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A humic substance analogue AQDS stimulates *Geobacter* sp. abundance and enhances pentachlorophenol transformation in a paddy soil

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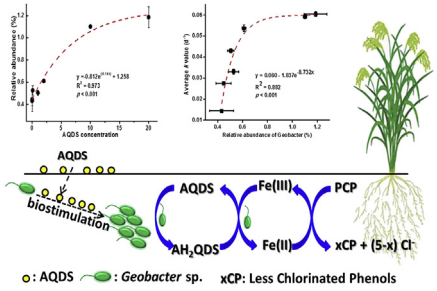
HIGHLIGHTS

- We report the enhancement mechanism of AQDS for PCP transformation in paddy soil.
- Higher dosages of AQDS (>2 mM) have significant effect on soil microbial community.
- Added AQDS enhances the amount of *Geobacter* sp. and active Fe(II) species.
- *k* value of PCP transformation is positive exponent relationship with AQDS dosage.

GRAPHICAL ABSTRACT



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ABSTRACT

Soil humic substances can be used as redox mediators in accelerating the biotransformation of organic pollutants, and humus-respiring bacteria are widely distributed in soils. However, the impact of humic substances on the soil microbial community during the biotransformation of organic pollutants is expected to be crucial while remains to be unclear. In this study, the biostimulation of indigenous microbial communities and the consequent effects on anaerobic transformation of pentachlorophenol (PCP) by a model humic substance, anthraquinone-2,6-disulfonate (AQDS), were systematically investigated in a paddy soil. The addition of AQDS was observed to increase the production of HCl-extractable Fe(II) and enhance the PCP transformation rates consequently. The pseudo-first-order rate constants of the PCP transformation showed a positive exponential relationship with the AQDS dosage. The terminal restriction fragment length polymorphism (T-RFLP) results indicated the substantial effect of added AQDS on soil microbial community. The enhanced abundance of *Geobacter* sp. was disclosed to be most critical for accelerated PCP transformation when with AQDS, in which *Geobacter* sp. functioned for promoting the generation of active Fe(II) and consequently enhancing the PCP transformation rates. The transformation rates of PCP were exponentially correlated with the abundance of *Geobacter* sp. positively. The

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findings are expected to improve the understanding of diversity and ubiquity of microorganisms in humic substances-rich soils for accelerating the transformations of soil chlorinated pollutants.

1. Introduction

Humic substances are important active heterogeneous polyelectrolytes in soils and contain multiple functional groups, including carboxylic acid, alcoholic and phenolic hydroxyl, sulfhydryl, quinone, and ketone groups (Stevenson, 1994; Hernández-Montoya et al., 2012). Due to their structured functional groups, humic substances are highly active in soil biogeochemical processes and greatly impact many prevailing soil reactions, such as methanogenesis, nutrient uptake, iron reduction, and transformation of organic or inorganic pollutants (Lovley et al., 1996; Kalinichev and Kirkpatrick, 2007; Van der Zee and Cervantes, 2009; Martinez et al., 2013). The most-reported role of humic substances in soil biogeochemical processes is acting as an electron shuttle, i.e. a simultaneous electron donor/acceptor for microorganism respiration and redox mediator, for reduction processes in the abiotic and biotic transformation of pollutants (Field et al., 2000; Martinez et al., 2013). Humic respiring bacteria have been isolated from a wide range of freshwater or marine environments and are mostly iron reducers related to the family Geobacteriaceae (Coates et al., 1998; Straub et al., 2005). Consequently, humic substances can be reduced by dissimilatory Fe(III)-reducing bacteria (such as *Geobacter* sp.) and shuttle electrons to Fe(III) oxides, and then enhance the formation of the active Fe(II) species (Lovley et al., 1996; Roden et al., 2010). Furthermore, humic substances also have been reported to greatly stimulate the degradation of organochlorine pesticides, such as chlorophenols, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)- ethane (DDT), and hexachlorocyclohexanes (HCH), by enhancing the formation of the active Fe(II) species under iron-reducing conditions (Lovley et al., 1996; Watanabe et al., 2009; Cao et al., 2012). Supplemented humins in soils were found to act as both electron donors and electron acceptors in extracellular electron transfer, so as to accelerate the microbial reductive dehalogenation of pentachlorophenol (PCP) (Zhang and Katayama, 2012). Compared with the incubation containing co-substrate in the absence of AQDS, previous study reported that the addition of humic substance analogue, anthraquinone-2,6-disulfonate (AQDS), together with co-substrate (e.g. lactate and glucose), enhanced the transformation rates of PCP by increasing the reduction potentials of the reaction system and accelerating rates of electron transfer (Chen et al., 2012; Xu et al., 2014).

In addition, previous reports also suggested that humic substances altered the structure and activity of soil microbial communities (Dong et al., 2009; Puglisi et al., 2009). For example, humic acid supplements in maize soil increased the content of bioavailable carbon, leading to changes in the structures of microbial communities in the plant rhizosphere (Puglisi et al., 2009). Humic acids buffered urea enhanced its bioavailability of urea, which consequently altered the composition and abundance of the soil microbial community (Dong et al., 2009). Due to the changed microbial community, the transformation processes of soil pollutants would be further affected consequently. Although humic substances have been reported to change the structure and activity of soil microbial communities, there is much less evidences for the involvement of humic substances in the microbial reductive dechlorination of organochlorine pesticides, especially the evidences for impacting the microorganisms that responsible for the enhanced reductive dechlorination in paddy soil.

