biofilm-coated sand matrix was dissected into four segments collected in separate ceramic cups. These cups were then dried at 120 °C for 14 h, cooled to room temperature, and weighed. To remove organic mass, the samples were then combusted at 800 °C for 24 h, cooled to room temperature, and re-weighed. The difference in mass represents the amount of biomass present in each column section. The distribution of biomass along the length of the biofilm-coated sand column after equilibration, but prior to Pd-NZVI injection, is presented in Fig. S1.†

### Pd-NZVI transport experiments

Clean sand-packed column experiments were conducted as described previously.<sup>7</sup> Briefly, a glass column (GE Life Science – C16/20 tube (i.d. 1.6 cm) with AC 16 adapter) was wet packed with 27 g sand that was pre-equilibrated overnight with the electrolyte of interest. The packed bed length was 8.1 cm. Next, the column was equilibrated with the electrolyte of interest for ~10 PVs at a flow rate of 0.9 mL min<sup>-1</sup> (approach velocity of  $7.5 \times 10^{-5}$  m s<sup>-1</sup>) in downward flow direction using a peristaltic pump (ISMATEC, Model ISM596D). The Pd-NZVI suspension (either RL- or CMC-coated) was then injected at the same flow rate and direction using the same pump. Nearly 3 PVs of Pd-NZVI suspension (prepared in the background electrolyte of interest) was injected followed by the flushing phase with ~4 PVs background electrolyte to ensure complete removal of mobile Pd-NZVI from the column pore space. The concentration of Pd-NZVI in the influent ( $C_0$ ) and effluent ( $C$ ) suspensions was monitored (at 10 s intervals) online at 508 nm using a UV-vis

spectrophotometer (Agilent 8453) equipped with a flow-through cell (1 cm path length).<sup>7</sup>

To ensure homogeneity and a constant injection concentration, the glass vial containing the Pd-NZVI suspension was continuously shaken at 400 rpm (INFORS HT Labotron bench-scale orbital shaker) throughout the experiment. Biofilm-coated sand column experiments were conducted in the same manner as the clean sand columns, with the exception that the sand was pre-coated with *P. aeruginosa* biofilm as described above. The equilibration, Pd-NZVI injection and post-injection procedures were the same as in the clean sand packed column experiments. Prior to conducting the nanoparticle transport experiment, the breakthrough behavior of an inert tracer (10 mM KNO<sub>3</sub>) was monitored (Fig. S2†). All column experiments were conducted at least in duplicate, the range of elution in duplicate runs is reported in Table S4.†

### Enhanced darkfield hyperspectral imaging

The presence of Pd-NZVI on biofilm-laden sand was detected and a qualitative spatial distribution confirmed using an enhanced darkfield transmission optical microscope (Olympus BX43) equipped with a hyperspectral imaging spectrophotometer (CytoViva, Inc., Auburn, AL). Grains of biofilm-coated sand (pre-exposed to Pd-NZVI suspension) were transferred to a glycerol bead on a glass slide and covered with a glass cover slip before capturing hyperspectral images. At least three hyperspectral images were collected for each treatment (biofilm-coated sand) exposed to either RL-coated or CMC-coated Pd-NZVI; treatments with no particle exposure served as a control (blank). To build the reference spectra library for analysis, hyperspectral images of the nanoparticle suspensions were also captured. The images were processed and analyzed using a hyperspectral imaging system software (ENVI 4.8 version). The heterogeneity of the Pd-NZVI suspension was accounted for by randomly selecting several particles (regions of interest) to build the spectral library. The spectral library was then filtered through the blank to eliminate matching profiles. Analysis was subsequently performed using the modified spectral library and a spectral angle mapping (SAM) coefficient of 0.08 to spectrally determine the spatial distribution of the nanoparticles.

