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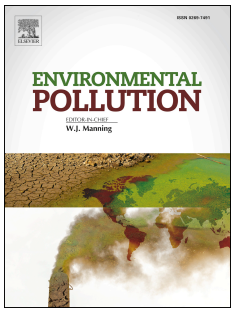

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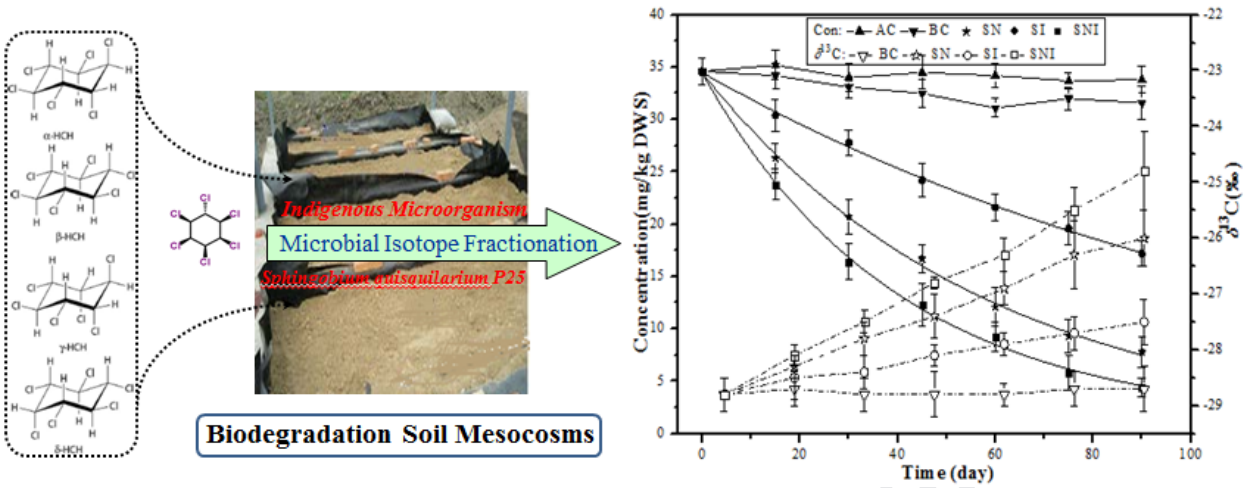

Assessment of Hexachlorcyclohexane Biodegradation in Contaminated Soil by Compound-specific Stable Isotope Analysis

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Abstract

Compound-specific isotope analysis (CSIA) was firstly applied to explore the biodegradation of hexachlorcyclohexane (HCH) isomers in contaminated soil. Concentrations and compound-specific carbon isotope ratio profiles of HCH in different specific *ex-situ* pilot-scale contaminated soil mesocosms were determined. The addition of nutrients and *Sphingobium spp*. significantly enhanced the degradation of HCH in contaminated soils within 90 days. Isomer specific biodegradation of HCHs was observed with α- and γ-HCH being more degradable than β and δ-HCH. Stable carbon isotope fractionation of HCH was observed and the $\delta^{13}C$ values shifted from -28.8 ± 0.3 ‰ to -24.8 ± 0.7 ‰ upon 87.3% removal, -27.9 ± 0.2 ‰ to -25.9 ± 0.5 ‰ upon 72.8% removal, -29.4 ± 0.3‰ to -19.9 ± 0.6‰ upon 95.8% removal, and −27.8 ± 0.5 ‰ to −23.6 ± 0.7 ‰ after 96.9% removal for α, β, γ, and δ-HCH, respectively. Furthermore, the enrichment factor ε for α, β, γ, and δ-HCH biodegradation in soil was obtained for the first time as -2.0‰, -1.5‰, -3.2‰, and -1.4‰, which could play a critical role in assessing *in situ* biodegradation of HCH isomers in field site soil. Results from ex-situ pilot-scale experiments clearly demonstrated that CSIA could be a promising tool to qualitatively and quantitatively evaluate *in situ* biodegradation of HCH in contaminated field site.

Keywords: Carbon isotope fractionation; Persistent organic pollutants (POPs); Bioremediation of Contaminated soil; Biodegradation; Biostimulation

Capsule:

CSIA could be applicable to qualitatively and quantitatively evaluate *in situ* biodegradation of HCH in contaminated soil.

1. Introduction

Hexachlorocyclohexane (HCH) isomers are extensively used persistent organic pollutants (POPs) and frequently detected in the environment (Kumar et al., 2005; Quintero et al., 2005). Due to their potentially negative effects and high persistence, HCH isomers were included to the Stockholm Convention's annexes in 2004, and the production of α-, β- and γ-HCH has been forbidden since 2009 (Vijgen et al., 2011). However, severe environmental contamination of HCH still exists because of their wide use in the past and the ongoing abuse of γ-HCH (lindane) in the world, especially in developing countries (Wang et al., 2009; Yang et al., 2018). Stockpiles from abandoned pesticide factories and leachates from dump sites have led to serious soil contamination by HCH (Bhatt et al., 2009).

Organic pollutants can provide energy and carbon source for microorganisms in contaminated soils (Sun et al., 2015). Biodegradation plays an important role in removing HCH in the soil subsurface (Bhatt et al., 2009), and is considered as an economical and effective substitute for physicochemical remediation of soil contaminated by HCH (Alvarez et al., 2012; Phillips et al., 2006). Previous laboratory and field studies have reported the biodegradation of HCH using various microbial consortium or isolated microbes under aerobic or anaerobic conditions (Badea et al., 2009; Bajaj et al., 2017; Bhatt et al., 2007; Murthy and Manonmani, 2007). For example, *Clostridium* was reported to degrade HCH isomers (Macrae et al., 1969), and HCH isomers were found to be degraded by *Pseudomonas* and *Sphingomonas* in pure cultures (Lal et al., 2010; Zhao et al., 2017) and agricultural soils (Xu et al., 2018a).

The evaluation of *in situ* biodegradation for organic pollutants in contaminated soil only based on the concentration is not convincing, because physical processes

(e.g., volatilization, sorption, dilution and dispersion) can contribute to the decrease in their concentration, leading to an overestimation of removal or biodegradation efficiencies for xenobiotic (Bombach et al., 2010; Illman and Alvarez, 2009). In general, molecules with heavy carbon isotopes (^{13}C) require more activation energies for bond breaking in the reactive sites than those contain light carbon isotopes (^{12}C), and tend to be decomposed slower than ^{12}C containing molecules. Thus, the $^{13}C/^{12}C$ isotope composition or carbon isotope ratio ($^{13}C/^{12}C$, most commonly given as $\delta^{13}C$) usually varies due to isotope fractionation in organic pollutants. Compound-specific stable isotope analysis (CSIA) was developed to distinguish the biodegradation from nondestructive processes by determining the carbon isotope fractionation of compounds. CSIA has already become a promising tool for characterizing *in situ* biodegradation of a wide variety of organic pollutants in the environment (Bombach et al., 2010; Braeckevelt et al., 2012; Elsner and Imfeld, 2016; Hofstetter and Berg, 2011; Steinbach et al., 2004). The aerobic and anaerobic biodegradation of HCH isomers in pure culture (Badea et al., 2009; Bashir et al., 2013) and in contaminated aquifer (Bashir et al., 2015; Liu et al., 2017) has been investigated by CSIA. However, the precise assessment on biodegradation of HCH in contaminated soils remains still unclear.