Zhang and Katayama (2012) demonstrated that the addition of solid-form humins could enhance the PCP transformation rate by changing the microbial community composition. The anaerobic biotransformation of PCP in paddy soil was accelerated in the presence of AQDS and lactate in our previous study by stimulating activities of the bacteria with potential dechlorinating abilities, although the microbial community had no significant from the incubation with lactate only (Chen et al., 2012, 2016). However, few reports are available on the impact of humic substances (acting as the sole co-substrate) on the abundances of indigenous microorganisms and changes in the microbial communities, which ultimately affecting the transformations of organochlorine pesticides in paddy soils.

Therefore, the present study was carried out to systematically investigate the effect of AQDS, one of the humic substances analogue, on the microbial community and the bacteria with potential dechlorinating abilities, such as dechlorinating- and iron-reducing bacteria, for PCP transformation, and consequently on the transformation rate of PCP in a paddy soil. PCP was selected as the target organochlorine pesticide because it has been widely used as a fungicide and wood preservatives, which have been reported to pose harmful effects on soil systems (Hong et al., 2005). This emphasis of the present study was focused on elucidating the microbial mechanism of the anaerobically reductive transformation of PCP enhanced by AQDS in a paddy soil by studying (i) the effects of AQDS amendment on changes of the soil microbial community, and (ii) the microbial mechanism of AQDS impacting on the reductive transformation of PCP.

2. Materials and methods

2.1. Soil sampling and its physicochemical properties

The soil sample used in this study was collected from a rice field located in Jiangmen City, Guangdong Province, China (22°22.352′N, 112°48.426′E). The method for soil collection was described previously (Chen et al., 2012). The basic physicochemical properties of the soils, including organic matter (29.5 g/kg), cation exchange capacity (CEC) (83.8 mmol(+)/kg), total K (10.97 g/kg) and total N (3.81 g/kg), were determined using the methods described previously (Chen et al., 2012). At the time of sample collection, no residual PCP in soil was detected.

2.2. Chemicals

PCP (98+% purity), AQDS (97+% purity), and 1,4-piperazinediethanesulfonic acid (PIPES, 98+% purity), were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. The other chemicals were all of analytical grade and obtained from Guangzhou Chemical Co., China. The PCP stock solution (376 μM) was prepared by dissolving PCP in 1.0 M NaOH solution.

2.3. Microcosm set-up for PCP transformation under anaerobic condition

All anaerobic incubation experiments were carried out in 20 mL sterile serum bottles. PIPES solution at a concentration of 30 mM buffered at pH 7.0 ± 0.1 was used as the reaction medium. These

bottles contained sterile and anoxic reaction suspension (10 mL), 2.0 g of wet soil (1 g dry soil), and 17 μM PCP. The 180 bottles used in this study were divided into nine treatment groups that received soil samples and a combination of AQDS (Table 1). Three controls were used, including a sterile soil slurry without AQDS (SC1) addition, sterile soil slurry with 2 mM AQDS (SC2) and soil slurry without AQDS addition (CK). The soil was sterilized by 50 KGy of γ-irradiation. Another set of controls without PCP, including Soil + PIPES and Soil + PIPES + 2 mM AQDS, tested whether AQDS could be utilized by the indigenous microorganisms. A set of abiotic controls, containing sterile soil samples, Fe(II) (1 mM), and AQDS (0, 0.01, 0.1, 1, 2, 10, and 20 mM) were also included. After all of the designated regents were added into the bottles and purged with O_2-free N_2 for 15 min, the bottles were sealed with autoclaved silicone-lined septa and aluminum crimps. The bottles were incubated then at 25 ± 1 °C in the dark in a Bactron Anaerobic/Environment Chamber II (Shellab, Shedon Manufacturing, Inc., Cornelius, OR, USA). At specific intervals (t = 0, 2, 4, 7, 15, and 30 days), the bottles were taken out to measure PCP, PCP metabolites and ferrous iron, and perform DNA extraction. For microbial community analyses, the soil samples were collected in the vials after incubation for 15 days and frozen to −20 °C. Each experiment was conducted in triplicate and included blanks, and analytical determinations were performed in duplicate.

2.4. Analyses of iron species and PCP and its intermediates

The HCl-extractable Fe(II) concentration, i.e., the microbially produced Fe(II) including the dissolved and adsorbed Fe(II), was determined using a 1,10-phenanthroline colorimetric assay after extracting reaction suspensions with 0.5 M HCl for 1.5 h and assaying the extract (Fredrickson et al., 1998). PCP concentrations were determined by high-performance liquid chromatography (HPLC) and the PCP transformation products were identified by gas chromatograph-mass spectrometry (GC-MS). The HPLC and GC-MS analytical procedures were the same as previous described (Fava and Piccolo, 2002; Chen et al., 2014). The recovery of the added standard in the extraction and determination process ranged from 95.6% to 103.8%.