### *P. aeruginosa* growth in the presence or absence of Pd-NZVI

The potential influence of the Pd-NZVI during bacterial growth and biofilm formation was investigated. An overnight culture was grown in LB at 37 °C (for 20 hours) with shaking at 130 rpm. The bacterial culture was then diluted 100 times (to an optical density at 600 nm, OD<sub>600</sub>, between 0.04–0.05) and distributed (900 μL aliquots) into a 24-well black microplate with clear bottom (PerkinElmer). Each well was loaded with 100 μL Pd-NZVI suspension from the stock prepared at two different Pd-NZVI concentrations (1.5 and 0.15 g L<sup>-1</sup>) that resulted in final Pd-NZVI concentrations of 0.15 or 0.015 g L<sup>-1</sup>. Selected wells without Pd-NZVI served as controls. To distinguish the effect of particle modifier, additional



experiments were conducted using the supernatant of the Pd-NZVI suspension containing free (unbound) surface modifiers. The Pd-NZVI was first separated from the unbound surface modifiers using a super magnet. The plates were incubated in a microplate reader (Tecan Infinite M200 Pro, Switzerland) at 37 °C with orbital shaking (1000 s, 1.5 mm amplitudes, wait time 1 min) and the planktonic growth ( $OD_{600}$ ) monitored at 30 min intervals for 24 h. All experiments were conducted in triplicate.

### Effect of Pd-NZVI on biofilm development

At the end of the 24 h growth period described above, the liquid (non-adherent cells) in each well was removed by gentle pipetting. The wells were then washed two times with deionized (DI) water and stained with 0.1% crystal violet (CV) (1 mL per well). After 15 min, the excess CV stain was removed, the wells were rinsed three times with DI water, and the CV was solubilized with 95% ethanol (1 mL per well)

for 15 min. Finally,  $OD_{570}$  values in each well were measured using the microplate reader and reported as the quantitative measure of biofilm formed. To account for the variations in growth, biofilm level ( $OD_{570}$ ) was normalized by the level of bacterial growth ( $OD_{600}$ ) at the end of growth period (24 h).

### Bacterial viability assay

Overnight cultures, diluted 100 times, were loaded into 24-well plates (1 mL per well) that were incubated at 37 °C for 24 h with orbital shaking at 100 rpm. After 24 h, the non-adherent cells in each well were removed by gentle pipetting. The thin layer of biofilm formed on the well surfaces was then exposed to 15 or 150 mg L<sup>-1</sup> RL-coated or CMC-coated Pd-NZVI suspension (1 mL per well) prepared in 10 mM NaCl. To avoid Pd-NZVI oxidation during exposure, the plates were kept in an anaerobic chamber at room temperature. At the end of exposure period (1 or 24 h), the suspension in each well was removed by gentle pipetting followed by



**Fig. 1** (a) Confocal micrograph of quartz sand coated with *P. aeruginosa* biofilm (overlay of dark field and Film Tracer™ Green Biofilm Cell Stain). (b) Representative orthogonal confocal image of biofilm-coated sand grain showing biofilm thickness on the sand surface. Three images per treatment (with z-stacking) were acquired to measure biofilm thickness using the CLSM image processing software. (c) Biofilm thickness on the quartz sand surface before column packing (pre-eq) with the sample collected from the glass bottle in which the biofilm was grown, and after column packing and equilibration (post-eq) with either 10 mM NaCl or 10 mM CaCl<sub>2</sub>. To collect sand grains for confocal imaging, the column was dissected into two segments (top and bottom) following the equilibration. The box plot is based on at least 60 independent thickness measurements per treatment. Statistically significant differences with respect to pre-equilibration is indicated by \*\* ( $p < 0.01$ , *t*-test) (n.s. – no significant difference).



staining with 200  $\mu\text{L}$  per well of 1:1 mixture of Live/Dead stain (LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> Bacterial Viability Kit, Life Technologies) for 15 min. The BacLight mixture consists of SYTO 9 and propidium iodide that stains cells with compromised membranes red and cells with intact membranes green. After 15 min contact time (in the dark), excess stains in the wells were removed, followed by imaging using fluorescence microscopy (IX-71, Olympus) at 200 $\times$  magnification. At least 30 images were acquired per treatment, and analyzed using ImageJ (ImageJ 1.43 m, NIH, USA) to determine the total area of compromised (red) and uncompromised (green) cells. The loss in cell viability was calculated as the ratio of red to total (red + green) area.