In the present study, CSIA was applied to explore the biodegradation of HCH in contaminated soil for the first time. Different *ex-situ* pilot-scale mesocosms for bioremediation of HCH contaminated soils were set up and conducted for 90 days. Concentrations and isomer-specific carbon isotope ratio profiles of HCH in all soil mesocosms at different time-intervals were measured. The stable carbon isotope fractionation during the microbial degradation of HCHs in ex-situ pilot-scale contaminated soils was determined. The present study aims to explore the

applicability of CSIA in evaluation in situ biodegradation in HCH-contaminated soil system at the field- scale.

2. Materials and methods

2.1. Soils

HCH contaminated soil samples were collected from an abandoned pesticide factory site in Wuhan, China (30°33′ N, 114° 14′ E) (Fig.1) by a shovel excavator. All the soils were air dried, homogenized, and stored at 4 °C in the dark before use. The physicochemical properties of the soils are given in Table 1.

2.2. Setup of *ex-situ* soil mesocosms

Pilot-scale *ex situ* mesocosms experiments were performed from April to June in 2015. The collected soil samples with varied HCH concentrations (10~240 mg/kg dry weight soil, DWS) were mixed to achieve a final HCH concentration of 55.2 mg/kg DWS for the *ex-situ* bioremediation mesocosms. The initial concentration of α-HCH, β-HCH, γ-HCH and δ-HCH was 34.6, 10.3, 7.1 and 3.2 mg/kg DWS, respectively. Five soil treatments were included: abiotic control (AC), biotic control (bulk soil with no action, BC), soil with nutrition (SN), soil with inoculation (SI), and soil with both nutrition and inoculation (SNI). The abiotic soils were obtained by three rounds of sterilization using an autoclave. The BC, SN, SI and SNI mesocosms were used to investigate the natural attenuation, biostimulation, bioaugmentation, and the combination of biostimulation and bioaugmentation of HCH in contaminated soils, respectively. For SN and SNI treatments, the nutrients including glucose (250 mg/kg DWS), $(NH_4)_2HPO_4$ (125 mg/kg DWS) and K_2HPO_4 (25 mg/kg DWS) were supplemented every 15 days. For SI and SNI treatments, 500 mL of bacterial inoculate containing 1.9×10^8 cfu/g soil of *Sphingobium quisquilarium P25* were added separately. The water content for all groups was controlled at about 35% by pot

watering every day. Each treatment was performed in three parallel pits of 2 m^2 × 20 cm depth, filled with approximately 200 kg of contaminated soil. All the fifteen pits were lined at the bottom with plastic sheet to prevent leaching of the contaminant. At 0, 15, 30, 45, 60, 75, and 90 d, five soil samples were collected from each pit and thoroughly mixed as a more representative sample for each pit to study the biodegradation of HCH. Aerobic strain *Sphingobium quisquilarium P25*, which was maintained on LB medium at 28 °C, was isolated from soil contaminated with HCH isomers and kindly provided by the Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research (UFZ).

2.3. HCH extraction and purification

Residual HCH in the soil was extracted through the accelerated solvent extraction (ASE) using the Dionex ASE 300. Briefly, 10 g soils were extracted in a 34 ml stainless steel vessel at 140 °C (heating time of 6 min) and a pressure of 1500 psi with dichloromethane methylene chloride (DCM) and acetone mixture (1:1, v/v). The detailed extraction procedure is described in Text S1.2. Preliminary experiments showed that the extraction by ASE had no significant influence on the carbon isotope ratios of HCH (Fig. S1).

2.4. Chromatographic analysis and CSIA

The residual HCH was quantified using gas chromatograph coupled to mass spectrometer (GC-MS). More detailed information on analysis of HCH is shown in Text S1.2. CSIA of HCH during the pilot-scale *ex situ* bioremediation of contaminated soils was performed by a gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS). The GC-C-IRMS contains a GC (6890 Series; Agilent Technology, USA) coupling with a MAT 252 mass spectrometer (Thermo Fisher, Scientific) by a GC/C III interface (Thermo Fisher Scientific). Briefly, the carbon

isotope ratio ($^{13}C/^{12}C$) of HCH was presented as $\delta^{13}C$ (‰) and calculated by the following Eq.1 (Coplen et al., 2006):

$$\delta^{13}C_{sample} = \frac{R_{sample}}{R_{standard}} - 1 \quad (1)$$

Where $R_{standard}$ and R_{sample} are the $^{13}C/^{12}C$ ratios of the international standard Vienna Pee Dee Belemnite (VPDB) and the sample, respectively. Each sample was performed in triplicate, with the uncertainty of analysis being ≤ ± 0.5‰.

2.5 Stable isotope analysis of biodegradation

To quantify the biodegradation of HCH in soils, the Rayleigh equation was used to establish the relationship between stable isotope ratios and concentrations of HCH isomers during biodegradation, and the fractionation factor (α) reveals the changes in concentration shifts and stable isotope ratios (Eq. (2)) (Elsner and Imfeld, 2016).

$$\frac{R_t}{R_0} = \left(\frac{C_t/C_0}{R_t+1/R_0+1}\right)^{\alpha-1} \quad (2)$$

Where C_0, R_0 and C_t, R_t are the chemical concentrations and the stable isotope ratios at the beginning of the biochemical reaction and at a given time, respectively. Generally, the abundance of ^{12}C in the natural environment is much higher than that of ^{13}C, then R + 1≈1, and Eq. (2) can be simplified as Eq. (3):

$$\frac{R_t}{R_0} = \left(\frac{C_t}{C_0}\right)^{\alpha-1} \quad (3)$$

Fractionation effects are negligible for most of the naturally reactive processes (i.e., α≈1), and the enrichment factor ε (‰) is usually used to provide the link between the changes in the concentrations (C_t/C_0) and the changes in stable isotope ratios (R_t/R_0) and defined as Eq. (4).

$$\varepsilon = (\alpha - 1) \times 1000‰ \quad (4)$$

Eq. (3) is described in the logarithmic formula (Eq. (5)):

$$ln\left(\frac{\delta_t^{13}C+1}{\delta_0^{13}C+1}\right) = \varepsilon_c ln\left(\frac{C_t}{C_0}\right) \quad (5)$$

