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A New Concept of Promoting Nitrate Reduction in Surface Waters: Simultaneous Supplement of Denitrifiers, Electron Donor Pool, and Electron Mediators

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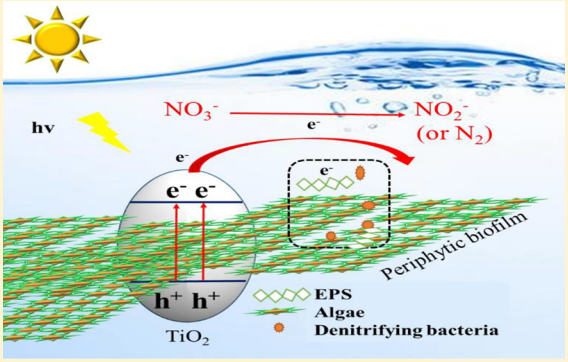
Supporting Information

ABSTRACT: The efficiency of biological nitrate reduction depends on the community composition of microorganisms, the electron donor pool, and the electron mediators participating in the biological reduction process. This study aims at creating an *in situ* system comprising of denitrifiers, electron donors, and electron mediators to reduce nitrate in surface waters. The ubiquitous periphytic biofilm in waters was employed to promote *in situ* nitrate reduction in the presence of titanium dioxide (TiO_2) nanoparticles (NPs). The nitrate removal rate in the periphytic biofilm and TiO_2 NPs system was significantly higher than the control (only periphytic biofilm or TiO_2 NPs). TiO_2 NPs optimized the community composition of periphytic biofilm for nitrate reduction by increasing the relative abundance of four dominant denitrifying bacteria. Periphytic biofilm showed a substantial increase in extracellular polymeric substance, especially the humic acid and protein content, due to the presence of TiO_2 NPs. The synergistic action of humic acid, protein, denitrifying bacteria of the periphytic biofilm, and TiO_2 NPs contributed to 80% of the nitrate reduction. The protein and humic acid, acting as electron mediators, facilitated the transfer of exogenous electrons from photoexcited TiO_2 NPs to periphytic biofilm containing denitrifiers, which enhanced nitrate reduction in surface waters.



INTRODUCTION

Photocatalytic reduction of nitrate is an alternative transformative technology to address the continuous excessive discharge of nitrate into surface water which poses environmental risks such as eutrophication and harmful algal blooms.[1,2] To the best of our knowledge, very little information is available in the literature concerning *in situ* nitrate reduction by photocatalysis in surface waters. This is because existing anionic competitors, such as SO_4^{2-} and CO_3^{2-}, preferentially occupy the active catalytic sites while inclusion hole scavengers (electron donor), such as formic acid, carry the risk of undesirable biofilm growth in surface waters.[3,4] In consideration of the huge nitrate loads of surface waters, it is very important to give priority to the development of *in situ* technologies for nitrate treatment.

Biological nitrate removal in surface waters depends on the combination of electron acceptors and electron donors which are usually provided by the organic matter present in water—represented by the chemical oxygen demand (COD).[4,5] In the

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field of *in situ* biological water treatment, the influent COD is capable of oxidizing the influent ammonium to nitrite and nitrate in the presence of electron acceptors, such as O_2, that enter the water through photosynthesis or aeration.[6] This process consumes large amounts of COD causing nitrate accumulation, while the remaining COD is insufficient for facilitating denitrification. To maintain the growth and activity of nitrifiers/denitrifiers and drive the denitrification step, an external carbon source should be added as the electron donor.[7] However, supplementing organic electron donors as a COD source is expensive, carries a high risk of turbidity increase, and causes excess biomass growth and unwanted N_2O emissions.[8,9]

It has been reported that microbial cells possess the ability to acquire electrons from natural solid donors to reduce the electron acceptors.[10] The use of environmentally benign and low-cost electron donors has attracted interest among the research community and environmental engineers. Titanium dioxide (TiO_2) nanoparticles (NPs) have been extensively studied for photocatalytic denitrification in the presence of hole scavengers under ultraviolet irradiation.[11] Utilization of photoexcited electrons derived from nanophotocatalysts, such as TiO_2 NPs, for biological nitrate reduction is promising for development of *in situ* nitrate remediation technologies.[12] However, previous studies have demonstrated that nanophotocatalysts were toxic to single cells or pure strains of microorganisms, such as *E. coli*[13,14] or algae.[15,16]

Fortunately, the ubiquitous periphytic biofilm in surface waters has a strong ability to grow successfully under a variety of environmental conditions, including those affected by the presence of NPs.[17] Periphytic biofilm, as a mixture of multispecies microbial aggregates, is composed of heterotrophic and phototrophic microorganisms which are embedded in a self-produced mucilage matrix of extracellular polymeric substances (EPS).[18] EPS are able to provide refuge for microorganisms, such as denitrifiers, defending the toxicity of NPs, and are also capable of serving as electron mediators for electron transfer.[19,20] In consideration of the high loadings of nitrate pollution in waterways in paddy fields or wetlands, a new concept of simultaneous supplement of denitrifiers and electron transfer mediators by periphytic biofilm and the electron donor pool derived from TiO_2 NPs to reduce nitrate in surface waters is proposed in this study.

The objectives of this study were (i) to test the new concept of promoting *in situ* nitrate reduction by simultaneous addition of denitrifiers, electron donor pool, and electron mediators in surface waters, (ii) to study the responses of periphytic biofilm in the presence of exogenous electron donor exciters, such as TiO_2 NPs, and (iii) to explore the mechanism of nitrate reduction in simulated surface waters.