## Results and discussion

### Biofilm thickness characterization

A representative confocal image of biofilm-coated sand is presented in Fig. 1a, which shows a homogeneous biofilm coating on the sand surface. An orthogonal confocal image shows a thin layer of biofilm around the sand (Fig. 1b). Measured biofilm thickness on the sand surface (after 24 hours growth) is shown in Fig. 1c. The thickness before column packing was  $3.7 \pm 0.17 \mu\text{m}$  (mean  $\pm$  95% confidence interval). After column packing and equilibration, the thickness decreased ( $p < 0.05$ ,  $t$ -test) with the mean of the top (inlet end) and bottom (outlet end) values as  $2.8 \pm 0.15 \mu\text{m}$  and  $2.5 \pm 0.05 \mu\text{m}$  respectively for 10 mM NaCl and CaCl<sub>2</sub> equilibrated columns (Fig. 1c). During equilibration, some of the loose biofilm likely dislodged from the sand surface, resulting in slightly thinner biofilm observed after equilibration. Additionally, no apparent difference in biofilm thickness was observed between the top or bottom column segments ( $p > 0.05$ ) suggesting a homogeneous biofilm distribution along the column. This result is consistent with a previous study<sup>14</sup> where the same biofilm forming approach was used, although the thickness reported was higher ( $\sim 4 \mu\text{m}$ ) than this study (2.5–2.8  $\mu\text{m}$ ). These differences may be attributed to the different quartz sand used (average grain size of 256  $\mu\text{m}$  in this study *versus* 760  $\mu\text{m}$  in the previous study<sup>14</sup>), likely leading to different packing arrangement and different surface roughness, and thus slightly different growth conditions. Owing to its smaller grain size, the available surface area for biofilm growth is much larger (3 times more for a given volume) in this study. Moreover, the approach velocity in this study ( $7.46 \times 10^{-5} \text{ m s}^{-1}$ ) was higher than in the previous study ( $6.22 \times 10^{-5} \text{ m s}^{-1}$ ).<sup>14</sup>

### Electrokinetic characterization and hydrodynamic diameter of Pd-NZVI

For RL-coated Pd-NZVI, the EPM values varied from  $-4$  to  $-2.5 \mu\text{m cm V}^{-1} \text{ s}^{-1}$  in 3–100 mM NaCl, and  $-2$  to  $-1 \mu\text{m cm V}^{-1} \text{ s}^{-1}$  in 1–30 mM CaCl<sub>2</sub> (Fig. S3<sup>†</sup>). The EPM values for CMC-coated Pd-NZVI varied from  $-4.5$  to  $-2.5 \mu\text{m cm V}^{-1} \text{ s}^{-1}$  in NaCl and  $-4$  to  $-1.5 \mu\text{m cm V}^{-1} \text{ s}^{-1}$  in CaCl<sub>2</sub> (Fig. S3<sup>†</sup>).

Overall, the EPM data shows that (i) in both monovalent and divalent salts, an increase in solution ionic strength results in lower absolute EPM values due to compression of the diffuse double layer and charge screening, (ii) at a given ionic strength, the divalent salt is more effective at masking the charge as noted by the significantly lower EPM values in CaCl<sub>2</sub> compared to NaCl.