Where the enrichment factor ε_c (‰) was given as the slope of the linear regression, and the errors are documented as 95% of the confidence interval (Bashir et al., 2013; Elsner et al., 2007). The percentage of biodegradation(B [%]) of organic pollutants is subsequently determined by Eq. (6) (Elsner and Imfeld, 2016).

$$B(\%) = \left(1 - \frac{C_t}{C_0}\right) \times 100 = \left[1 - \left(\frac{\delta_t^{13}C+1}{\delta_0^{13}C+1}\right)^{1/\varepsilon_c}\right] \times 100 \quad (6)$$

2.6 Data analysis

Analysis of variance (ANOVA) and post hoc Tukey's test were performed to investigate difference in the concentrations and carbon isotope data between different treatments using SPSS 20.0 (IBM SPSS, USA). A minimal level of statistical significance for differences in values was considered to be $p < 0.05$. All graphs were drawn by Origin Pro 2016 (Origin Lab, USA).

3. Results and discussions

3.1 Attenuation of HCH in different soil mesocosms

The kinetics of residual HCH and the degradation rate in all the experimental soil mesocosms within 90 days are shown in Fig.2. The degradation of HCH in BC mesocosm was not appreciable with a degradation rate of 6.3% at 90th day. After sterilization, degradation of HCH in AC was rather limited and did not exceed 1.1% within 90 days, indicating that microorganisms in soil played a role in HCH dissipation. Similarly, Sun et al. (2015) found that indigenous microorganisms (e.g., *Clostridium, Pseudomonas and Sphingomonas*) are able to metabolize HCH in aged contaminated soils. The degradation rates of HCH were consistently higher in SN and SI mesocosm than that in BC mesocosm ($P < 0.05$). For example, after 90 days of bioremediation, the residual concentrations of HCH were 51.7 ± 2.5, 12.0 ± 3.0 and

193 31.3 ± 3.1 mg/kg DWS in BC, SN and SI mesocosms, respectively, suggesting that
194 the addition of nutrients or *Sphingobium quisquilarium P25* significantly accelerated
195 the attenuation of HCH in soils. The inorganic nutrients plays a key role in the
196 microbial activity for microorganisms in soil. The increase in degradation rate of
197 HCH by nutrition was likely due to the fact that a number of indigenous HCH
198 degrading microorganisms existed in the HCH-contaminated soils and the addition of
199 glucose, $(NH_4)_2HPO_4$ and K_2HPO_4 as nutritional supplements may stimulate the
200 activity of such microorganisms, leading to a higher removal efficiency for HCH.
201 This result is in accordance with findings in some previously studies that
202 biostimuation of indigenous HCH-degrading microorganisms is effective for
203 bioremediation of HCH (Dadhwal et al., 2009; Garg et al., 2016). HCH-degrading
204 *Sphingomonads* were detected at chronically HCH contaminated sites (Boltner et al.,
205 2005; Mohn et al., 2006), thus biostimulation could be a good proposition for
206 remediation of HCH contaminated soil. Exogenous HCH degradation bacteria
207 inoculation (i.e., *Sphingobium quisquilarium P25*) showed a synergistic effect with
208 the indigenous HCH degrading microorganisms on removal of HCH based on the
209 removal rates of HCH in BC and SI mesocosm. In addition, the removal efficiency of
210 HCH in SI mesocosm (43.3% at 90 d) was much lower than that in SN mesocosm
211 (78.3% at 90 d), indicating that biostimulation (addition of nutrients) was more
212 effective in HCH degradation than bioaugmentation (amendment of *Sphingobium*
213 *quisquilarium P25*). This may be attributed to the stimulation of indigenous
214 microorganisms and the low bioavailability of inoculated *Sphingobium quisquilarium*
215 *P25* without enough nutrients. The removal efficiency of HCH in SNI mesocosm was
216 significantly higher than SN and SI mesocosms, with a degradation rate of 86.4% in
217 SNI at 90 d. The increase may be attributed to a combined effect of the nutrient

supplement and the inoculum of *Sphingobium quisquilarium P25*, suggesting that the combination of biostimulation and bioaugmentation is an effective approach for the decontamination of HCH in the contaminated soil sites.

3.2 Stable carbon isotope fractionation during HCH biodegradation in soil

To better understand the biodegradation of HCH, the kinetics of each isomer (*α*, *β*, *γ*, and *δ*-HCH) and their $\delta^{13}C$ values were determined in contaminated soil over 90 days (Fig.3). The carbon isotope ratios of α, β, γ, and δ-HCH kept almost unchanged throughout the whole experiment in abiotic controls (data not shown), indicating no significant carbon isotope fractionation of HCH occurred during the physiochemical process.

3.2.1 *α*-HCH and γ-HCH

In all the pilot-scale *ex situ* bioremediation mesocosms (BC, SN, SI and SNI), the dynamics of carbon isotope ratios for *α*-HCH within 90 days was shown in Fig.3. Except for the BC mesocosm, the $\delta^{13}C$ values of α-HCH increased while the concentration decreased during the whole experiment. For example, the $\delta^{13}C$ value of α-HCH increased from -28.8 ± 0.3 ‰ to -26.0 ± 0.5 ‰ with a removal rate of 77.5% in the SN mesocosms, from -28.8 ± 0.3 ‰ to -27.5 ± 0.4 ‰ with a removal rate of 50.3% in the SI mesocosms, and from -28.8 ± 0.3 ‰ to -24.8 ± 0.7 ‰ with a removal rate of 87.3% in the SNI mesocosms. These results indicate that biodegradation caused a stable carbon isotope fractionation of α-HCH. Meanwhile, as indicated by Fig.4, biodegradation of α-HCH in contaminated soil under biostimulation (SN), bioaugmentation (SI), and biostimulation combined with bioaugmentation (SNI) was well fitted to the first order kinetics ($R^2 > 0.98$) with biodegradation rate constants (*k*) of 0.017 d^{-1}, 0.008 d^{-1} and 0.023 d^{-1}, respectively. Similar to α-HCH, γ-HCH was also easily biodegraded in soil, with removal rates of 90.1%, 69.0% and 95.8% at 90 d

in SN, SI and SNI mesocosms, respectively (Fig.3). The biodegradation of γ-HCH coincided well with first order kinetic model ($R^2 > 0.98$) and the biodegradation rate constants (k) were 0.026 d^{-1}, 0.014 d^{-1} and 0.035 d^{-1} for SN, SI and SNI, respectively (Fig.4). The $\delta^{13}C$ of γ-HCH exerted very high ^{13}C enrichment from −29.4 ± 0.3 ‰ to −22.0 ± 0.7 ‰, −29.4 ± 0.3 ‰ to −25.7 ± 0.5 ‰ and −29.4 ± 0.3 ‰ to -19.9 ± 0.6 for SN, SI and SNI respectively (Fig.3). It could be demonstrated that α-HCH and γ-HCH showed strong microbial degradability and higher ^{13}C enrichment with much more stable carbon isotope fractionation during their biodegradation in soil. Thus, CSIA is applicable for revealing the biodegradation of α-HCH and γ-HCH in contaminated field soil and assessing the biodegradation rate.