MATERIALS AND METHODS

Preparation of Photocatalyst Suspension and Periphytic Biofilm Collection. Analytical grade TiO_2 NPs (Aladdin, China) were used in this study. The stock suspensions of TiO_2 NPs were prepared by adding 1000 mg of TiO_2 NPs to 300 mL of deionized water. The contents were agitated and mixed well for 30 min and thereafter diluted to 1000 mL in an Erlenmeyer flask. This suspension was diluted to required concentrations for individual experiments. Periphytic biofilms used in this study were collected from the authors' biofilm culture system.[21]

Nitrate Removal by Periphytic Biofilm in the Presence of TiO_2 NPs. Bench scale experiments were performed for 7 days to stimulate growth of periphytic biofilm for nitrate removal. This 7 days duration was based on the lifecycle of periphytic biofilm used (21 days). Periphytic biofilm was collected and used in experiments after 14 days of growth. 1 g (w/w) of periphytic biofilm was added into 250 mL of flasks containing 100.0, 95.5, 95.0, and 90.0 mL of Woods Hole (WC) media[22] and sodium nitrate (see Supporting Information). Either 0, 0.5, 5.0, or 10.0 mL of TiO_2 NPs suspensions were added to the individual flasks to achieve a final volume of 100 mL. The initial concentrations of TiO_2 NPs in the flasks were 0 (control), 5.0, 50.0, and 100.0 mg L^{-1}. The initial nitrate concentration was 124.0 mg L^{-1}. All treatments and the control were sealed with sterile sealing films in a standard light-dark cycle of 12 h illumination (150 W Xe-high pressure lamp, intensity 20 W m^{-2}) followed by 12 h of total darkness at a temperature of 28 ± 1 °C. On days 0, 1, 3, 5, and 7, the water was sampled to analyze the pH and the residual concentrations of nitrate and nitrite.

To distinguish whether TiO_2 NPs itself affects nitrate removal in the absence of periphytic biofilm, another set of control experiment was conducted (details provided in the Supporting Information). To investigate the influence of the light response of TiO_2 NPs on nitrate removal in this system, fluorescence lamp experiments (λ > 420 nm) were carried out in the control and treatments while filtration UV experiments were conducted in the presence of TiO_2 NPs (details provided in the Supporting Information).

Characterization of Periphytic Biofilm and EPS. Periphytic biofilm of the control and 100 mg L^{-1} TiO_2 treatment were collected to analyze the activity of nitrate reductase (NaR) and catalase (CAT) and community composition on day 7. NaR and CAT activities were determined using the reagent kit method (Nanjing Jiancheng Bioengineering Institute, China). The periphytic biofilm was also collected on day 7 and sized after drying. Thereafter, TEM, scanning electron microscopy (SEM, JEOL Co, Ltd., Japan), and energy dispersive X-ray spectroscopy (EDX, Oxford Instruments Link ISIS) were coupled to obtain the micrographs of periphytic biofilm and determine the distribution of NPs and corresponding elements on the biofilm surface. The diversity levels of bacterial communities within the periphytic biofilm matrix were analyzed on day 7 (the end of the experiments) by MiSeq sequencing technology (details provided in the Supporting Information).

The EPS extraction procedure was based on an alkaline method.[23] The total protein content in EPS was measured using the Coomassie brilliant blue staining method.[24] Polysaccharose content in EPS was estimated using the Anthrone method.[25] Humic acid in EPS was analyzed by determining the absorbance at 260 nm, using a double UV−vis spectrophotometer (UV-2450 Shimadzu, Japan).[26]

To evaluate whether EPS played an important role as hole scavengers for nitrate removal, EPS were extracted from periphytic biofilm. The extract was then added to nitrate solution containing TiO_2 NPs under stimulated solar light irradiation (details provided in the Supporting Information). A single-chamber, three-electrode electrochemical quartz cell system was used to distinguish the role of EPS, TiO_2 NPs, and periphytic biofilm for nitrate removal (details provided in the Supporting Information).

Characterization of TiO_2 NPs. Experiments were carried out to detect TiO_2 NPs loading in solution and how they were embedded in EPS (details provided in the Supporting

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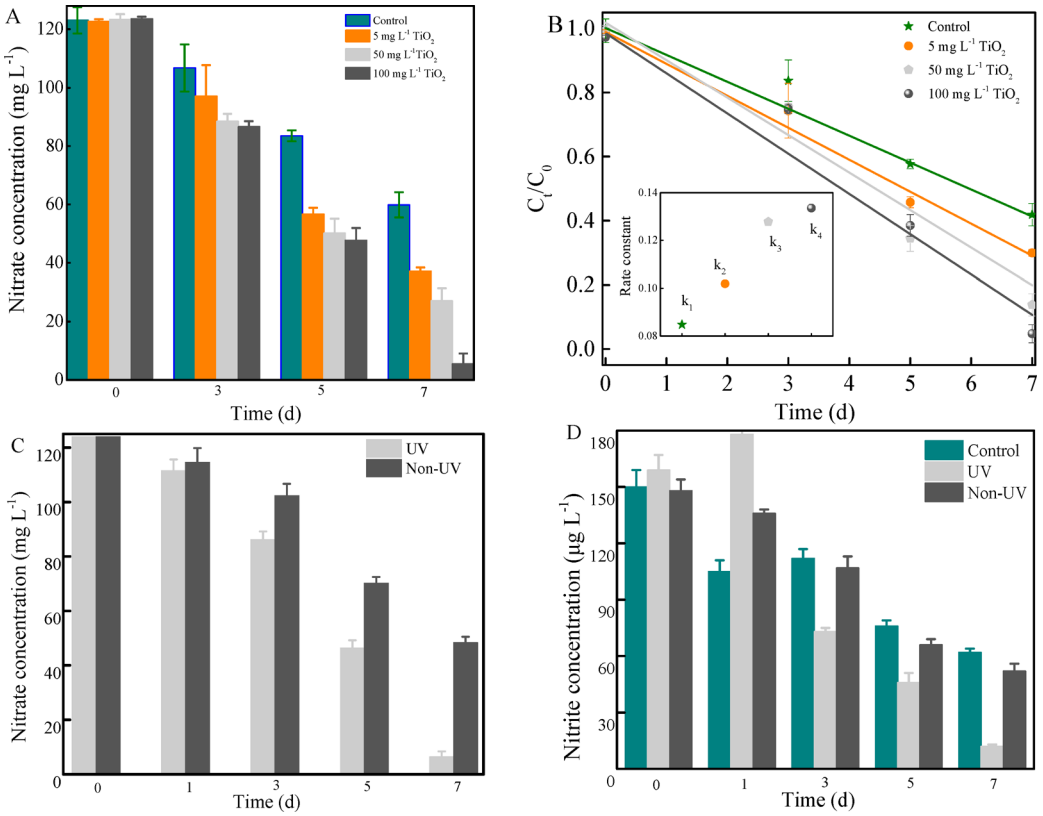