The stability or the aggregation state of Pd-NZVI used in the transport experiments was assessed by measuring the hydrodynamic diameters of Pd-NZVI using dynamic light scattering ( $d_{\text{DLS}}$ ) (Fig. S4 and Table S2,<sup>†</sup> influent  $d_{\text{DLS}}$ ). Broadly, the sizing data correlates with the EPM data, *i.e.*, the extent of aggregation generally increased with the decreased absolute EPM (electrostatic destabilization). For RL-coated Pd-NZVI in NaCl, the average  $d_{\text{DLS}}$  value ranged from  $402 \pm 20 \text{ nm}$  at 3 mM to  $518 \pm 20 \text{ nm}$  at 30 mM (difference statistically significant,  $p < 0.001$ ), whereas in CaCl<sub>2</sub> a larger  $d_{\text{DLS}}$  is observed over the same range of ionic strength (IS); the average  $d_{\text{DLS}}$  ranged from  $700 \pm 35 \text{ nm}$  at 3 mM to  $1590 \pm 64 \text{ nm}$  at 30 mM. Likewise, for CMC-coated Pd-NZVI in NaCl, the average  $d_{\text{DLS}}$  ranged from  $435 \pm 35 \text{ nm}$  at 3 mM to  $1510 \pm 51 \text{ nm}$  at 30 mM, whereas the particle size was considerably larger in CaCl<sub>2</sub>: average  $d_{\text{DLS}}$  ranged from  $2317 \pm 132 \text{ nm}$  at 3 mM to  $2285 \pm 177 \text{ nm}$  at 30 mM (Fig. S4 and Table S2,<sup>†</sup> influent  $d_{\text{DLS}}$ ). It is worth noting that these values should be interpreted with caution as the DLS measurements may be biased by the sedimentation of larger aggregates present in the suspension.

Overall, the sizing data indicate that the extent of aggregation increases with IS. This result corroborates our previous work where an IS dependent aggregation behavior was demonstrated for both RL- and CMC-coated Pd-NZVI over a broad range of IS (10–500 mM NaHCO<sub>3</sub>).<sup>7</sup> At a given IS, CaCl<sub>2</sub> is more effective at inducing agglomeration as depicted by larger  $d_{\text{DLS}}$  values in CaCl<sub>2</sub> (Fig. S4<sup>†</sup>). The presence of Ca<sup>2+</sup> notably reduces the absolute EPM of Pd-NZVI (due to charge screening) resulting in reduced particle–particle repulsive interaction which favors aggregation. Moreover, Ca<sup>2+</sup>-induced bridging mechanism between the carboxylic group containing coating molecules of Pd-NZVI can lead to further aggregation. This result is consistent with a prior study that reported increased NZVI aggregation in the presence of CaCl<sub>2</sub>.<sup>26</sup>

### Transport of Pd-NZVI suspended in monovalent salt solution

The results of Pd-NZVI transport experiments in biofilm-coated sand in the presence of monovalent salt (NaCl) are presented in Fig. S5 and Table S4.<sup>†</sup> For RL-coated Pd-NZVI, particle elution ( $C/C_0$ ) systematically decreases with an increase in IS; the average  $C/C_0$  varies from 0.80 at 3 mM to 0.12 at 100 mM NaCl (Table S4<sup>†</sup>). For CMC-coated Pd-NZVI, although the elution does vary slightly from 3–10 mM ( $C/C_0 = 0.85$ – $0.86$ ), a drastic reduction in elution occurs at 30 mM ( $C/C_0 = 0.28$ ) and 100 mM ( $C/C_0 = 0.18$ ) (Table S4<sup>†</sup>). The elution in biofilm-coated sand is compared to experiments



conducted in clean sand from our previous study,<sup>23</sup> in which the same sand was used. Overall, at a given IS, reduced elution is observed in biofilm-coated sand compared to clean sand (*i.e.*,  $C/C_{0,\text{biofilm}} < C/C_{0,\text{clean}}$ ), except for one treatment (CMC-coated Pd-NZVI at 10 mM NaCl) (Table S4†). To be more representative of field conditions, additional experiments conducted at higher Pd-NZVI concentration ( $1 \text{ g L}^{-1}$ ) show that RL and CMC are effective at improving Pd-NZVI transport even in biofilm-coated sand ( $C/C_0 = 0.60 \pm 0.02$  for RL-coated and  $0.77 \pm 0.01$  for CMC-coated Pd-NZVI) (Fig. S6a†).