3.2.2 β-HCH and δ-HCH

The isomers β-HCH and δ-HCH were more resistant to degradation as both β-HCH and δ-HCH were only degraded in the SN and SNI mesocosms (Fig. 3), and thus $\delta^{13}C$ values of β-HCH and δ-HCH were only measured in this two mesocosms. As shown in Fig.3, in the SN and SNI mesocosms, the $\delta^{13}C$ of β-HCH increased from -27.9 ± 0.2 ‰ at the beginning to -26.2 ± 0.3 ‰ at 90 d in SN mesocosm with 66.0% of β-HCH removed and increased from -27.9 ± 0.2 ‰ to -25.9 ± 0.5 ‰ at 90 d in the SNI mesocosm the maximum removal rate was 72.8%. The concentration of δ-HCH (< 3.2 mg/kg DWS) was very low in all tested soil, then no reliable $\delta^{13}C$ values were obtained due to the low detection level of δ-HCH after 60 days. As shown in Fig.3, there was an increase of the $\delta^{13}C$ of δ-HCH from -27.8 ± 0.5 ‰ on day 0 to -23.7 ± 0.6 ‰ on day 60 and an increase from -27.8 ± 0.5 ‰ on day 0 to -23.6 ± 0.7 ‰ on day 60 in the SN and SNI mesocosms, respectively. The removal rate of δ-HCH was 96.9% and 93.8% in the SN and SNI mesocosms, respectively. However, a small amount of β-HCH (10.3 - 9.5 mg/kg DWS) and δ-HCH (3.2 - 2.7 mg/kg DWS) were

degraded in the SI mesocosms with little carbon isotope fractionation. This was likely attributed to their resistance to the inoculated aerobic strain *Sphingobium quisquilarium P25* or the low activity of the added inoculum of *Sphingobium quisquilarium P25* in SI treatment (Raina et al. 2008). Meanwhile, β- and δ-HCH biodegradation in contaminated soil did both also follow first order kinetics (R^2 > 0.99). The biodegradation rate constants (k) of β-HCH were 0.012 d^{-1} and 0.015 d^{-1} for SN and SNI, respectively (Fig.4), while the biodegradation rate constants (k) of δ-HCH were 0.047 d^{-1} and 0.052 d^{-1} for SN and SNI, respectively (Fig.4). All the results demonstrate that although β-HCH and δ-HCH were less biodegradable, stable carbon isotope fractionation occurred in the case of biodegradation occurring in soil.

3.3 Biodegradation assessment of HCH isomers in soil

The biodegradability of α and γ-HCH was much higher than β and δ-HCH in contaminated soil, suggesting that isomer specific biodegradation was observed for HCH and the variation in molecular structure may lead to the discrepancy. This finding was consistent with observations from some previous studies (Lal et al., 2010; Mehboob et al., 2013). Interestingly, similar to the contaminated soils, α and γ-HCH were found to be more appreciably degraded than β and δ-HCH in the SI treatment, indicating that the addition of nutrient did not alter the biodegradation selectivity for HCH by indigenous soil microorganisms. However, in the SN and SNI treatments, the biodegradability of these four main HCH isomers was following the order of δ-HCH > γ-HCH >α-HCH >β-HCH (Table 2), demonstrating that the degradation selectivity was significantly influenced by the inoculation of *Sphingobium quisquilarium P25*, and the biogradation mechanisms between the indigenous HCH-degrading microorganisms and the *Sphingobium quisquilarium P25* were different. Thus, only the SN and SNI mesocosms were selected to determine the enrichment factor ε for α,

β, γ, and δ-HCH biodegradation in soil. The relationship between the $\delta^{13}C$ and residual concentrations of α, β, γ, and δ-HCH in the SN and SNI soils together was established by Eq. (5) and showed in Fig.5. A significant negative linear correlation was found between ln $[(\delta_t\ ^{13}C + 1)/(\delta_0\ ^{13}C + 1)]$ and ln $(C_t\ /C_0)$, with correlation coefficients > 0.97. The enrichment factor ε for α, β, γ, and δ-HCH biodegradation in soil was determined to be -2.0‰, -1.5‰, -3.2‰, and -1.4‰, respectively. Previous studies have reported the enrichment factors of α-HCH (aerobic conditions: -1.0‰ ~ -1.7‰; anaerobic conditions: -3.7‰) and γ-HCH (aerobic conditions: -1.5‰ ~ -1.7‰; anaerobic conditions: -3.4‰ ~ -3.9‰) during the biodegradation in pure culture (Badea et al., 2009; Bashir et al., 2013). The ε_c values of α-HCH and γ-HCH obtained in the present study were lower than that by aerobic biodegradation and greater than that by anaerobic biodegradation in pure culture, indicating a possible synergistic effect caused by both aerobic and anaerobic biodegradation of HCH in contaminated soil. However, α-HCH biodegradation was more appreciable under aerobic condition than that under anaerobic condition, steps except for the isotope sensitive carbon bond cleavage were likely rate-limiting in the aerobic biodegradation of α-HCH, resulting in a masking effect for the carbon isotope fractionation (Aeppli et al., 2009; Bashir et al., 2013). Therefore, the ε_c value of α-HCH was much lower than γ-HCH in the present study.

3.4 Implications for environmental studies

Stable carbon isotope fractionation was found in the biodegradation of HCH isomers in soils, indicating that the indigenous microorganisms preferred to metabolize the light isotope molecules of these four HCH isomers in the contaminated soils. (Elsner and Imfeld, 2016; Xu et al., 2018b). CSIA can be applied to qualitatively and quantitatively evaluate the biodegradation of HCH in field soils. The

enrichment factor ε was determined to be -2.0‰, -1.5‰, -3.2‰ and -1.4‰ for biodegradation of α, β, γ, and δ-HCH in soil, respectively. These enrichment factors could be introduced to assess *in-situ* biodegradation of HCH in the field site and even with no need to determine HCH concentration. Moreover, the biodegradation rate constants (k_t) could also be estimated by the changes of $\delta^{13}C$ using a modified Rayleigh-equation as following:

$$k_t = -\frac{1}{\varepsilon \cdot t} ln\left(\frac{\delta_t^{13}C+1}{\delta_0^{13}C+1}\right) \quad (7)$$

The time-resolved CSIA has the potential to predict the attenuation of HCH isomers in contaminated field soils.