2.5. Microbial analyses

To investigate the effect of AQDS on the microbial community and key bacteria, i.e., iron-reducing and dechlorinating bacteria that are responsible for the dechlorination of PCP in the soil, three microbial techniques, including terminal restriction fragment length polymorphism (T-RFLP), 16S rRNA clone library, and real-time quantitative PCR (qPCR), were used in the present study.

The total soil DNA was extracted from 0.25 g of soil (in triplicate) using the PowerSoil™ DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA concentrations were determined with a Qubit 2.0 Fluorometer (Invitrogen, USA). Bacteria 16S rRNA genes were amplified by PCR from total DNA by using primers 27F (5′ end labeled with 6-carboxyfluorescein) and 1492R (Hongoh et al., 2003).

The labeled PCR products were purified with a commercial PCR purification kit (OMEGA Biotek, USA) and digested with *Alu*I restriction endonucleases (TaKaRa Biotechnology, China) at 37 °C for 6 h according to the manufacturer's protocol. Terminal restriction fragments (T-RFs) were separated on a 3730xl Genetic Analyzer (Applied Biosystems, USA). The data were analyzed using the Peak Scanner software V1.0 (Applied Biosystems, USA). Only fragments with an intensity above a baseline threshold (50 fluorescent units) were recorded to reduce data "noise". The relative abundance of individual T-RFs was calculated as the percentage of the total peak area in a given T-RFLP profile, and those that were <5% for each fragment were excluded from further analysis. Each measurement was performed in quadruplicate.

Clone libraries were constructed to phylogenetically identify T-RFs. The purified PCR products were ligated into vector pCR2.1-TOPO according to the manufacturer's instructions (Invitrogen, USA). Selected clones were grown in 1.5 mL of Luria–Bertani (LB) medium amended with 50 μg/mL of ampicillin. Clones were screened for inserts with PCR primers of M13F and M13R and subsequently sequenced on an ABI 3730xl sequencer. Quality filtering and chimera removal were performed with mothur software (Schloss et al., 2009). The high-quality sequences were taxonomically classified using the RDP pipelines. The nucleotide sequences of the clones retrieved in this study have been deposited with GenBank under accession numbers KP138717–KP138795.

In this study, the abundance of bacterial population and the bacteria with potential dechlorinating abilities, such as dechlorinating- and iron-reducing bacteria, were quantified using specific primers (Table S1) with MyiQ™2 Two Color Real-Time PCR Detection System (Bio-Rad). The reaction mixtures (final volume 25 μL) contained 12.5 μL of SYBR Green Mix (TaKaRa Bio. (Dalian) Co., Ltd.), pairs of primers (62.5 nM each), and 0.5 μL of soil DNA. Each measurement was performed in triplicate. The PCR conditions were the same as described previously (Smits et al., 2004; Himmelheber et al., 2009; Nelson et al., 2011). The melting curve analysis was performed at 1 °C/30 s from 55 to 95 °C. At each run, a standard curve was included, and all samples were related to this standard curve. Calibration curves were obtained using serial dilutions of plasmids carrying a single, cloned target gene. Copy numbers were calculated based on the plasmid size, insert lengths and Avogadro number (Whelan et al., 2003), and the relative abundances of target genes were normalized to the total bacterial community.

2.6. Statistical analyses

A statistical analysis of the experimental data was performed using the SPSS 20.0 statistical software. The differences among the transformation kinetics of PCP in all non-sterile treatments were examined by one-way analyses of variance (ANOVA) on ranks followed by Fisher's least-significant-difference (LSD) test with the levels of significance at $P < 0.05$. The relation between AQDS dosage and the abundance of T-RF was examined by two-tailed Pearson correlation analyses (significance level: $P < 0.05$). T-RF profiles of 16S rDNA amplified directly from the soil samples of different batches were used to construct principal component analysis (PCA) (Dollhopf et al., 2001). The symbols and error bars in the given results represent the averages and standard deviations, respectively.

Table 1
Treatment methods, first-order rate constants (k) of PCP transformation under anaerobic conditions at 25 °C and pH 7.0 (30 mM PIPES) in paddy soil.

Treatment methods	k (d^{-1})	R^2
Sterile soil + PIPES (SC1)	/	/
Sterile soil + PIPES + 2 mM AQDS (SC2)	/	/
Soil + PIPES (CK)	0.014 ± 0.0004^{e}	0.719
Soil + PIPES + 0.01 mM AQDS	0.028 ± 0.0005^{d}	0.980
Soil + PIPES + 0.1 mM AQDS	0.033 ± 0.0012 ^{c}	0.990
Soil + PIPES + 1 mM AQDS	0.043 ± 0.0008^{b}	0.986
Soil + PIPES + 2 mM AQDS	0.054 ± 0.0006^{a}	0.976
Soil + PIPES + 10 mM AQDS	0.059 ± 0.0011^{a}	0.963
Soil + PIPES + 20 mM AQDS	0.060 ± 0.0011^{a}	0.950

a, b, c, d and e within the same column indicate the significance of difference ($P < 0.05$).