A semi-quantitative comparison of nanoparticle transport potential is made *via* the calculated attachment efficiency ( $\alpha_{\text{pc}}$ )<sup>27,28</sup> (Fig. 2). The  $\alpha_{\text{pc}}$  values for columns packed with clean sand were obtained from our previous work.<sup>23</sup> Overall, in both treatments, the  $\alpha_{\text{pc}}$  values increase with an increase in solution IS (Fig. 2a and b). As explained in the section on electrokinetic characterization, an increase in solution IS results in lower absolute EPM and thus reduced repulsive electrostatic forces that favor the likelihood of particle

attachment onto the collector surfaces. Another important observation is that, at a given IS, the biofilm-coated sand results in greater  $\alpha_{\text{pc}}$  values. For RL-coated Pd-NZVI, we observe 2 to 6 fold increase in  $\alpha_{\text{pc}}$  in biofilm-coated sand *versus* bare sand; the increase is  $\sim 2$  fold for CMC-coated Pd-NZVI (Fig. 2a and b). Likewise, at  $1 \text{ g L}^{-1}$  Pd-NZVI, a 1.6 fold (RL-coated) to 16 fold (CMC-coated) increase in  $\alpha_{\text{pc}}$  is observed in biofilm-coated sand (Fig. S6b†). The implication of these calculations is that the transport potential of these nanoparticles is potentially reduced in granular groundwater environments that contain biofilm. Therefore, (Pd-) NZVI transport studies conducted in clean sand may overestimate the transport potential of these reactive nanoparticles in natural environments. Lerner *et al.*<sup>17</sup> also reported a larger  $\alpha_{\text{pc}}$  value for the transport of poly(acrylic) acid-stabilized NZVI in columns packed with biofilm-coated glass beads ( $\alpha_{\text{pc}} = 0.03$ ) *versus* clean glass beads ( $\alpha_{\text{pc}} = 0.004$ ) at 25 mM NaCl.<sup>17</sup> However, the difference in  $\alpha_{\text{pc}}$  was negligible at 1 mM NaCl ( $\alpha_{\text{pc}} = 0.006$  and 0.005, respectively, in clean and biofilm-coated media).



**Fig. 2** Calculated particle-collector attachment efficiency ( $\alpha_{\text{pc}}$ ) in monovalent salt (NaCl) for (a) RL-coated and (b) CMC-coated Pd-NZVI in clean and biofilm-coated sand column. The  $\alpha_{\text{pc}}$  values in clean sand were adopted from our previous work.<sup>23</sup> The  $\alpha_{\text{pc}}$  values in divalent salt ( $\text{CaCl}_2$ ) for (c) RL-coated and (d) CMC-coated Pd-NZVI in clean and biofilm-coated sand columns. Error bars represent standard deviations. The dashed lines are included to guide the eye. The circular red and triangular blue symbols on Fig. 2a and b correspond to the transport experiment conducted at higher Pd-NZVI concentration ( $1 \text{ g L}^{-1}$ ) in biofilm coated-sand and clean sand (from our previous work<sup>23</sup>), respectively. For all other conditions, the particle concentration was  $0.15 \text{ g L}^{-1}$ .