4. Conclusions

The stable carbon isotope fractionation was firstly determined during the biodegradation of α, β, γ, and δ-HCH in contaminated soil. Accordingly, CSIA may be applicable for qualitatively and quantitatively evaluating HCH biodegradation during the bioremediation of HCH-contaminated soil. Additionally, relationship between the residual concentrations and the stable carbon isotope fraction of each HCH isomer in contaminated soil was established. The enrichment factor ε for the biodegradation of α, β, γ, and δ-HCH in soil obtained in this study would help us to gain a more scientific evaluation on in situ biodegradation of HCH in contaminated field soil.

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442 Table 1 Main physicochemical property of the contaminated soil

Soil Property	Values(Mean value ± SD)	Soil Property	Values (Mean value ± SD)
TOM (%)	23.4 ± 0.5	K (mg/kg)	16.9 ± 3.4
TOC (%)	1.7 ± 0.2	Ca (mg/kg)	67.6 ± 8.2
pH	6.7 ± 0.7	Soil Texture	Clay-loam
Salinity (mS/cm)	2.3 ± 0.2	Sand (%)	24.1 ± 7.5
TN (mg/kg)	49.3 ± 1.7	Clay (%)	31.5 ± 6.3
TP (mg/kg)	0.59 ± 0.1	Silt (%)	44.4 ± 10.4

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445 Table 2 Comparison of biodegradation HCH isomers by the concentration analysis
446 and CSIA

HCH Isomers	Half-life (days)			Enrichment factor ε (‰)
	NA	SI	NS	
α-HCH	40.8	86.6	30.1	-2.0
β-HCH	57.8	-	46.2	-1.5
γ-HCH	26.7	49.5	19.8	-3.2
δ-HCH	14.7	-	13.3	-1.4

447 - : not determined

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459 Figure Captions

460 Fig.1 Location of sampling sites in the abandoned pesticide plant in Wuhan, China.

461 Fig.2.Concentration (black symbols) and degradation rate (white symbols) of HCH in
462 soil samples from the AC (upper triangular), BC (lower triangular), SN (star), SI
463 (circle), and SNI (square) mesocosms during the 90 days bioremediation period.
464 The error bars indicate standard deviation of triplicate analysis.

465 Fig.3 Concentrations (black symbols) and carbon isotope ratios ($\delta^{13}C$) (white symbols)
466 of α-HCH , γ-HCH, β-HCH, and δ-HCH in soil samples from the AC (upper
467 triangular), BC (lower triangular), SN (star), SI (circle), and SNI (square)
468 mesocosms during 90 days bioremediation period. The error bars indicate mean
469 ± SD; n = 3 independent treatments.

470 Fig.4 Pseudo first order kinetics (black symbols) for the biodegradation of α-HCH,
471 γ-HCH, β-HCH, and δ-HCH by SN (star), SI (circle), and SNI (square)
472 treatments.

473 Fig.5 Double logarithmic plot according to the Rayleigh equation (Eq. 5) to reveal the
474 relationship between the carbon isotope ratios and residual concentrations of
475 α-HCH (square), β-HCH (circle), γ-HCH (triangle), and δ-HCH (star) by
476 biodegradation in contaminated soil.

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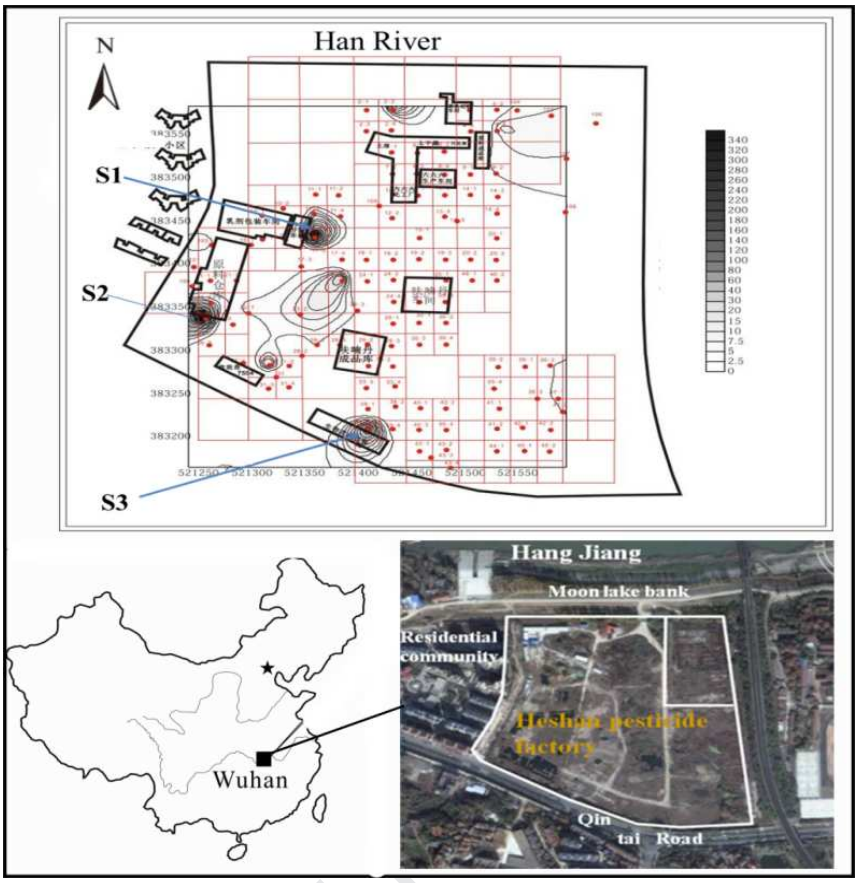