3. Results

3.1. Anaerobic transformation of PCP with supplemented AQDS

The transformation of PCP in the paddy soil with different concentrations of AQDS were provided in Fig. 1a, which indicated that PCP transformation generally follows the pseudo-first-order kinetics rate law. The rate constants (k) values were then obtained from the pseudo-first-order model fitting and listed in Table 1. No obvious change in the PCP concentration were observed in the sterilized treatments (SC1 and SC2) after a 30-day cultivation period, which indicated that abiotic processes did not contribute to the transformation of PCP in paddy soils. However, approximately 20.8% of the PCP was transformed in the non-sterile paddy soil (CK treatment) with a transformation rate constant value of 0.014 d^{-1}, suggesting the microbial transformation of PCP by the soil microorganisms. The presence of AQDS further enhanced the transformation rates of PCP, and the rate increased gradually with the increase of AQDS concentrations from 0 mM to 20 mM. After incubation for 30 d, 37.4%, 40.7%, 49.6%, 59.2%, 64.6% and 65.9% of the initial PCP were transformed when the soils were supplemented with 0.01, 0.1, 1, 2, 10, and 20 mM AQDS, with the k-values of 0.028, 0.033, 0.043, 0.054, 0.059, and 0.060 d^{-1}, respectively. Compared with the kinetic without AQDS treatment (CK), the reaction in the soil suspension with AQDS proceeded at a faster rate ($P < 0.05$) (Table 1). These results suggested that PCP could be transformed in the paddy soil, and the supplemented AQDS was a critical factor for enhancing the transformation rate. To obtain deep insight into the effect of AQDS on PCP transformation processes, the relationship between AQDS concentrations and the PCP transformation rate was further studied. As shown in Fig. 1b, the k values of the PCP transformation had a positive exponential relationship with the concentration of added AQDS ($R^2 = 0.884$, $p < 0.001$). To accelerate the PCP transformation process through the supplement of AQDS in the soil, 2 mM would be the most efficient concentration of AQDS, while higher dosages would not significantly increase the transformation efficiency of PCP any further (Table 1, Fig. 1b).

The transformation of PCP in the sterile soil with 1 mM Fe(II) and different concentrations of AQDS were provided in Fig. S1. About 63.49 ± 1.26% of PCP were transformed in all treatments after incubation for 30 days and there were no significant difference among the treatments with different concentrations of AQDS. These results indicated that PCP could be chemically transformed by Fe(II) in the sterile soils, which was consistent with the previous study (Li et al., 2008).

The intermediates of the PCP transformation after incubation for 30 days were determined by GC-MS and the results were shown in Fig. S2. The results indicate that the PCP transformation is a reductive transformation in which PCP is reductively dechlorinated to tetrachlorophenol (TeCP) and trichlorophenol (TCP).

3.2. Fe(II) generation with AQDS amendment during PCP transformation

The produced 0.5 M HCl-extracted Fe(II), including the forms of adsorbed and dissolved Fe(II), are the active iron species with the highest reduction potential for PCP transformation (Fredrickson et al., 1998; Chen et al., 2014). The changes in the produced 0.5 M HCl-extractable Fe(II) during the PCP transformation are shown in Fig. 2. Only 2.74 mM and 2.87 mM of HCl-extractable Fe(II) were generated after 30 d incubation in the sterile treatments SC1 and SC2, respectively, and no obvious changes were observed throughout the PCP transformation process. However, the concentration of HCl-extractable Fe(II) in all of the non-sterile treatments increased markedly in the first 7 days and then stayed relatively constant, which indicates that most of the Fe(II) species were microbially generated by the soil microorganisms. Overall, the higher concentration of AQDS resulted in a higher production of HCl-extractable Fe(II).

3.3. Effect of AQDS on soil microbial community structure and the abundance of bacteria responsible for PCP reductive transformation

To investigate the changes in the microbial community and its further effects on the reduction of Fe(III) and transformation of PCP in the soil system with supplemented AQDS, reaction samples incubated for 15 days were analyzed by T-RFLP targeting the bacterial 16S rRNA genes. Furthermore, clone libraries were constructed from bacterial 16S rRNA genes to identify the phylogenetic taxonomy of the dominant T-RFs. A total of 12 T-RFs were discovered across all samples, and only those with relative abundance >5% were analyzed (Table 2, Fig. 3). Seven T-RFs, including 147, 191, 220, 233, 236, 242, and 249 bp, were detected as the major peaks in the T-RFLP profile. The most abundant fractions in all of the enrichments were 233 and 236 bp T-RFs, with relative abundances of 18.31% ± 1.94% and 21.12% ± 1.36%, respectively. Clones representing the T-RFs of 233 and 236 bp, which had 98% and 99% sequence homology with iron-reducing bacterium, were affiliated to *Burkholderiales* family and unclassified bacteria, respectively (Wang

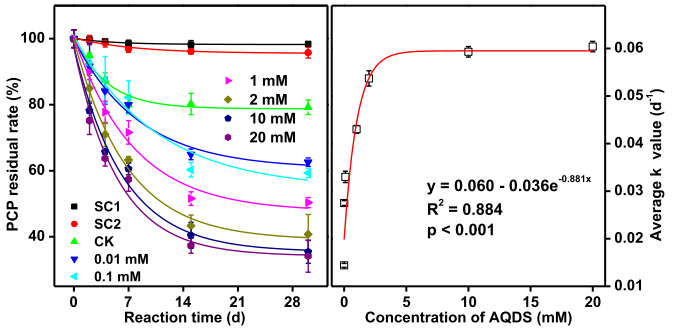

Fig. 1. (**a**) Kinetics of PCP transformation in different concentration of AQDS under anoxic conditions at 25 °C and pH 7.0 (PIPES): sterile control (SC1 and SC2), control, 0.01 mM, 0.1 mM, 1 mM, 2 mM, 10 mM, and 20 mM AQDS. Error bars show standard deviation from triplicates. (**b**) The relationship between the k-values of PCP transformation and the concentrations of AQDS added.

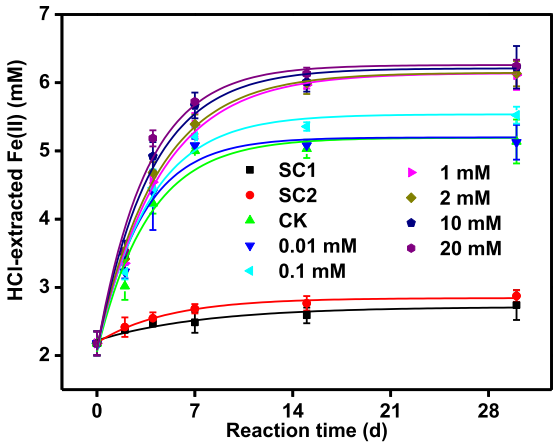

Fig. 2. The generation of HCl-extractable Fe(II) during PCP transformation in the paddy soil supplemented with different concentrations of AQDS. Error bars show the standard deviation from triplicates.

et al., 2009; Bruun et al., 2010). This indicates that iron-reducing bacteria were the dominant species in the microbial community of PCP transformation. The T-RFs of 147 bp, 191 bp and 242 bp were affiliated to *Clostridium* genus, the T-RF of 220 bp represented *Lachnospiracea_incertae_sedis* genus, and the T-RF of 249 bp represented *Veillonellaceae* genus. To further confirm the effect of AQDS on the microbial community, the relationship between AQDS concentrations and the abundance of the dominant T-RFs was examined. The results showed that the AQDS concentrations were positively correlated with relative abundance of T-RF 249 bp ($r = 0.790$) ($P < 0.05$) significantly. Clone representing the T-RFs of 249 bp had 98% similarity to *Pelosinus* sp. UFO1 strain, which has been reported previously to reduce AQDS, Fe(III), Cr(VI) and U (VI) (Ray et al., 2011). This result indicated that T-RF 249 bp played an important role in the reduction of AQDS and Fe(III), which might have potential ability for PCP degradation.

In addition, the changes in the microbial communities in all treatments were analyzed using principal component analysis (PCA) of T-RFLPs (Dollhopf et al., 2001) (Fig. 4). Based on the visual inspection of Fig. 4, there was a clear difference in the T-RFLP profiles between microcosms that depended on the concentrations of AQDS. PCA revealed two different groups; in which the first group (group A) consisted of high dosage AQDS treatments (2, 10, and 20 mM AQDS), and the second group (group B) consisted of relative low dosage AQDS treatments (CK, 0.01, 0.1, and 1 mM AQDS). Group A exhibit a noticeable separation from group B in the first principal component (PC1), which explained 55.3% of the variation in the data among different treatments. The differences in the structures of bacterial community within group A or B could be explained by the highest second principal component (PC2), which represented 14.6% of the variation in the data.

To further explore the effect of AQDS on the bacteria (e.g., iron-reducing and dechlorinating bacteria) involved in the transformation of PCP, 16S rRNA gene copy numbers of the total bacteria, *Dehalococcoides*, *Dehalobacter*, *Desulfitobacterium*, *Shewanella*, and *Geobacter* were studied using a real-time qPCR (Fig. 5a). The 16S rRNA gene copy numbers of total bacteria, *Shewanella* sp., and *Geobacter* sp. per gram of incubated soil sample in all non-sterile treatments ranged from 2.56×10^{10} to 4.34×10^{10}, 0.93×10^{4} to 4.12×10^{4}, and 1.35×10^{8} to 3.96×10^{8}, respectively. No dechlorinating bacteria, such as *Dehalococcoides* sp., *Dehalobacter* sp. and *Desulfitobacterium* sp., were quantified or detected in the reaction systems. Notably, the relative abundance of *Geobacter* sp. ranged from 0.43% to 1.19% (Fig. 5b). Compared with *Geobacter* sp., the relative abundance of *Shewanella* sp. ranged from 0.0000362% to 0.000123%, which is too low for further analysis. These observations agree with the T-RFLP results that the iron-reducing bacteria were the major species in the microbial community of PCP transformation, and the results also indicated that *Geobacter* sp. may be a good candidate for PCP transformation.

To further disclose the role of *Geobacter* sp. in PCP degradation, two treatments without PCP (Soil + PIPES and Soil + PIPES + 2 mM AQDS) were set up to study the abundance of *Geobacter* sp. As shown in Fig. S3, numbers of *Geobacter* sp. 16S rRNA gene copies increased 2 orders of magnitude in PCP-treated cultures (Soil + PIPES + PCP and Soil + PIPES + 2 mM AQDS + PCP), with numbers essentially identical with total bacterial rRNA gene copies. In cultures without PCP (Soil + PIPES and Soil + PIPES + 2 mM AQDS), the supplemented AQDS could increase the 16S rRNA gene copies of total bacteria and *Geobacter* sp., which indicated the biostimulation effect of AQDS. Furthermore, Fig. 5b shows the exponential relation between the relative abundance of *Geobacter* sp. with the AQDS dosages ($R^2 = 0.973$, $p < 0.001$). After being incubated for 15 days, the reaction systems with 0.01, 0.1, 1, 2, 10,

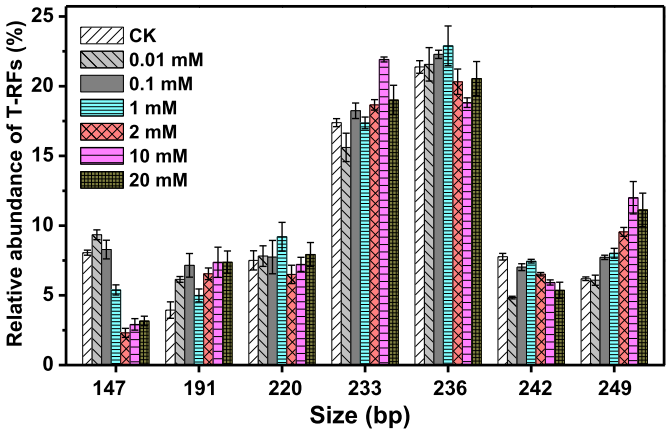

Fig. 3. T-RFLP analysis of bacteria retrieved from non-sterile soil incubated for 15 days.

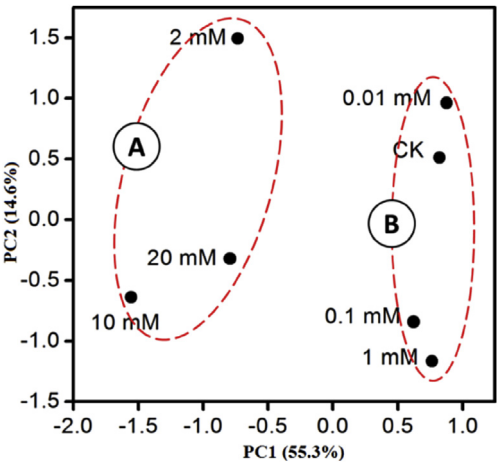

Fig. 4. Principal component analysis (PCA) of microbial communities retrieved from non-sterile soil incubated with different concentrations of AQDS for 15 days.

Table 2
Summary of dominant T-RFs lengths present in all treatments.

Peak number	T-RFs length (bp)	T-RFs abundance (%)							Predicted genus association	Accession number
		CK	0.01 mM	0.1 mM	1 mM	2 mM	10 mM	20 mM		
1	147	8.06	9.34	8.29	5.39	2.31	2.92	3.16	unclassified_*Clostridium*	JX222919
2	191	3.95	6.14	7.15	5.00	6.55	7.37	7.38	*Clostridium XI*	DQ811926
3	220	7.51	7.82	7.74	9.21	6.51	7.22	7.93	*Lachnospiracea_incertae_sedis*	HQ660788
4	233	17.39	15.61	18.24	17.37	18.66	21.92	19.01	unclassified_*Burkholderiales*	FJ269052
5	236	21.38	21.57	22.28	22.90	20.32	18.82	20.55	unclassified_Bacteria	GQ339156
6	242	7.77	4.84	7.02	7.45	6.52	5.92	5.37	*Clostridium XlVa*	JX133664
7	249	6.19	6.09	7.72	8.02	9.55	12.00	11.13	*unclassified_Veillonellaceae*	DQ295866

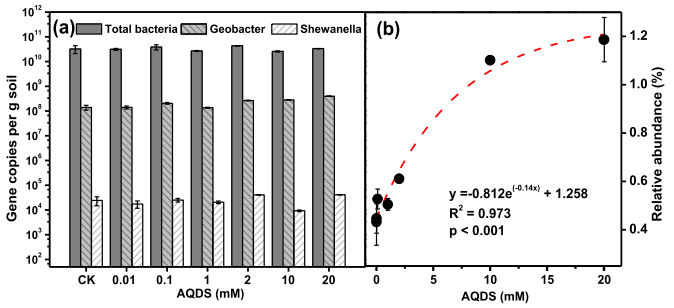

Fig. 5. (**a**) DNA copies of the 16S rRNA genes of total bacteria, *Geobacter* sp. and *Shewanella* sp. from non-sterile soil incubated for 15 days. (**b**) The relationship between AQDS concentration and the relative abundance of *Geobacter* sp. from non-sterile soil incubated for 15 days. The relative abundances of the target genes were normalized to the total bacterial community.

and 20 mM AQDS yielded increases of the relative abundance *Geobacter* sp. by 1.03, 1.17, 1.21, 1.41, 2.55, and 2.74 times, respectively, comparing with the system without AQDS added (CK treatment). In addition, the relative abundance of *Geobacter* sp. also shows positive exponential relationship with the PCP transformation kinetic constants ($R^2 = 0.882$, $p < 0.001$) (Fig. 6). The transformation rate of PCP increased when with a higher abundance of *Geobacter* sp. Overall, the addition of AQDS could enhance the relative abundance of *Geobacter* sp., and accelerate the transformation process of PCP. And additionally, higher concentrations of AQDS resulted in a greater abundance of *Geobacter* sp. and thereby a higher transformation rate of PCP.

4. Discussion

AQDS has been reported to stimulate the reduction of chlorinated organic compounds/iron oxides by acting as an electron mediator between the bacteria and chlorinated organic compounds/iron oxides (Lovley et al., 1999; Van der Zee and Cervantes, 2009). The biotransformation of carbon tetrachloride in anaerobic sludge was also reported to be accelerated in the presence of AQDS, and the enhancement was attributed to the quinone-respiring bacteria in the sludge (Cervantes et al., 2004). The present study found that AQDS could increase the relative abundance of *Geobacter* sp., and accordingly increase the formation of active iron species which was responsible for the accelerated PCP transformation. The effect of AQDS on the microbial community and the

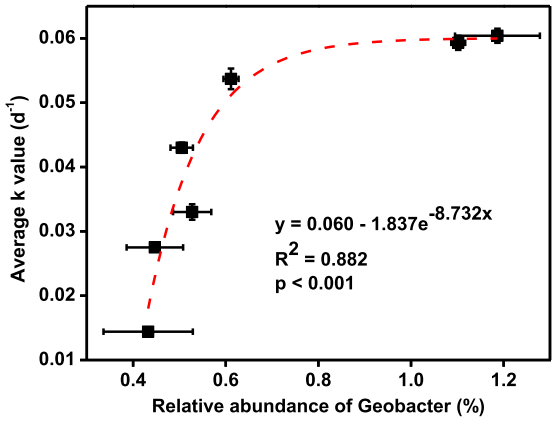

Fig. 6. The relationship between *k*-values of PCP transformation and the relative abundance of *Geobacter* sp. from non-sterile soil incubated for 15 days. The relative abundances of the target genes were normalized to the total bacterial community.

bacteria with potential dechlorinating abilities and the generation of active iron species during the PCP transformation were further elucidated here.

Microorganisms in the soils were important for the reductive dechlorination of organochlorine pesticides (Yoshida et al., 2007). The soil indigenous microorganisms played a crucial role in the microbial reduction of iron, so as to result in the generation of Fe(II) species and even in the directly reductive dechlorination of PCP (Chen et al., 2014). As our results indicated, PCP was stable and underwent little abiotic degradation in soils in the sterile treatments, whereas the indigenous microorganisms in the soils led to the obvious transformation of PCP in non-sterile treatments (Fig. 1a). Furthermore, AQDS supplement has been previously reported for accelerating the microbial transformation rates of organochlorine pollutants by pure bacteria incubation (Li et al., 2009; Cao et al., 2012). The enhanced PCP transformation rates when with supplemented AQDS were mainly attributed to the changed soil microbial communities compositions by AQDS, which exerted an important effect on the bacterial community structure in paddy soil (Fig. 3). For example, the relative abundance of T-RFs of 191 bp and 249 bp, being identified as hydrogen-producing bacteria (Hernández-Montoya et al., 2012) and normal species in anaerobic soil, respectively, were obviously increased in all AQDS-supplemented treatments. Furthermore, the AQDS concentrations were significantly correlated with the relative abundance of T-RF 249 bp positively, which is capable of AQDS reduction. These results suggested that the abundances of hydrogen-producing and AQDS-reducing bacteria were biostimulated by the supplemented AQDS.

Additionally, Fig. 4 indicates that the bacterial community structure was not significantly changed further when the concentrations of supplemented AQDS were higher than the optimal AQDS dosage. According to previous reports, the excess supply of humic substances was not necessary in stimulation experiments due to recycling of humic substances through chemical and microbial reactions (Lovley et al., 1996; Luu and Ramsay, 2003; Kalinichev and Kirkpatrick, 2007). As shown by the PCA analysis of T-RFs (Fig. 4), the treatments with relatively low concentrations of AQDS (0.01, 0.1 and 1 mM) were grouped with CK treatment (0 mM AQDS), while all the treatments with the concentrations of AQDS higher than 2 mM were clustered into group B. The results of the relationship between the *k*-values of PCP transformation and the concentration of AQDS added also suggested the excess supplement of AQDS would not significantly accelerate the PCP transformation any further (Fig. 1b).

The T-RFLP results in Fig. 3 show that iron-reducing bacteria (233 and 236 bp T-RFs) were the most abundant species in all of the treatments when with different AQDS concentrations, indicating that the iron-reducing bacteria were universal and most abundant in paddy soils, which were consistent with previous studies (Wang et al., 2009; Hori et al., 2010). Iron-reducing bacteria such as *Geobacter* sp. are the key soil components for the degradation of organic pollutants (Martinez et al., 2013). According to Hori et al. (2010), *Geobacter* sp. accounted for approximately 85% of the iron-reducing community in an Italian paddy soil when adding acetate as the electron donor and ferrihydrite as the electron acceptor. The qPCR results show that the relative abundances of *Shewanella* sp. and *Geobacter* sp. ranged from 0.93 to 4.12×10^4 copies per of gram soil and 1.35 to 3.96×10^8 copies per gram of soil in all treatments, respectively (Fig. 5a). Furthermore, comparing to the treatment without PCP, the addition of PCP enhanced the abundance of *Geobacter* sp. (Fig. S3), which indicated the involvement of *Geobacter* sp. in PCP transformation. Fig. 6 shows that the *k*-values of the PCP transformation were exponentially correlated with the relative abundance of *Geobacter* sp. ($p < 0.001$, $R^2 = 0.882$). Based on the above results, the iron-reducing bacteria, especially

Geobacter sp., should be the main iron-reducing bacteria community in this studied paddy soil, and they play a critical role in the microbial transformation of PCP.

The relative abundance of *Geobacter* sp. was exponentially correlated with the concentrations of supplemented AQDS positively (Fig. 5), which indicates that *Geobacter* sp. was able to use AQDS as the energy source to support its growth (Cervantes et al., 2002). Humus-respiring bacteria that are capable of reducing AQDS have been identified from various anaerobic environments and are mostly iron reducers related to the *Geobacter* sp. (Straub et al., 2005). Hence, the supplemented AQDS could enhance the abundance of *Geobacter* sp. and further increase the production of active Fe(II) species. The Fe(II) species formed by iron-reducing bacteria were also reported to be active reducers for the reductive transformation of organochlorine pollutants (e.g., carbon tetrachloride, DDT and PCP) in anaerobic environments (Kim and Picardal, 1999; Li et al., 2009). In the paddy soil studied here, Fe(II) was produced from the dissimilatory iron reduction by the soil indigenous microorganisms, especially when being stimulated by AQDS. The trends of the generation of HCl-extractable Fe(II) during the reaction time were similar to those of the PCP transformation (Figs. 1 and 2), which suggested the involvement of HCl-extractable Fe(II) in the transformation of PCP. In addition, the supplemented AQDS posed similar effects of positive exponential relationships on the relative abundance of *Geobacter* sp. (Fig. 5b) and the *k*-values of PCP transformation (Fig. 1b), which suggesting the involvement of *Geobacter* sp. in the PCP transformation in the paddy soil.

Previous studies demonstrated that both chemical and microbial reactions simultaneously contributed to the transformation of PCP in soils (McAllister et al., 1996; Li et al., 2008). The results of the present study showed that iron-reducing bacteria were the main microorganisms in soil, while typical dechlorinating bacteria, such as *Dehalococcoides* sp., *Dehalobacter* sp. and *Desulfitobacterium* sp., were not detected. This indicates that the direct microbial dechlorination was not the prevailing dechlorination pathway of the PCP transformation in soils, whereas the produced reductant of Fe(II) species was responsible for the PCP transformation.

To further evaluate the mechanism of the PCP transformation with AQDS supplements, sets of experiments were conducted using sterile soils with 1 mM Fe(II) and different concentration of AQDS. The average value of PCP transformation ratios were 63.49 ± 1.26% and there was no significant difference among all the treatments after incubation for 30 days (Fig. S1), indicating the possibility of PCP transformation by Fe(II) in the sterile soils. Previous studies also demonstrated that addition of Fe(II) in soil colloid and iron-containing minerals would increase the abundance of surface-bound Fe(II), which led to enhanced transformation rates of organic contaminants (Elsner et al., 2004; Li et al., 2008; Xu et al., 2014). More importantly, the transformation rates of PCP (average 63.49 ± 1.26%) in the sterile soils with Fe(II) and different concentrations of AQDS were approximates to that of microbial process in the paddy soil with 20 mM of AQDS (65.88 ± 1.26%) (Figs. 1 and S2). These results indicated that active Fe(II) species in soil were the main driven force for the transformation of PCP.

5. Conclusions

In this study, the effect of AQDS on the microbial community and the bacteria responsible for anaerobic transformation of PCP in soils were systematically investigated. AQDS was confirmed to be crucial for PCP transformation in paddy soils. With the addition of AQDS, the relative abundance of *Geobacter* sp. was largely enhanced, and accordingly increased the production of Fe(II) species from dissimilatory iron reduction of soil iron minerals, which was responsible for the accelerated PCP transformation. PCP could also be chemical transformed by sterile soil with Fe(II) and different concentrations of AQDS. In addition, the PCP transformation rate and the relative abundance of *Geobacter* sp. were both found to be exponentially dependent on the concentrations of the supplemented AQDS in the paddy soil. Therefore, it is concluded that AQDS can accelerates the transformation of chlorinated contaminants by stimulating the indigenous AQDS-utilizing bacteria in the paddy soil.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.06.061.

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