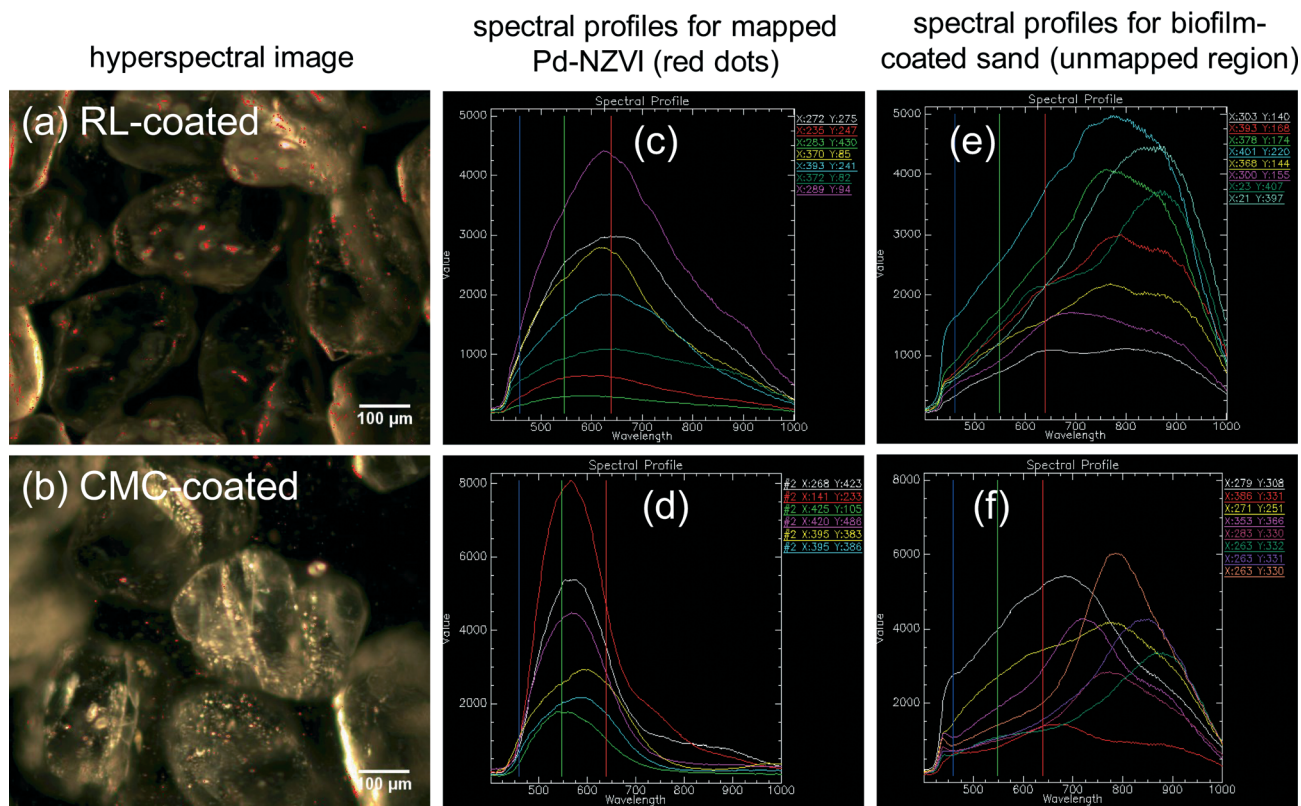


Fig. 3 Hyperspectral images of biofilm-coated sand exposed with (a) RL-coated and (b) CMC-coated Pd-NZVI. Red dots represent mapped Pd-NZVI particles on the biofilm matrices. The representative spectral profiles correspond to the mapped (c) RL-coated and (d) CMC-coated Pd-NZVI. The representative spectral profiles for the unmapped region (biofilm-coated sand only) are also presented (e, f).