Fig.1

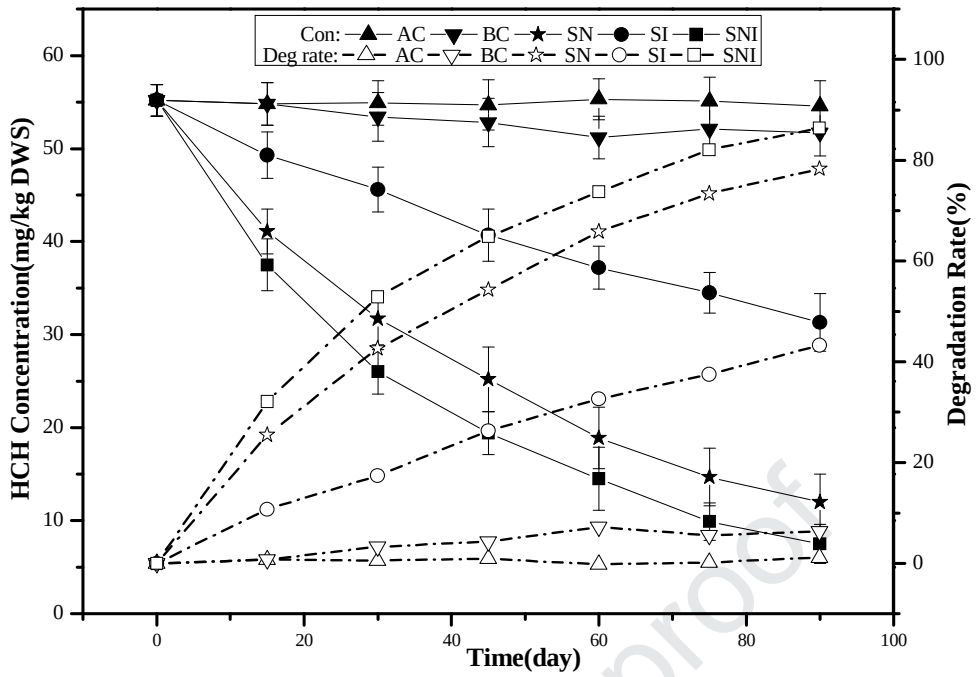

Fig.2.

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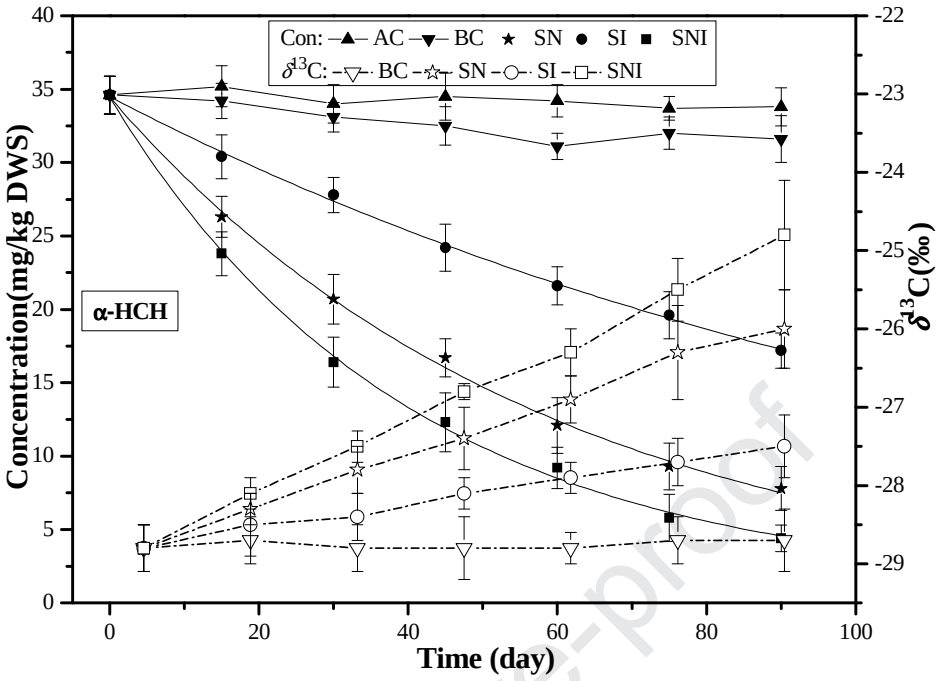

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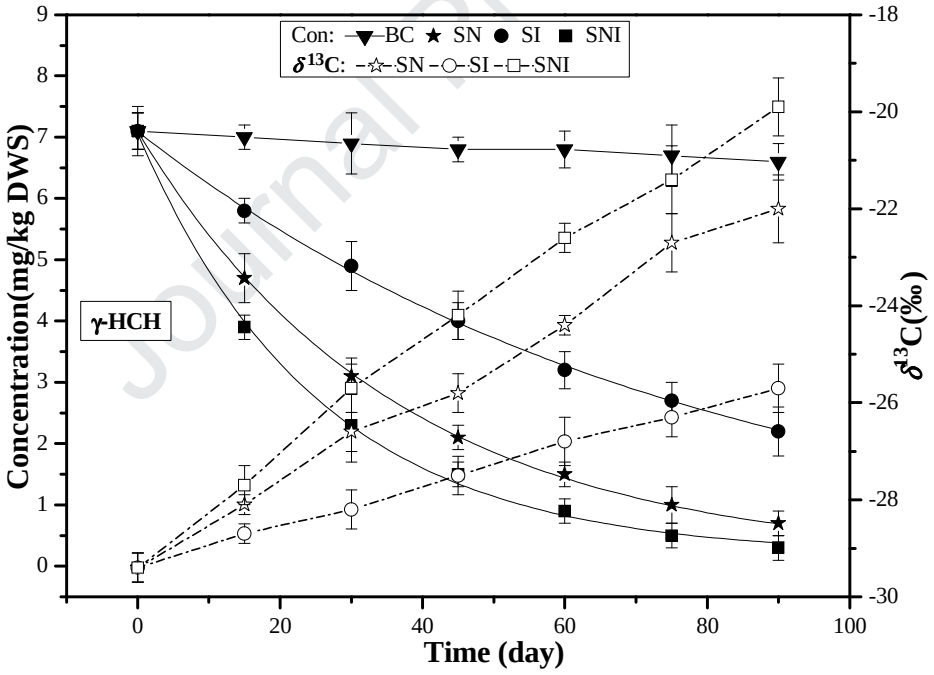

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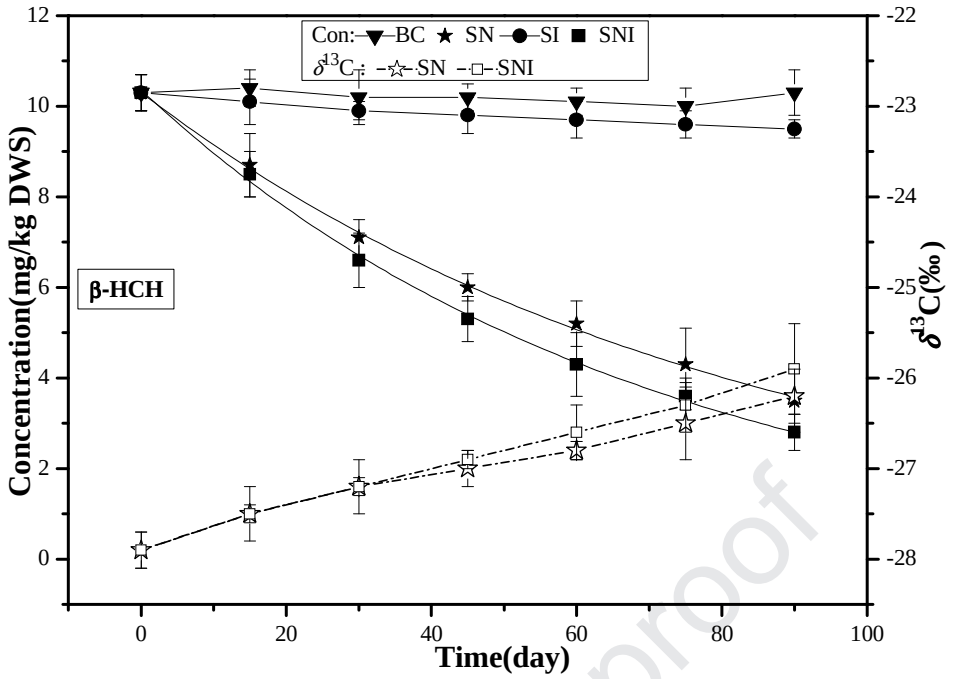