Figure 1. (A) Nitrate removal by periphytic biofilm in the presence of TiO_2 at different concentrations. (B) Nitrate removal rate constant by periphytic biofilm in the presence of TiO_2 at different concentrations. (C) Effects of light irradiation (filtration of ultraviolet from the light (Non-UV)) on nitrate removal. (D) Nitrite production in the presence of TiO_2 (UV) and in the absence of ultraviolet irradiation (non-UV) compared to the control (CK) when the nitrate was removed by periphytic biofilm.

Information). The micrographs of TiO_2 NPs were observed under transmission electron microscope (TEM) (Zeiss 900, Japan). X-ray diffraction (XRD) patterns of the TiO_2 NPs were measured using a Siemens D-501 diffractometer fitted with a Ni filter and a graphite monochromator (LabX XRD-6100, Japan). All the reagents used in this study were of analytical grade.

Analytical Techniques and Statistical Analyses. Nitrate concentration was determined spectrophotometrically according to the Brucine−sulfanil colorimetric method,[27] using a double UV−vis spectrophotometer (UV-2450 Shimadzu, Japan). The pH was measured using a pH meter (PHSJ-4F Leici, Shanghai, China). Nitrite concentration was determined by the Griess Assay reaction which involves a diazo-coupling procedure.[28] The Griess reagents (sulfanilamide and *N*-(1-naphthyl)ethylenediamine) reacted with nitrite sequentially to form a diazo-compound which was detected at 530 nm using a double UV−vis spectrophotometer (UV-2450 Shimadzu, Japan).

Variance partition analysis (VPA) was used to identify the factors that play a major role in nitrate reduction by means of R software. Statistically significant differences between the treatment and the control were evaluated using ANOVA. The probability p value was set at 0.05 for all analyses. All the figures were drawn using Origin 9.0 and R software.

RESULTS AND DISCUSSION

Photocatalytic Property of TiO_2 NPs. TiO_2 NPs had cluster-shaped particles with the particle size distribution ranging between 10 and 40 nm (Figure S1a). The diffraction peaks were 2θ = 25.3°, 37.8°, 48.1°, 53.9°, and 55.1°, which correspond to the 101, 004, 200, 105, and 211 crystal planes, respectively (Figure S1b). These results clearly showed that the crystal phase of TiO_2 NPs used in this study was the anatase phase.

Nitrate Removal in the Presence of TiO_2 NPs. The nitrate removal by periphytic biofilm improved when the concentration of TiO_2 NPs in suspension was higher than 50 mg L^{-1} (Figure 1A). When TiO_2 NPs dosage increased from 0 to 100 mg L^{-1}, the maximum nitrate removal efficiency increased from 58.1% to 95.2%, and the rate constant increased from 0.085 to 0.134 day^{-1} (Figure 1B). Nitrate removal rate constant in treatments with TiO_2 NPs were significantly faster than in the control ($p < 0.05$). To examine whether TiO_2 NPs directly affected the nitrate removal, a separate experiment was conducted under Xe-lamp irradiation in the absence of periphytic biofilm (Figure S2a). The residual nitrate concentrations in the control and 5, 50, and 100 mg L^{-1} TiO_2 NPs treatments were not significantly different during the experimental time of 14 h ($p > 0.05$). This observation clearly indicates that the TiO_2 NPs themselves did not affect nitrate removal.

The fluorescence lamp irradiation experiments showed no significant differences in nitrate removal efficiency in the control and treatment (Figure S2b). The nitrate removal rate constants were 0.086 day^{-1} for control and 0.090 day^{-1} for treatment. Filtration of UV light significantly decreased the nitrate removal efficiency by periphytic biofilm in the presence of TiO_2 NPs ($p < 0.05$). The nitrate removal rate constant was 0.136 day^{-1} in the UV treatment and 0.088 day^{-1} in the non-

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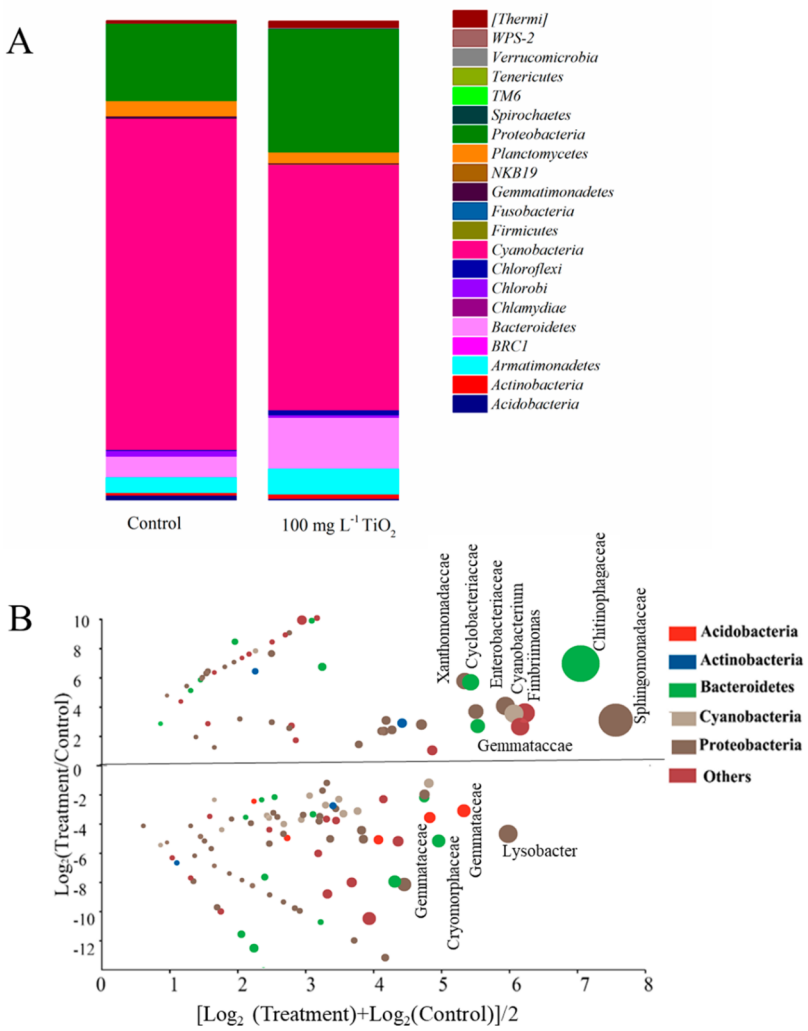