### Effect of coated Pd-NZVI on *P. aeruginosa* growth

Bacterial growth curves in the absence or presence of Pd-NZVI are presented in Fig. 4a and b. Growth kinetics are stimulated (higher OD<sub>600</sub> values) at lower Pd-NZVI concentration (15 mg L<sup>-1</sup>) for both RL- and CMC-coated Pd-NZVI. The presence of small amount of iron and/or the associated surface modifiers appear to favor the bacterial growth. In contrast, a markedly inhibited growth rate is observed at higher Pd-NZVI concentration (150 mg L<sup>-1</sup>). Murugesan *et al.*<sup>30</sup> also reported a comparable trend whereby the bacterium (*Sphingomonas* sp. PH-07) was able to grow well at lower Pd-NZVI concentration (<0.1 g L<sup>-1</sup>) at 25 °C. However, an inhibited bacterial growth was observed when Pd-NZVI concentration was greater than 0.1 g L<sup>-1</sup>.<sup>30</sup> Based on our previous work<sup>7</sup> a notable fraction (>80%) of free surface modifier is likely to remain in the suspension; therefore to elucidate its effect, additional growth experiments were conducted in the presence of free (unbound) surface modifier. The supernatant of CMC- and RL-coated Pd-NZVI suspension was collected by separating the NZVI with a super magnet. The experiments show comparable growth as with the control (Fig. 4e and f), suggesting that the surface modifiers (RL and CMC) had no adverse effects on bacterial growth. For the bare Pd-NZVI, the growth was not affected at a lower concentration (15 mg L<sup>-1</sup>), however, a marked inhibited bacterial growth was observed

at a higher Pd-NZVI concentration (150 mg L<sup>-1</sup>) (Fig. 4g). The presence of dissolved Fe during growth experiment cannot be ruled out, thus the inhibitory effect observed here might also be linked to the presence of dissolved ions. The toxicity of dissolved Fe<sup>2+</sup> and Fe<sup>3+</sup> ions to phytoplankton *P. subcapitata* has previously been reported.<sup>26</sup> Moreover, NZVI toxicity to *E. coli* has been associated with oxidative stress in the presence of released Fe ions due to oxidation.<sup>31</sup>

### Effect of surface-modified Pd-NZVI on formation of *P. aeruginosa* biofilm

A quantitative estimate of biofilm formed over a 24 h growth period in the absence (control) or presence of RL- and CMC-coated Pd-NZVI (at 15 and 150 mg L<sup>-1</sup>) is presented in Fig. 4c. Biofilm levels (OD<sub>570</sub>) were normalized to the extent of bacterial growth (OD<sub>600</sub>, determined separately) to decrease the potential bias in the measurements due to differences in growth rate.<sup>32,33</sup> Biofilm formation decreased in the presence of RL-coated Pd-NZVI, by 23% and 57% (compared to control) at 15 and 150 mg L<sup>-1</sup>, respectively. For CMC-coated Pd-NZVI, biofilm formation was slightly decreased (by 3%) at 15 mg L<sup>-1</sup> but increased (by 29%) at 150 mg L<sup>-1</sup> (Fig. 4c). Biofilm formation was notably reduced in the presence of the unbound surface modifier (collected as supernatant of 150 mg L<sup>-1</sup> RL- and CMC-coated Pd-NZVI





**Fig. 4** (a, b) Bacterial growth curves measured as  $OD_{600}$  in the absence (control) or presence of (a) RL-coated and (b) CMC-coated Pd-NZVI at 15 and 150 mg L<sup>-1</sup>. (c) Growth normalized quantitative estimate of the biofilm formed at the end of growth period (24 hours) measured using crystal violet assay ( $OD_{570}/OD_{600}$ ). (d) Bacterial viability measured with LIVE/DEAD assay after 1 or 24 h exposure with Pd-NZVI at 15 and 150 mg L<sup>-1</sup>. Error bars represent standard deviations. (e) Bacterial growth curves in the absence (control) or presence of free surface modifiers (supernatant) separated using super magnet for (e) RL-coated and (f) CMC-coated Pd-NZVI at 15 and 150 mg L<sup>-1</sup>. (g) Bacterial growth curves in the absence (control) and presence of bare Pd-NZVI at 15 and 150 mg L<sup>-1</sup>.



suspensions) and bare Pd-NZVI at both 15 and 150 mg L<sup>-1</sup> (Fig. S9†). Therefore, biofilm inhibition appears to correlate with Pd-NZVI concentration at 150 mg L<sup>-1</sup> with RL coating (both suspension and supernatant) and with CMC coating (supernatant). Using a microtiter dish assay, Davey *et al.*<sup>34</sup> also reported reduced *P. aeruginosa* biofilm formation in the presence of rhamnolipid.

### Effect of deposited Pd-NZVI on viability of biofilm cells

The viability of matured (24 h) biofilm cells after 1 h or 24 h exposure to CMC- or RL-coated Pd-NZVI is presented in Fig. 4d (representative Live/Dead fluorescence microscopy images are presented in Fig. S10†). At 15 mg L<sup>-1</sup> Pd-NZVI, with 1 h exposure, the average viability >96%, and a similar high viability (>98%) was observed at 150 mg L<sup>-1</sup>. Inspection of Fig. 4d indicates some loss of viability at 24 h; however, the effect is not statistically significant. Additional biofilm viability experiments conducted using 1 g L<sup>-1</sup> coated Pd-NZVI and its supernatant (free surface modifier) did not indicate any loss in viability (Fig. S11†). Therefore, overall, the viability assay suggests that exposure to either RL- or CMC-coated Pd-NZVI does not impact the membrane integrity of biofilm cells. As described previously for aggregated silver nanoparticles,<sup>35</sup> the Pd-NZVI suspension likely aggregated during the exposure period, and it is likely that the aggregated clusters did not penetrate the matrix of extracellular polymeric substances (EPS) as readily, thus limiting their impact on cell viability.

## Environmental implications

This study highlights how the processes of nanoparticle aggregation and retention (by deposition and physical straining) are of importance in determining the suitability of NZVI-based remediation in field application, in the presence of natural biofilms. It is important to note that injection of large quantities of NZVI suspension likely displaces natural groundwater, especially near the injection point and may alter the influence of the natural water chemistry. This work shows that the transport potential of RL and CMC-modified Pd-NZVI (at 150 mg L<sup>-1</sup>) is drastically reduced in the presence of *P. aeruginosa* biofilm. We observe a marked increase in nanoparticle attachment efficiency in the presence of biofilm, indicating that surface-modified Pd-NZVI can exhibit decreased transport in biofilm-laden groundwater environment. As expected, the variation in water chemistries influenced Pd-NZVI aggregation behavior, with drastic aggregation occurring in the presence of divalent salt. Particle retention was particularly significant ( $C/C_0 \sim 0.15$ ) for a markedly aggregated suspension (CMC-coated Pd-NZVI in divalent salt) in biofilm-coated sand. Transport experiments conducted at higher Pd-NZVI concentration (1 g L<sup>-1</sup>) showed that RL and CMC are effective for improving Pd-NZVI transport even in biofilm-coated sand but with a reduced transport potential when compared to clean sand. An important observation is the difference in measured particle size at the

column effluent and influent that confirmed the contribution of physical straining of large aggregates as an important mechanism for the heightened Pd-NZVI retention in biofilm-coated column. This phenomenon was enhanced at higher IS that induced substantial aggregation and leads to the conclusion that physical straining is expected to be an important mechanism during field injection. Another key contribution of this study is the use of hyperspectral imaging to confirm the presence of Pd-NZVI in complex biofilm-laden matrices. This technique may be relevant in field application to directly characterize unlabeled samples (solid and liquid) collected from the field and confirm the presence of injected nanoparticles.

This study shows that the toxicological impact of Pd-NZVI on bacteria varies if the latter is in a planktonic or a sessile state; the surface modifiers (RL and CMC) alone are non-toxic to the bacterial cells. Although the planktonic growth data indicate inhibitory effect at higher concentration (150 mg L<sup>-1</sup>) for both bare and coated (RL- and CMC-) Pd-NZVI, biofilm formation was only impacted by RL-coated Pd-NZVI. However, viability staining suggests negligible impact of the deposited surface-modified Pd-NZVI onto matured biofilm matrices (viability >95%) for both RL- and CMC-coated Pd-NZVI. Given that the injected surface-modified Pd-NZVI nanoparticles for remediation application are likely to be retained more in the presence of biofilm, a systematic study on the prolonged exposure of biofilms to the reactive nanoparticles coupled with nanoparticle aging/oxidation is of interest for further study.

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