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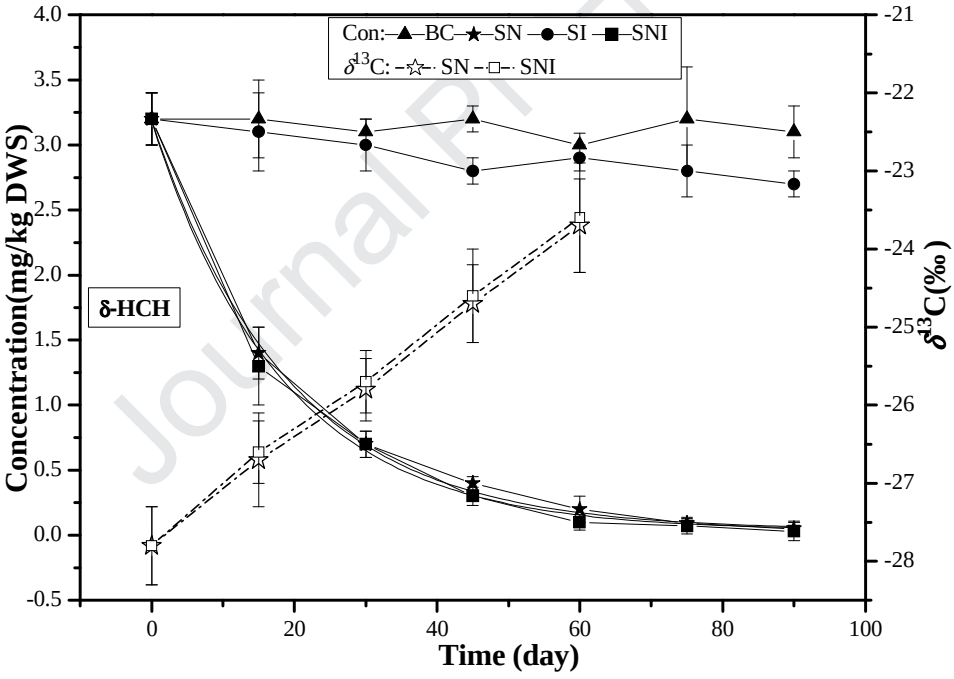

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497 Fig.3

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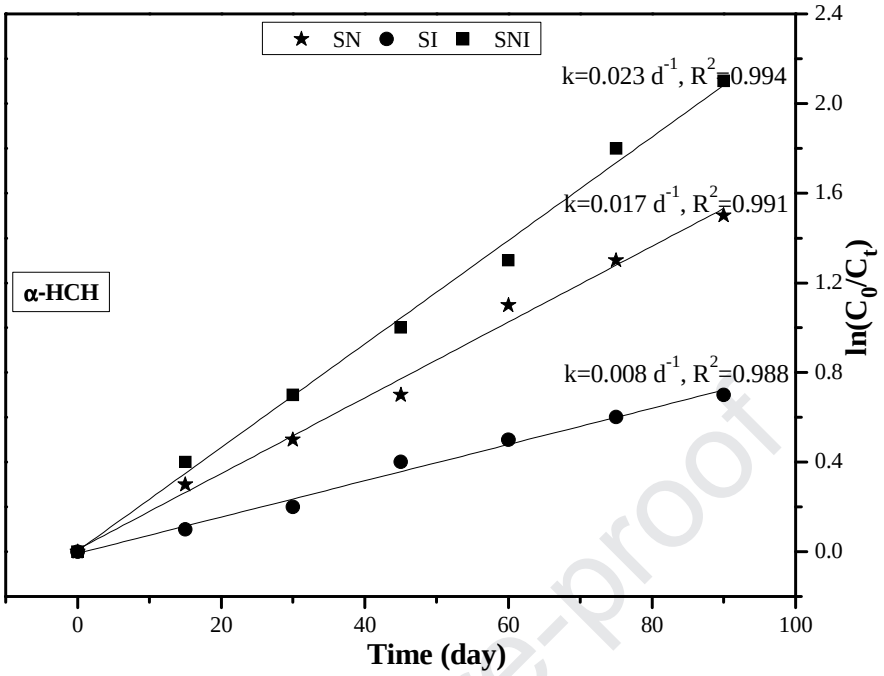

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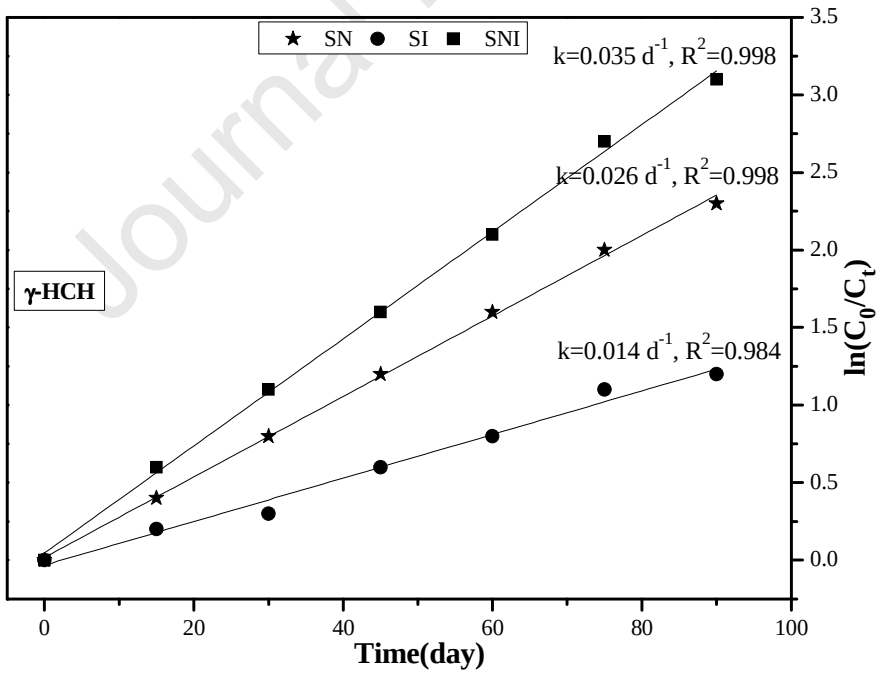