Figure 2. (A) Taxonomic composition of microbial community at the phylum level. (B) Differences in microbial community at the family level between the control and the treatment. The *x*-coordinate indicates the relative abundance of bacteria. The *y*-coordinate represents the objective response rate (ratio of different bacterial abundances in the treatment to that in the control). Positive value means the relative abundance of the bacteria in the TiO_2 treatment is higher than in the control, and the negative value means the relative abundance of the bacteria in the TiO_2 treatment is lower than in the control. All the compared species groups were significantly different ($p < 0.05$).

UV treatment. Nitrate removal efficiency of the UV treatment was significantly higher (33.8%) than that of the non-UV treatment in the presence of TiO_2 NPs from day 3 onward ($p < 0.05$) (Figure 1C). Thus, the response of TiO_2 NPs to light irradiation played an important role in promoting nitrate removal.

Nitrite concentration was also measured during the experimental period because nitrite is an important intermediate product during biological nitrate removal. The nitrite concentration decreased with time in both the control (150 to 62 $\mu g\ L^{-1}$) and non-UV treatment (149 to 56 $\mu g\ L^{-1}$). Nitrite production in the UV treatment increased on the first day (150 to 178 $\mu g\ L^{-1}$), and thereafter it decreased rapidly over the next 6 days (178 to 12 $\mu g\ L^{-1}$). The addition of TiO_2 NPs promoted nitrite production on the first day, but nitrite production was inhibited for the rest of the experiment compared to the control and non-UV treatment (Figure 1D). These results showed TiO_2 NPs contributed to periphytic biofilm by preventing the production of nitrite in the UV treatment. Previous research proved that high concentrations of nitrite may cause emissions of NO/N_2O.[29,30] Nitrate removal in the presence of TiO_2 NPs and periphytic biofilm may have a high selectivity for N_2 because of the low concentration of nitrite. In biological nitrate removal, it is essential to provide external electron donors to convert nitrate to nitrogen gas.[31] This result suggested that TiO_2 NPs may provide their photoexcited electrons for periphytic biofilm to reduce nitrate under Xe-lamp irradiation. With the external electron donor supply, periphytic biofilm could remove nitrate with high N_2 selectivity and few byproducts. These results demonstrated the feasibility of combining a photocatalytic system and a periphytic biofilm for *in situ* removal of nitrate from water.

Response of Periphytic Biofilm to TiO_2 NPs. The growth of periphytic biofilm in the absence (control) and in presence (treatment) of TiO_2 NPs was very similar under the experimental conditions investigated in this study (Figure 2A,B). Bacterial biofilms are sensitive to NPs exposure. Previous studies have reported that NPs have the capacity to modify the broader functions of biofilm and significantly shift the bacterial community structure.[32,33] The results of the bacterial composition analysis (Table S1) showed that the

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Simpson index and Shannon index of bacterial community were 4.79 and 0.81 for the control, respectively, and 5.30 and 0.90 for the 100 mg L^{-1} TiO_2 NPs treatment, respectively. This clearly demonstrates that the invariability and abundance of the bacterial community increased simultaneously in the presence of TiO_2 NPs.

The microbial community composition of periphytic biofilms at the phylum and family levels was determined. Figure 2A shows that 21 phyla were determined, mainly comprising of the phyla *Armatimonadetes*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, and *Proteobacteria* (ranging from 92.7 to 98.0%). Although the first three dominant communities at the phylum level (*Cyanobacteria*, *Proteobacteria*, and *Bacteroidetes*) were the same in the control and the treatments, the proportions of these three communities changed (77.5%, 17.7%, and 4.8% for the control and 57.9%, 29.6%, and 12.5% for the treatment, respectively). Compared to the sensitivity of single species bacteria to TiO_2 under intense UV irradiation,[34] the periphytic biofilms presented good adaptation to TiO_2 under Xe-lamp irradiation. This is because multispecies aggregates, such as periphytic biofilm, can resist unfavorable conditions including TiO_2 exposure because of their interspecies interactions and "collective function".[35] In addition, periphytic biofilm is able to produce more EPS and change their community composition to defend against the toxicity derived from NPs.[36]

Figure 2B compares the microbial composition in the control and treatment. The abundance of *Sphingomonadaceae* and *Xanthomonadaceae* (phylum *Proteobacteria*) and *Chitinophagaceae* and *Cyclobacteriaceae* (phylum *Bacteroidetes*) increased significantly in the presence of TiO_2 NPs compared to the control sample ($p < 0.05$). These results suggested that these four types of bacteria adapted to the TiO_2 NPs conditions well. The *Sphingomonadaceae*, *Xanthomonadaceae*,[37] *Chitinophagaceae*,[38,39] and *Cyclobacteriaceae*[40] have been reported as autotrophic denitrifiers. Autotrophs are able to acquire electrons derived from natural solid donors to reduce electron acceptors.[10] Researchers have proved that *Sphingomonadaceae* and *Xanthomonadaceae* could use the exogenous electrons derived from a semiconductor (e.g., pyrite) to accelerate denitrification.[37] TiO_2 NPs were able to optimize the community composition of multispecies biofilm for specific biofilm-mediated bioremediation processes. These four types of denitrifying bacteria may contribute to nitrate reduction through denitrification or by using the photogenerated electrons produced by TiO_2 NPs to catalyze nitrate reduction in the presence of TiO_2 NPs.

CAT is an important enzyme that indicates the activity of microorganisms against unfriendly habitats.[41] Thus, the CAT activity of periphytic biofilm was determined at the end of the experiment, i.e., after day 7 (Figure 3A). The CAT activity in the treatment was significantly higher than that in the control ($p < 0.05$), further implying that periphytic biofilms were capable of growing well in the presence of TiO_2 NPs. The growth of periphytic biofilm in the absence (control) and in the presence (treatment) of TiO_2 NPs were almost similar under the experimental conditions investigated in this study. The relative abundance of denitrifying bacteria increased significantly in the presence of TiO_2 NPs. However, the NaR also decreased slightly at the end of the experiments (Figure 3A). It is noted that determining activity of NaR required a dark environment which means that TiO_2 will not provide its photoexcited electrons under these circumstances. This

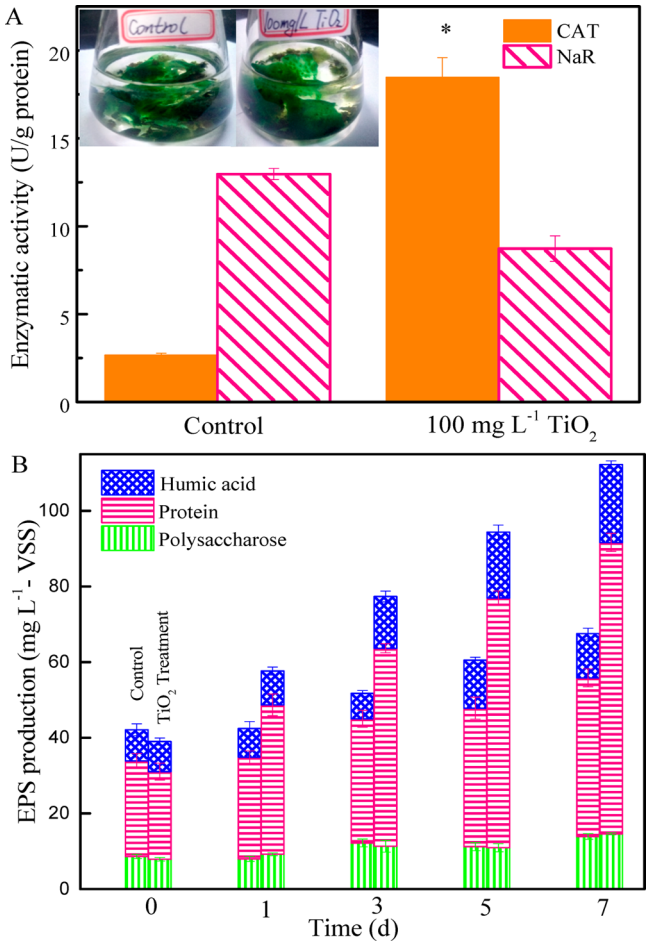

Figure 3. (A) Comparison of catalase (CAT) and nitrate reductase (NaR) concentrations between the control and the treatment (100 mg L^{-1} TiO_2) at the end of the experiment. (B) The humic acid, protein, and polysaccharose contents of extracellular polymeric substance (EPS) extracted from periphytic biofilm; the left bar is the EPS in the control, and the right bar is the EPS in the treatment. Asterisk (∗) indicates a significant difference between the control and the treatment ($p < 0.05$).

denitrifying bacterium may require exogenous electron donor supply to function in denitrification.

To investigate the changes in the composition of EPS, the humic acid, protein, and polysaccharose contents of the periphytic biofilm were determined (Figure 3B). The total EPS concentration (sum of humic acid, protein, and polysaccharose) in the TiO_2 NPs treatment was higher than that in the control ($p < 0.05$). The addition of TiO_2 NPs significantly enhanced the secretion of protein, from 42.2 mg L^{-1} in the control to 76.3 mg L^{-1} in the treatment, on day 7 ($p < 0.05$). After day 3, the concentration of humic acid in the TiO_2 NPs treatment was also significantly higher than that in the control ($p < 0.05$).

Distribution of TiO_2 NPs in Periphytic Biofilm. SEM images showed that the surface of the periphytic biofilm was rough and porous both in the control and in the presence of TiO_2 NPs (Figure S3a,b), while the surface of periphytic biofilm in the presence of TiO_2 NPs was smooth and compact after EPS removal (Figure S3c). The corresponding EDS spectra detected no Ti element on the surface of the periphytic biofilm in the control (Figure S3d). About 1.61 wt % of Ti was distributed on the surface of periphytic biofilm in the TiO_2

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NPs treatment (Figure S3e) while only 0.53 wt % of Ti was detected after EPS was removed (Figure S3f). TEM images showed the morphologies of TiO_2 NPs in periphytic biofilm after 7 days of exposure. There were no obvious particles in periphytic biofilm in the absence of TiO_2 NPs. The TiO_2 NPs were distributed on the surface of periphytic biofilm while some of these particles may permeate into the cell (Figure 4A,B). Some of the TiO_2 NPs were spherical (Figure 4C), and

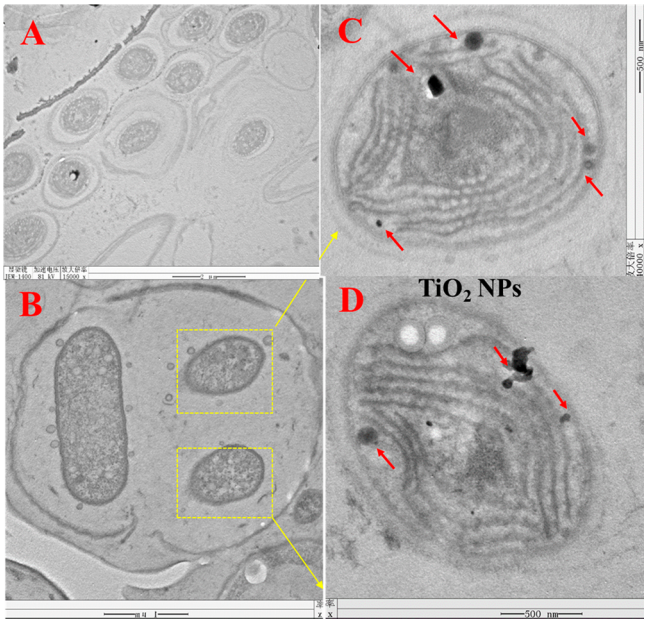

Figure 4. Transmission electron microscope images of periphytic biofilm in the control (A) and in the TiO_2 NPs treatment (B), (C), and (D).

others were irregular (Figure 4D). The diameter of TiO_2 NPs distributed in the periphytic biofilm ranged from 20 to 50 nm. This study also demonstrated that most TiO_2 NPs maintained their initial particle size in the EPS matrix of periphytic biofilm (on the surface of periphytic biofilm). Our previous studies proved that nanomaterials promoted the production of EPS, which in turn played an important role in defending against nanoparticle toxicity. TEM, SEM-EDS, and synchrotron radiation X-ray technology determined that the majority of nanomaterials were distributed in the EPS matrix while few entered into the cells.[36,42]

There are many studies about TiO_2 NPs in disinfection of pure strains of microorganisms, such as bacteria or algae.[15,16] Although these investigations about the application of TiO_2 NPs represent a step forward, the potential interactions between TiO_2 NPs and microbial aggregates are still unknown. This study demonstrated that periphytic biofilm possesses the ability to adapt to the environment in the presence of TiO_2 NPs through changing community composition and structure and increasing secretion of catalase. Periphytic biofilm enhanced the production of EPS which helped prevent most NPs penetrating into the cells of microbes in the periphytic biofilm. Moreover, the distribution of NPs, such as nano Pd crystallites, on the cell surface in the EPS may facilitate nitrate removal by denitrifying biofilm.[43]

Mechanism of Nitrate Removal in the Presence of TiO_2 NPs. In general, the nitrate removal is attributed to denitrification for which the main contributors are the denitrifying-functional bacteria and fungi.[44] The addition of TiO_2 NPs also increased the pH in this study. The high pH (8.0−10.0) conditions used in this study (Figure S4) did not provide the physical environment needed for the survival of fungi.[45] Since nitrate removal was not solely due to nitrate-reducing bacteria or fungi, other possible mechanisms were considered. Previous studies have showed the conversion of nitrate to nitrite with undesirable production of nitrite in the presence of TiO_2 NPs and humic acid under irradiation by a 150 W Xe high-pressure lamp.[11,46] Herein, we hypothesized that the TiO_2 NPs present made the main contribution to the enhanced nitrate reduction. Three-electrode electrochemical quartz cells were used to investigate the possible pathway of photogenerated electrons produced by TiO_2 NPs. The results from amperometric $I-t$ curves show that irrespective of the nature of the treatments (with TiO_2 NPs, periphytic biofilm, and periphytic biofilm + TiO_2 NPs), no current was produced under the dark condition of the light/dark cycle (Figure 5A). Under the Xe-lamp irradiation, strong (3.0−7.0 mA m^{-2}) and weak (0.9−2.9 mA m^{-2}) currents were detected in the periphytic biofilm + TiO_2 NPs and TiO_2 NPs only treatments, respectively. No current was determined in the cell that contained only the periphytic biofilm (Figure 5A). On the other hand, treatments with TiO_2 NPs alone were not able to reduce nitrate. Periphytic biofilm alone was able to remove nitrate, although the nitrate removal efficiency was much lower than that of the periphytic biofilm + TiO_2 NPs treatment (Figure 5B). The addition of TiO_2 NPs showed a light response coupling with an improved nitrate reduction efficiency, indicating that the periphytic biofilm was capable of reducing nitrate using the photogenerated electrons derived from TiO_2 NPs. In this study, the nitrate removal rate increased by ∼1.9 and 1.6 times in the presence of UV compared to the control and non-UV treatment, respectively. Thus, the light response of TiO_2 NPs contributed to an improvement in the nitrate removal rate by ∼84%. To distinguish whether TiO_2 NPs in solution or embedded in the EPS made the main contribution to nitrate removal, TiO_2 NPs loading in solution was carried out. Results show that there was a more significant relationship between TiO_2 NPs embedded in EPS and removal efficiency of nitrate (R^2 = 0.901, $p < 0.05$) than the relationship between TiO_2 NPs loading in solution and removal efficiency of nitrate (R^2 = 0.454, $p < 0.05$) (Figure S5). To explore the hole scavengers in this system for nitrate removal, the EPS extract was then added to nitrate solution containing TiO_2 NPs under stimulated solar light irradiation. Results showed 16.3% of nitrate reduction occurred in the presence of both EPS and TiO_2 NPs. When the concentration of sodium alginate increased from 5 to 20 mg L^{-1}, the nitrate removal rate increased from 12.6 to 23.9%. The nitrate removal rate did not increase when the concentration of bovine serum albumin increased. Nitrate removal efficiency fluctuated, ranging from 2.2 to 5.1% in the presence of humic acid. On the basis of these results, it is possible that the polysaccharose content can serve as a hole scavenger (Figure S6).

The Pearson's r correlation coefficient was used to compare the dimensionally homogeneous quantitative variables (e.g., nitrate, nitrite, EPS, polysaccharose, protein, humic acid, and pH) using the R software. There was an insignificant negative relationship between the nitrate residues and humic acid in the control (r = −0.836, $p > 0.05$) but a significant negative relationship between nitrate and humic acid in the treatment (r

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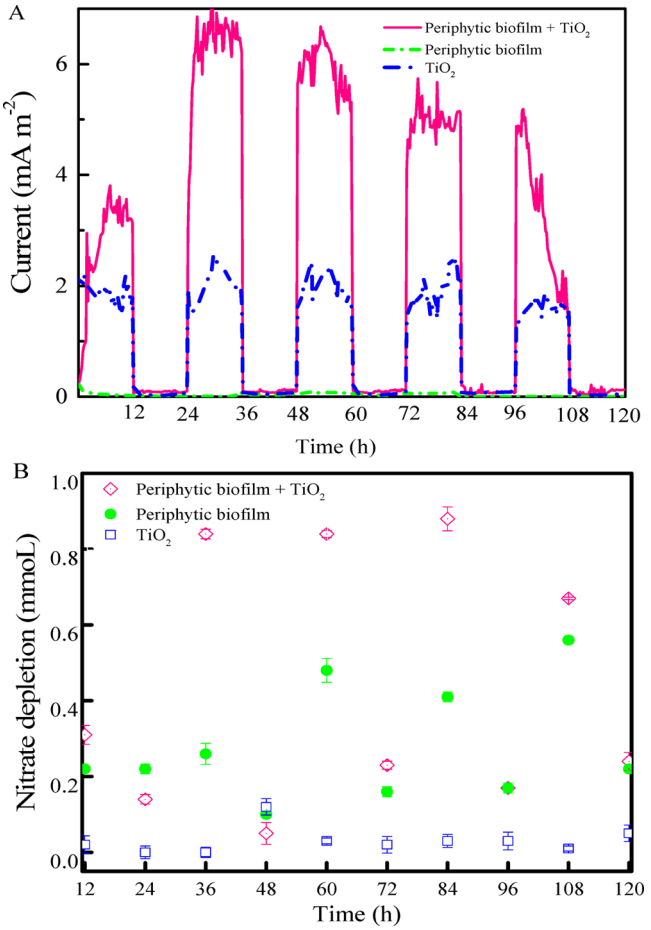

Figure 5. (A) Amperometric $I-t$ curves recorded from a three-electrode electrochemical quartz chamber with a light on/off cycle (12/12 h) at light intensity of 20 W m^{-2}. A tin-doped In_2O_3 (ITO) glass electrode (7.1 cm^2) served as the working electrode while a platinum plate (1 cm^2) and a Ag/AgCl (KCl saturated) electrode were used as the counter and reference electrodes, respectively. The working electrode was poised at +200 mV versus Ag/AgCl throughout the incubation using a CHI1040C potentiostat (Chenhua Shanghai China), and the current was recorded versus time. Periphytic biofilm means a ITO working electrode with the incubating periphytic biofilm, TiO_2 means a TiO_2 decorated ITO electrode without periphytic biofilm, and periphytic biofilm + TiO_2 means a TiO_2 decorated ITO electrode with the periphytic biofilm in the electrochemical cell. (B) Nitrate consumption in the electrochemical chambers.

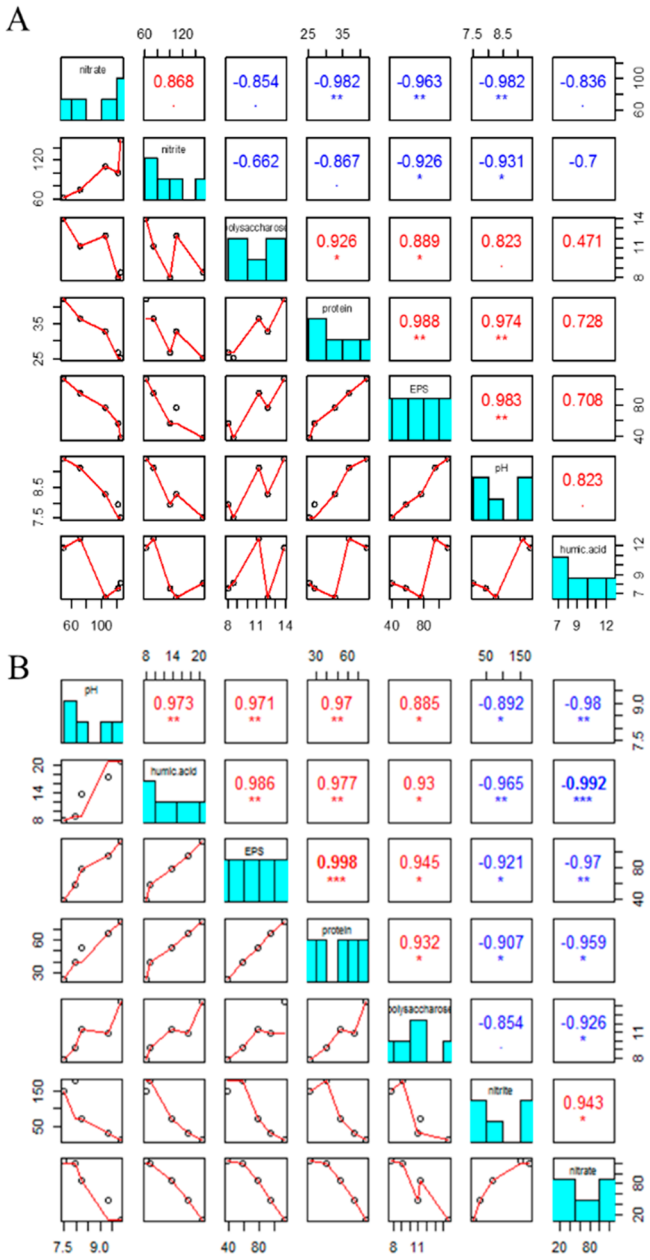

Figure 6. Pearson analysis among the nitrate, nitrite, polysaccharose, protein, humic acids, extracellular polymeric substance (EPS), and pH in the control (A) and in the treatment (B), *significant difference at the <0.05 probability level, **significant difference at <0.01 probability level, ***significant difference at the <0.001 probability level. These seven factors are located on the diagonal. The point of intersection between every pair of factors located in the bottom left of the figure represents the Pearson correlation coefficient between these two factors. The correlation coefficient is showed by the point of intersection between every pair of factors located in the upper right of the figure represents the Pearson correlation coefficient between these two factors.

= −0.992, $p < 0.01$) (Figure 6A,B). Similarly, a negative relationship was observed between humic acid and nitrite production in the treatment ($r = -0.935$, $p < 0.05$), but not in the control (Figure 6A,B). Our results suggest that the production of humic acid was enhanced by TiO_2 NPs which promoted nitrate reduction and inhibited nitrite production by the periphytic biofilm. The presence of TiO_2 NPs enhanced the reduction of nitrate to nitrite by the periphytic biofilm, but it caused a decrease in the NaR activity. The synergistic action of TiO_2 NPs, the EPS, and bacteria on nitrate removal was investigated using VPA analysis. The results show that the synergistic action of TiO_2 NPs, humic acid, protein, and bacteria (*Sphingomonadaceae*, *Xanthomonadaceae*, *Chitinophagaceae*, and *Cyclobacteriaceae*) contributed to an improvement in nitrate removal by ~80% (Figure 6C). Nitrate reduction by bacterial denitrification processes requires electron donors, but the natural environment, such as surface water, has a low background concentration of electron donors.

In the first step of denitrification (eq 1), two electrons and two protons are required for conversion of nitrate to nitrite. These electron donors, related to the physiological donor ubiquinol, an electron transfer mediator, are usually ineffective,

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while two protons often originate from the cytoplasm resulting from the movement of free electrons.[47,48]

$$NO_3^- + 2H^+ + 2e^- \xrightarrow{NaR} NO_2^- + H_2O \quad -89.2\ kJ/mol \tag{1}$$

Previous studies have also confirmed that microorganisms, such as *Shewanella loihica* PV-4, possess the ability to utilize photogenerated conduction-band electrons derived from α-Fe_2O_3.[49] In this study, the electrons generated during the photoexcitation of TiO_2 NPs accelerated the reaction shown in eq 1, leading to denitrification and decrease in H^+ concentration. The decreased in H^+ concentration was also mirrored in the changing pH values, where the pH increased with an increase in the initial TiO_2 NPs concentration. Periphytic biofilm could be a sanctuary for the denitrifying bacteria due to the increased production of EPS.[36,42] EPS is able to act as an electron transit medium, benefiting the microbial extracellular electron transfer process.[20] Among the components of EPS, humic acid is capable of serving as a mediator to help extracellular electron transfer.[50] Amperometric $I-t$ curves were measured in the three-electrode electrochemical quartz cells in the presence or absence of EPS. Results showed the current intensity decreased (from 4.843−5.911 to 1.796−3.221 mA m^{-2}) after EPS was removed from the periphytic biofilm and TiO_2 NPs system (Figure S7A). LSV results of EPS from periphytic biofilm showed two peaks at −0.409 and −0.106 V (Figure S7B). These peaks represented the presence of Fe related humic acid.[51] Therefore, in this study, the inclusion of TiO_2 NPs promoted the production of EPS (i.e., humic acid) which played an important role in electron transfer between TiO_2 NPs surfaces and denitrifying bacterial communities present in the periphytic biofilm as electron mediators.

Practical Implications of This Work. Although the study of *in situ* nitrate reduction in natural water systems based on photocatalysis is still in its infancy, to our best knowledge, this is the first time an *in situ* coupling system of simultaneously supplying denitrifiers, electron donor pool, and electron mediators in surface waters which promote nitrate reduction has been created. From a practical viewpoint, it is advisible to stimulate the periphytic biofilm with TiO_2 NPs for nitrate removal. Nitrate removal depends on the NaR activity if the denitrification step is dominated by denitrifying bacteria.[52,53] Interestingly, in this study, the synergistic effect of TiO_2 NPs, protein, humic acid, and nitrate-denitrifying bacteria contributed to the majority of the nitrate removal despite a decrease in NaR activity. The results obtained from this study clearly show the application of TiO_2 NPs as a promising alternative to enhance nitrate reduction in biofilm-based wastewater treatment systems or photocatalyst applications which are ineffective under sunlight illumination.

The potential risks of TiO_2 NPs being released into the natural environments should be considered. Fortunately, the immobilization of TiO_2 NPs on glass substrate, quartz fiber filters, and porous titanium sheets have been successfully applied for wastewater treatment,[54] which provide a possible way to reduce TiO_2 NPs leaching and mobility in practice. Moreover, the ubiquitous multispecies microbial aggregates (i.e., periphytic biofilm) produced more EPS which is able to entrap the TiO_2 NPs in the biofilm matrix, preventing the release of TiO_2 NPs into water. Future research is needed, however, before large scale application.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b01605.

Materials and Methods: the composition of WC culture medium, experimental details about the influence of light irradiation of TiO_2 NPs on nitrate removal, hole scavengers and TiO_2 NPs distribution, high-throughput sequencing, electrochemical analysis, and TiO_2 NPs distribution; parameters from MiSeq sequencing (Table S1); variation partition analysis result (Table S2); characteristics of TiO_2 NPs, nitrate removal by only TiO_2 NPs, distribution of TiO_2 NPs in periphyton matrix, variation of pH in the presence of TiO_2 NPs, relationship between TiO_2 NPs in solution or embedded in EPS and nitrate removal, and the hole scavengers for nitrate removal (Figures S1−S6); electrochemical analysis of periphytic biofilm and EPS (Figure S7) (PDF)

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Notes

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