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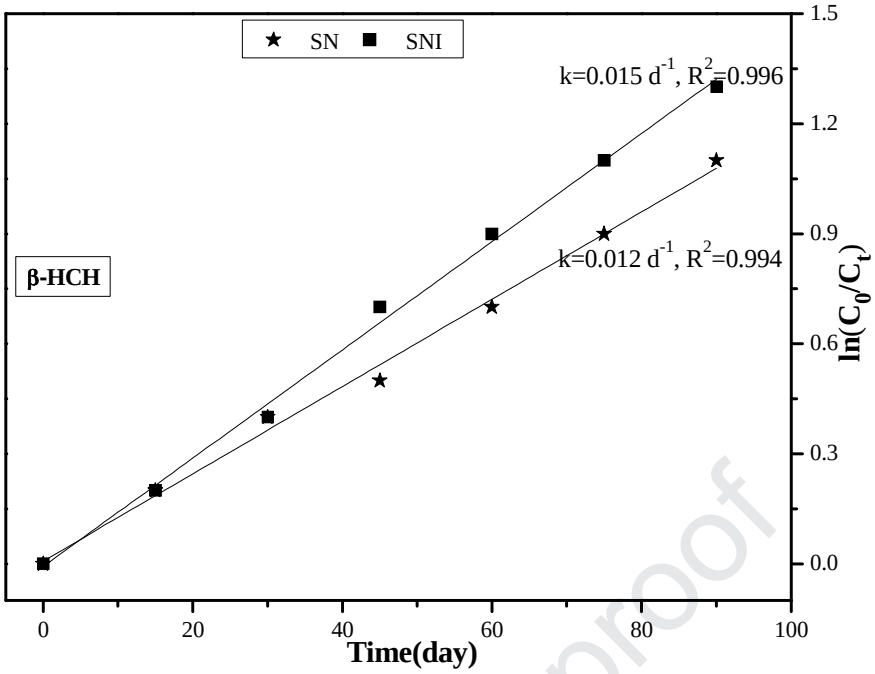

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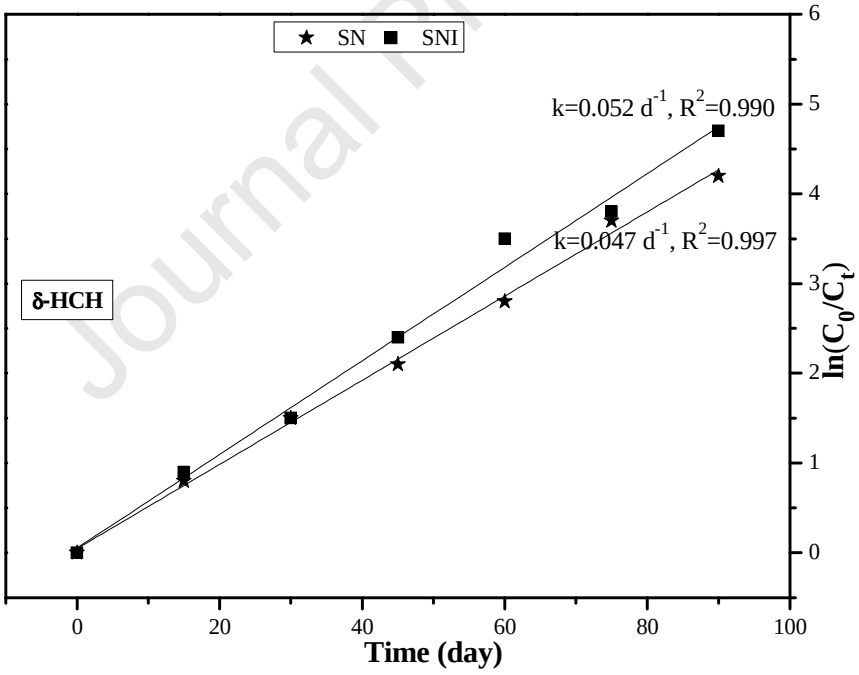

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508 Fig.4

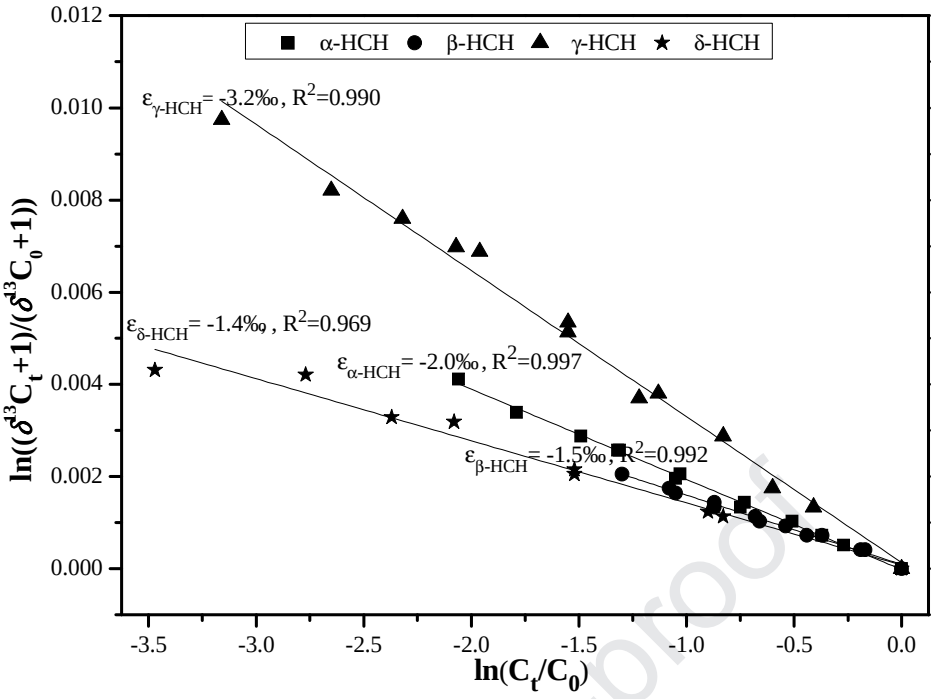

Fig.5

Highlights

- CSIA was used to assess HCHs biodegradation in contaminated soil for the first time
- Addition of nutrients and Sphingobium spp. facilitated the degradation of HCHs
- Isomer specific biodegradation of HCHs was observed in HCHs-contaminated soils
- Stable carbon isotope fractionation occurred for HCHs biodegradation in soil
- Enrichment factors ε_c for HCHs biodegradation in soil were obtained

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: