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1 **Dehalogenation of α -hexachlorocyclohexane by**
2 **iron sulfide nanoparticles: study of reaction**
3 **mechanism with stable carbon isotopes and pH**
4 **variations**

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20

21 **Abstract**

22 The biodegradation of hexachlorocyclohexanes (HCHs) is known to be accompanied by isotope
23 fractionation of carbon ($^{13}\text{C}/^{12}\text{C}$), but no systematic studies were performed on abiotic degradation
24 of HCH isomers by iron (II) minerals. In this study, we explored the carbon isotope fractionation
25 of α -HCH during dechlorination by FeS nanoparticles at different pH values. The results of three
26 different experiments showed that the apparent rate constants during dehalogenation of α -HCH by
27 FeS increased with pH. The lowest apparent rate constant value α -HCH during dehalogenation by
28 FeS was 0.009 d^{-1} at pH value of 2.4, while the highest was 1.098 d^{-1} at pH 11.8. α -HCH was
29 completely dechlorinated by FeS only at pH values 9.9 and 11.8, while the corresponding apparent
30 rate constants were 0.253 d^{-1} and 1.098 d^{-1} , respectively. Regardless of the pH used, the 1,2,4-
31 trichlorobenzene (1,2,4-TCB), 1,2-dichlorobenzene (1,2-DCB), and benzene were the dominant
32 degradation products of α -HCH. An enrichment factor (ϵ_C) of $-4.7 \pm 1.3\text{ ‰}$ was obtained for α -
33 HCH using Rayleigh model, which is equivalent to an apparent kinetic isotope effect (AKIEC)
34 value of 1.029 ± 0.008 for dehydrohalogenation, and of 1.014 ± 0.004 for dihaloelimination,
35 respectively. The magnitude of isotope fractionation from this study suggests that abiotic isotope
36 fractionation by FeS must be taken into account in anoxic sediments and aquifers contaminated
37 with HCH isomers, when high concentrations of FeS are present in the above-mentioned anoxic
38 environments.

39

40 **Keywords:**

41 dehalogenation, HCH, CSIA, FeS, dehydrohalogenation, reductive dechlorination

42

43 **1. Introduction**

44 The abiotic transformation of emerging and priority pollutants has been less studied compared to
45 biodegradation. This is the case of abiotic reductive dehalogenation, a process that can mimic the
46 anoxic transformation of halogenated contaminants (i.e. chlorinated ethenes, chlorinated ethanes,
47 hexabromocyclododecane (HBCD), etc) in the environment (Wacławek et al., 2019). The
48 contaminants can be abiotically transformed by iron (II) minerals (He et al., 2015) formed either
49 by microbial activity or occurring in nature as part of the site geochemistry. The minerals capable
50 of abiotic contaminant transformation include iron sulfides (mackinawite (FeS), pyrite (FeS₂),
51 greigite (Fe₃S₄)) and additional iron (II) minerals such as magnetite (Lee and Batchelor, 2002),
52 green rust (Ayala-Luis et al., 2012), and phyllosilicate clays (biotite (Kriegman-King and Reinhard,
53 1992) and vermiculite (Bae and Lee, 2012)). Among the most important of these iron (II) minerals
54 is iron sulfide (FeS), a mineral with reductive properties that appears naturally in various anoxic
55 environments (in concentrations often exceeding 10 µmol/g dry weight (Li et al., 2016)), being
56 associated with sulfate-reducing bacteria that grow in anoxic aquifers and sediments. The natural
57 formation of FeS involves two steps. The first step is biotic and consists in HS⁻ ion generation by
58 sulfate-reducing microorganisms from sulfate, as well in parallel Fe(II) generation from naturally
59 occurring Fe(III) oxyhydroxides by iron-reducing microorganisms. The second step results in the
60 rapid precipitation of poorly crystalline and reactive iron sulfide species. Considering that the
61 accidental pollutions or the unsound disposal practices may lead to the spreading of organohalogen
62 contaminants into environment, those compounds can be transformed, in anoxic sediment and
63 aquifers, by biotic or abiotic processes. Beside biodegradation by various bacteria, the
64 organohalogen contaminants can also be abiotically transformed, in the presence of the reactive
65 iron sulfide species, as electrons are donated from the iron sulfide to the halogenated contaminants
66 (i.e. HCHs, HBCDs, etc).

67 FeS nanoparticles have also been synthesized under laboratory conditions by various methods and
68 they were used in the elimination of different organohalogen contaminants. Previous studies have
69 reported the capacity of FeS to reductively dehalogenate various halogenated organic pollutants,
70 such as hexachloroethane (Butler and Hayes, 1998), trichloroethylene (Butler et al., 2013; He et
71 al., 2010), γ -hexachlorocyclohexane (γ -HCH) (Liu et al., 2003) and hexabromocyclododecane
72 (HBCD) (Li et al., 2016). (Nie et al., 2020) recently showed that the degradation of
73 trichloroethylene by biotic FeS was six time faster than its degradation by abiotic FeS. The higher
74 reductive activity of the biogenic FeS detected by (Nie et al., 2020) was attributed to a higher
75 monosulfide (HS^- ion) content and to more structural Fe^{2+} on the surface.

76 Compound specific isotope analysis (CSIA) of carbon isotopes is using the preferential
77 transformation of lighter isotopomers during a biotic or abiotic reaction, therefore leading to an
78 enrichment of heavier isotopes in the residual phase of substrate (Elsner and Imfeld, 2016). In order
79 to relate the change of bulk isotope ratios of chemicals to the extent of the target compound
80 degradation, the Rayleigh model has been extensively used in environmental studies (Julien et al.,
81 2018). The carbon, hydrogen and chlorine based CSIA is currently available for more simple
82 organic contaminants, such as BTEX (Elsner and Imfeld, 2016; Lesser et al., 2008; Vogt et al.,
83 2016) and chlorinated ethenes (Kuder et al., 2013; Marco-Urrea et al., 2011), and these concepts
84 are already applied in the monitoring of contaminated sites (Hunkeler, 2008). Conversely, the
85 concepts and applications of CSIA for larger molecules including chemical classes such as
86 pesticides, flame retardants, and other emerging products are still nascent areas of research and
87 urgently need further development (Elsner and Imfeld, 2016). Hexachlorocyclohexane (HCH)
88 belongs to the organochlorine compounds and its γ -isomer (Lindane) is a globally applied broad-
89 spectrum insecticide (Muller and Kohler, 2004). HCH has been classified as persistent organic
90 pollutant by the Stockholm Convention due to his adverse effects on humans and environment
91 (Wacławek et al., 2019). HCH consists of eight isomers (α -, β -, γ -, δ -, ϵ -, η -, ν -, and ι -HCH), of

92 which only the α -HCH is a chiral compound. CSIA has been applied in the past to characterize the
93 biodegradation of HCH isomers using sulfate-reducing bacteria (Badea et al., 2009), fermentative
94 microorganisms (Badea et al., 2011), a Dehalococcoides strain (Liu et al., 2020a), aerobic cultures
95 (*Sphingobium* spp (Bashir et al., 2013), and *Sphingobium indicum* Strain B90A (Liu et al., 2019)),
96 some studies involving also multi-element (C-Cl) CSIA (Liu et al., 2020a).

97 This study aims to investigate the mechanism and pathway of α -HCH dehalogenation by FeS
98 nanoparticles using the carbon isotope fractionation under anoxic conditions, in order to be able to
99 differentiate this process from biotic degradation in the field. To this end, the influence of pH on
100 the degradation rate were included, degradation products were identified and a mass balance of
101 substrate and products was established.

102 **2. Materials and Methods**

103 *2.1. Chemicals*

104 $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ (99% analytical purity) was purchased from Honeywell (Seelze, Germany), while
105 sodium sulfide hydrate (with 65.6 % Na_2S content) was purchased from Sigma–Aldrich (Munich,
106 Germany). α -HCH (99%) and hexachlorobenzene (HCB) (99%), 1,2,4-trichlorobenzene (1,2,4-
107 TCB) (99%), 1,2-dichlorobenzene (1,2-DCB) (99%) and benzene (99%) were obtained from
108 Sigma–Aldrich (Munich, Germany). Anhydrous Na_2SO_4 and hydrochloric acid (HCl) was
109 purchased from Sigma–Aldrich (Munich, Germany). Dichloromethane (DCM) (99.8 %) was
110 purchased from Carl Roth (Karlsruhe, Germany).

111 *2.2. Synthesis of FeS nanoparticles*

112 FeS nanoparticles were synthesized by adding 250 mL solution of 0.2 M Na_2S over 250 mL
113 solution 0.2 M FeSO_4 , under gaseous N_2 flow. The experiment was performed up to 6 h on a water
114 bath heated by a combined hot-plate magnetic-stirrer device (Biosan, Riga, Latvia) at 30 °C and
115 mixed at 1000 rpm to homogenize the FeS nanoparticles. FeS precipitate was divided in 11 parts
116 (in 50 mL Eppendorf conical tubes) and FeS nanoparticles were centrifuged at 6000 rpm for 10

117 minutes and afterwards washed with N₂-flushed water. The FeS precipitate was frozen with liquid
118 nitrogen and then freeze dried for about 48 h at about -80 °C. The dried powder of FeS was
119 collected and stored in small Eppendorf plastic tubes of 2 mL sealed with teflon band until use.
120 The FeS nanoparticles were characterized by determination of specific surface, X-Ray diffraction
121 (XRD), Raman spectroscopy. Scanning electron microscopy (SEM), as well by Fourier-transform
122 infrared spectroscopy (FTIR). The Raman and XRD peaks showed that mackinawite is the main
123 crystalline phase of FeS nanoparticles, while the specific surface of the FeS was 32.58 m²/g,
124 which is similarly with the specific surface reported by (Liu et al., 2003) (see Supplementary
125 Material).

126 2.3. Degradation experiments

127 The dehalogenation experiments were performed in 300 mL anaerobic bottles crimped by
128 polytetrafluoroethylene (PTFE)-coated butyl septa. In the first experiment, the dehalogenation
129 reaction was performed in duplicates by adding 165 mL buffer mixture solution K₂HPO₄0.1
130 M/ KH₂PO₄ 0.1 M (in a ratio of about 94 to 6) over 1.5 g FeS nanoparticles (final pH of 8.10 ±
131 0.04). The bottles were firstly flushed with nitrogen and then α-HCH was added from an acetone
132 solution (3.43 mM α-HCH), to a theoretical concentration of approximately 20.7 μM (see Tab.2).
133 Also, a control bottle was prepared in duplicates by spiking 165 mL deionized water with α-HCH
134 of the same concentration of 20.7 μM. In the first experiment, the dechlorination reaction was
135 performed for 22 days in an incubator at 30 °C and 125 rpm. In the second experiment, the
136 dehalogenation reaction was performed in three bottles by adding 165 mL deionized water over
137 1.5 g FeS nanoparticles (FeS concentration of 9.0 g/L). The pH of the three aquatic solutions was
138 adjusted with 2-3 mL of 1M NaOH / 1M HCl to three final pH values: 2.4, 5.3 and 11.8. In this
139 case also, the bottles were firstly purged with nitrogen and then α-HCH was added from the same
140 acetone solution (3.43 mM α-HCH), to a concentration of approximately 20 μM. In the 2nd
141 experiment, the dechlorination reaction was performed for 32 days in an incubator at 30 °C and

142 125 rpm. The third experiment was carried out in 120 mL bottles screwed gas-tight by a butyl
143 septum by mixing 1g of FeS nanoparticles with 110 mL N₂ flushed-water (FeS concentration of
144 9.0 g/L) and adjusting the pH to 9.9. In the 3rd experiment, the bottle was firstly purged with
145 nitrogen and then spiked with α -HCH in acetone to a concentration in water of about 30 μ M. In all
146 three experiments, control bottles without FeS nanoparticles were used. At regular intervals, 14 mL
147 aliquots of α -HCH solution were taken with syringes for extraction with 1 mL DCM that contains
148 hexachlorobenzene (HCB) as internal standard, as previously described (Badea et al., 2009). The
149 extraction and analysis of slurry samples of FeS nanoparticles was performed by ultrasonication
150 and was similarly with the one of aliquots (see Supplementary Material).

151 *2.4. Control experiment*

152 In order to demonstrate that the decrease in concentration of α -HCH and the formation of the
153 degradation products in the three experiments performed with FeS nanoparticles are attributable to
154 the dehydrohalogenation processes that are taking place on the surface of FeS, an additional control
155 experiment (experiment no. 4) was performed at three different pH values. The control experiments
156 were performed using two 300 mL anaerobic bottles crimped by polytetrafluoroethylene (PTFE)-
157 silicon septa. The first control bottle was preparing by dissolving HgCl₂ in 250 mL deionized water
158 to a final concentration of 82 mg/L HgCl₂ in order to prevent any biodegradation of α -HCH, while
159 the pH was adjusted to 7.0 by adding 0.4 mL 1M NaOH. In the second control bottle, the pH of
160 250 mL deionized water was adjusted to 9.99 using 2-3 mL of 1M NaOH / 1M HCl. In the third
161 control bottle, the pH of 250 mL deionized water was adjusted to 11.7 with 0.9 mL 1M NaOH. In
162 all three control bottles, 1 mL of solution 3.92 mM of α -HCH was added in both control bottles to
163 final concentration of about 15.6 μ M. Here too, 14 mL aliquots of α -HCH solution were taken with
164 syringes for extraction with 1 mL DCM that contains hexachlorobenzene (HCB) as internal
165 standard, the last sample for first and third control bottles (pH 7.0 and 11.7 respectively) being

166 taken after 17 days and the last one for the second control bottle (pH of 9.9) after 10 days. The
167 control experiment was performed up to 17 days in an incubator at 30 °C and 125 rpm.

168 2.5. GC-MS analysis

169 For the three degradation experiments with FeS, the GC-MS measurements were carried out on a
170 Varian 450 GC-240 MS (Varian, Inc, USA), equipped with an ion trap mass analyzer configured
171 in positive electron ionization mode (70 eV). To identify and characterize the structure of the
172 degradation products, the ion trap mass analyzer was configured in scan mode. The samples (1 μ L
173 DCM in volume) were injected at 260 °C into a split injector, with the split ratio adjusted to 20,
174 while the flow rate of carrier gas (helium) was 1 mL/min. The α -HCH and its degradation products
175 were separated on a CP-Sil 8 CB (8% phenyl polysilphenylene-siloxane) capillary column (30 m
176 \times 0.25 m \times 0.25 μ m) using the following temperature program: 40 °C initial temperature (held for
177 3 min), 4 °C min⁻¹ to 120 °C (0 min), 20 °C min⁻¹ to 200 °C (0 min) and finally 10 °C min⁻¹ to 280
178 °C (and held for 5 min).

179 In the experiment 4 (control experiment), the GC-MS measurements were carried out on a Perkin
180 Elmer Clarus 580 GC (Waltham, Massachusetts, USA), equipped with an Clarus SQ-8 quadrupole
181 mass analyzer configured in positive electron ionization mode (70 eV). The samples (1 μ L DCM
182 in volume) were injected into a split injector, with the split ratio adjusted to 10, while the flow rate
183 of carrier gas (helium) was 1 mL/min. The α -HCH and its degradation products were separated on
184 a DB5 MS (5% phenyl polysilphenylene-siloxane) capillary column (30 m \times 0.25 m \times 0.25 μ m)
185 using the same temperature program as described in the paragraph above.

186 2.6. GC-C-IRMS analysis

187 The carbon isotope composition of α -HCH was analyzed using a gas chromatograph (Thermo
188 Trace 1310 GC, Thermo Scientific) coupled via a CONFLOW IV (Thermo Scientific, Germany)
189 to a Delta V Advantage mass spectrometer (Thermo Scientific, Germany), as described previously

190 (Zamane et al., 2020). The oxidation furnace of the interface (Thermo Scientific, Germany)
191 containing Pt, Ni, and CuO was set to 1000 C° in order to completely combust α -HCH. The furnace
192 was conditioned for 120 minutes with O₂ before each batch run of samples. The flow rate of carrier
193 gas (helium) was 2.0 mL/min, while the injector was operated in split mode at 280°C. A DB-5MS
194 GC column (60 m × 0.25 mm, 0.25 μ m film thickness) was used for chromatographic separation
195 of α -HCH from HCB. The temperature program of the GC oven was described previously (Badea
196 et al., 2011) (see Supplementary Material).

197 2.7. Carbon stable isotope calculations

198 The carbon isotope signatures were reported in δ -notation (‰) relative to the Vienna Pee Dee
199 Belemnite (V-PDB) (Coplen, 2011), as follows:

$$200 \quad \delta^{13}\text{C} [\text{‰}] = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000 \quad (1)$$

201 where the ¹³C/¹²C ratio of the sample (R_{sample}) is reported relative to the ¹³C/¹²C ratio of the VPDB-
202 standard (R_{standard}).

203 The Rayleigh equation was used to quantify isotope fractionation upon degradation (equation 2):

$$204 \quad \ln\left(\frac{R_t}{R_0}\right) = (\alpha - 1) \ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

205 The C_0 and C_t and are the concentrations of the substrate at times 0 and t, while the R_0 and R_t are
206 the ¹³C/¹²C ratios of the substrate at times 0 and t, respectively. The α is the isotope fractionation
207 factor, and it can further used to calculate the isotope enrichment factor ε (equation 3):

$$208 \quad \varepsilon = (\alpha - 1) \times 1000 \quad (3)$$

209 The error of the isotope enrichment factor was given as 95% confidence interval (CI) and
210 determined using a regression analysis as described elsewhere (Elsner et al., 2007).

211 The apparent kinetic isotope effect (AKIE) was calculated using equation 4 according to (Elsner et
212 al., 2005)

$$213 \quad AKIE \approx \frac{1}{1 + \left(\frac{n}{x} \cdot z \cdot \frac{\varepsilon}{1000} \right)} \quad (4)$$

214 where x is the number of reactive positions, z is the number of positions in intramolecular
215 competition and n is the number of atoms of the molecule of a target element. The error propagation
216 method was used to estimate uncertainty of the AKIE, as described elsewhere (Fischer et al., 2010).

217 **3. Results and discussions**

218 *3.1. Dehalogenation of α -HCH by iron sulfide nanoparticles*

219 α -HCH was dechlorinated in all degradation experiments at reaction times of up to 32 days. The
220 main degradation products formed at all pH during dehalogenation were (Fig. 1, pathway (A)):
221 1,2,4-trichlorobenzene (1,2,4-TCB), 1,2-dichlorobenzene (1,2-DCB), benzene and, as intermediate,
222 pentachlorocyclohexene (PCCH), tentatively identified by GC-MS by comparing its mass
223 spectrum with those of the NIST library and with the previous degradation studies of α -HCH
224 (Suar et al., 2005). (see Fig. S9, Supplementary Material). In the control bottles, no degradation
225 products were detected. For example, in the experiment 1, no degradation products were detected
226 and the isotope composition of α -HCH remained constant during the whole experiment indicating
227 that the α -HCH from the control bottle is not biotically or abiotically transformed. Nevertheless, in
228 the experiment 1, the concentration of α -HCH varied from 16.7 μ M at the beginning of the
229 experiment to 7.5 μ M at the end of the experiment. This loss of α -HCH is probably attributable to
230 the volatilization of α -HCH due to the low final volume of aquatic phase (about 54 mL) comparing
231 with the initial one of 166 mL (with results in a large head-space volume), as well to the sorption
232 processes. Small amounts of α -HCH might have been adsorbed onto the plastic syringes during
233 sampling procedures. Also in the experiment no.4 without FeS nanoparticles, the evolution of α -

234 HCH concentration in the control bottles varied according to the initial pH values. In the control
235 bottle with the pH of 7.0, no degradation products were detected and the concentration of α -HCH
236 evolved from 15.6 μM at the beginning of the experiment to 16.6 μM after 17 days (at the end of
237 the experiment), showing no clear trend (see Fig.2). In control bottle with pH value of 9.9, the
238 concentration of α -HCH decreased from 15.4 μM at the beginning of the experiment to 11.9 μM
239 after 10 days (see Fig S7, Supplementary Material). In the control bottle with pH value of 9.9, the
240 pseudo-first-order rate constant was calculated to 0.090 d^{-1} which is slightly higher than the pseudo-
241 first-order rate constant of 0.069 d^{-1} recorded by (Ren et al., 2006) during alkaline hydrolysis of
242 α -HCH performed at pH 9.28 and 25°C , but was lower than the rate constant of 0.384 d^{-1} (0.0064
243 h^{-1}) recorded by (Zhang et al., 2014) during alkaline hydrolysis of α -HCH performed at pH 9.78.
244 In the control bottle with pH value of 11.7, the degradation rate was the fastest from all control
245 bottles, since the concentration of α -HCH decreased from 15.5 μM at the beginning of the
246 experiment to 0.3 μM after just one day (24 h), afterwards its concentration being below the
247 detection limit of the GC-MS method.

248 The above-mentioned degradation products (PCCH, 1,2,4-TCB, 1,2-DCB, benzene) showed that
249 the dehydrochlorination was the main degradation pathway of α -HCH by FeS. The proposed
250 degradation pathway from this study assumed a simultaneous elimination of two chlorine and two
251 hydrogen atoms from PCCH to produce 1,2,4-TCB, followed by a further reductive dechlorination
252 of 1,2,4-TCB to 1,2-DCB and finally to benzene ((Fig. 1, pathway (A)), in contrast with the
253 hydrolysis pathway recently proposed by (Kannath et al., 2019) (Fig. 1, pathway (B)) which
254 suggest an attack of the hydroxyl ion on the molecules of different HCHs isomers. The degradation
255 pathway from this study is also similar to a dehydrochlorination biotic pathways of α -HCH by
256 *Sphingomonas paucimobilis* B90A proposed by (Suar et al., 2005) and (Lal et al., 2010) (Fig. 1,
257 pathway (C)) , but the abiotic dehalogenation reaction is going beyond 1,2,4-TCB with the
258 formation of 1,2-dichlorobenzene and benzene as new degradation products.

259 The proposed degradation pathway was supported by the absence of monochlorobenzene (MCB)
260 as degradation product. The vicinal dehaloelimination (vicinal reductive dechlorination) of α -HCH
261 by FeS nanoparticles might be just a minor degradation pathway. In contrast with the previous
262 studies (Badea et al., 2011), as the key intermediate of such pathway, the 3,4,5,6-tetrachloro-1-
263 cyclohexene (TCCH, Fig. 1 pathway (D)) was detected just in the FeS slurry samples (see Fig. S13,
264 Supplementary Material), but not in the aquatic ones. Since no reference standards of PCCH and
265 TCCH were available for calibration, the concentrations (in μM) were calculated only for the 1,2,4-
266 TCB, 1,2-DCB and benzene. The trend of degradation products in aquatic samples was influenced
267 by the rate of α -HCH dechlorination which was influenced by the pH on the solutions. The ratio
268 between final concentration of 1,2,4-TCB to 1,2-DCB varied from about 7.3 at pH 8.1 to about
269 32.9 at pH 11.8, indicating that the formation of 1,2-DCB by reductive dechlorination of 1,2,4-
270 TCB is favored in heterogenous systems at more neutral pH, as previously found (Mackenzie et
271 al., 2005). α -HCH was degraded completely in aquatic solutions only at pH 11.8 after 11 days, his
272 concentration decreasing from 5.0 μM at the beginning of the 2nd experiment to 0.003 μM after 3
273 days and to further 0.002 μM after 7 days, afterwards the α -HCH being below the detection limit
274 of the GC-MS method. In comparison, in the same 2nd experiment, the α -HCH concentrations
275 decreased from 8.7 μM at the beginning of the experiment to 5.0 μM (pH 5.3) and respectively
276 from 8.4 μM to 5.6 μM (pH 2.4), after 32 days of degradation, showing a high influence of pH on
277 the degradation rate of α -HCH (see Fig. 2). In the 1st experiment, performed at pH 8.1, the α -HCH
278 concentration decreased from $9.0 \pm 0.4 \mu\text{M}$ at the beginning of the experiment to $5.6 \pm 0.1 \mu\text{M}$,
279 after 22 days, at the end of the experiment. (Fig. 2). In the 3rd experiment performed at pH 9.9, the
280 apparent degradation rate of α -HCH was slightly slower comparing with the one at pH 11.8 (see
281 the apparent rate constants from Tab. 1), the α -HCH concentration decreasing from 5.9 μM at the
282 beginning of the experiment to 0.5 μM after 4 days and to 0.041 μM after 19 days, at the end of the
283 experiment (Fig. 2). This influence of pH on the degradation rate was also observed by (Liu et al.,

284 2003) in the degradation study of γ -HCH isomer with FeS nanoparticles. In order to explain the
285 influence of pH on degradation rate, (Butler and Hayes, 1998) suggested that the dehalogenation
286 rate of chlorinated chemicals (i.e. hexachloroethane) at alkaline pH is higher comparing with the
287 reaction rate at acidic pH, possible due to deprotonation of species from the surface of FeS
288 (deprotonated species have a higher reactivity). Nevertheless, in our study, the “apparent”
289 degradation rate of α -HCH might be overestimated and might differ from the “true” degradation
290 rate due to the sorption of α -HCH on the surface of FeS nanoparticles. This assumption is supported
291 by the above-mentioned values of measured aquatic concentrations at the beginning of the
292 experiments 1, 2 and 3 which were all lower than the initial spiked aquatic concentrations of about
293 20 μ M (respectively around 30 μ M for 3rd experiment). For the experiment 1, only about 43.7 %
294 (1.49 μ moles out of 3.44 μ moles) of the total quantity of spiked α -HCH was detected in first aquatic
295 samples taken, the rest being adsorbed on surface of FeS nanoparticles. To quantitatively determine
296 the influence of pH on the degradation rate, the kinetics of α -HCH degradation by FeS was assessed
297 by considering the dehalogenation reaction to follow a pseudo first order kinetic. Therefore, the
298 apparent rate constants (k_a) for the dehalogenation of α -HCH were calculated at different pH values
299 (Tab.1), in the three different experiments, having all of them the same level of FeS to liquid ratio
300 (the same FeS concentration of about 9 g/L) in the respective bottles. In the acidic pH domain, the
301 apparent rate constant value showed only a small increase from 0.009 d⁻¹ at pH 2.4 to 0.014 d⁻¹ at
302 pH 5.3 (both in 2nd experiment). In the alkaline pH domain, the apparent rate constants increased
303 from 0.018 \pm 0.002 d⁻¹ at pH 8.1 (1st experiment) to 0.25 d⁻¹ at pH 9.9 (3rd experiment), while the
304 highest apparent rate constant of 1.1 d⁻¹ was recorded at pH 11.8 (2nd experiment). The value of
305 apparent rate constant recorded at pH 11.8 (1.1 d⁻¹) was higher than the rate constant of 0.384 d⁻¹
306 (0.0064 h⁻¹) recorded by (Zhang et al., 2014) during alkaline hydrolysis of α -HCH performed at
307 pH 9.78. These increases in k_a values clearly showed that the apparent rate constants during
308 dehalogenation of α -HCH by FeS increased with pH, which was also shown by (Liu et al., 2003)

309 for γ -HCH isomer. By comparing the apparent rate constant of 0.25 d^{-1} recorded during
310 dehalogenation of α -HCH by FeS at pH 9.9 (3rd experiment), with the pseudo-first-order rate
311 constant of 0.090 d^{-1} recorded during hydrolysis of α -HCH in the control bottle with same pH value
312 of 9.9 (4th experiment), it can be concluded that the kinetics of α -HCH dehalogenation of by FeS
313 at moderate alkaline pH values (up to 10) is different from the one of hydrolysis. Nevertheless, at
314 very high pH values (higher than 10) the contribution of hydrolysis can become significant.

315 At the end of experiment 1, the concentrations of α -HCH and of its main degradation products
316 in the FeS slurry were: $0.840 \pm 0.008 \text{ }\mu\text{mol/g}$ for α -HCH, $0.008 \pm 0.003 \text{ }\mu\text{mol/g}$ for 1,2,4-TCB and
317 $0.0009 \pm 0.0004 \text{ }\mu\text{mol/g}$ for 1,2-DCB, respectively (see Tab. 2). These values can be used to
318 calculate a mass balance. In this respect, the number of moles from the FeS slurry for α -HCH (1.26
319 μmol), 1,2,4-TCB ($0.012 \text{ }\mu\text{mol}$) and 1,2-DCB ($0.0013 \text{ }\mu\text{mol}$) must be considered, taking into
320 account the quantity of FeS nanoparticles (1.5 g). As in many degradation studies, the mass balance
321 is taking into account firstly the final aquatic concentrations of the compounds which were ranged
322 between $5.6 \pm 0.1 \text{ }\mu\text{M}$ for α -HCH (the number of moles of α -HCH was $0.3024 \text{ }\mu\text{mol}$), $0.844 \pm$
323 $0.045 \text{ }\mu\text{M}$ for 1,2,4-TCB ($0.0455 \text{ }\mu\text{mol}$) and $0.115 \pm 0.098 \text{ }\mu\text{M}$ for DCB ($0.0062 \text{ }\mu\text{mol}$),
324 respectively (see Tab. 2). The number of moles for each compound were calculated taking into
325 account the final volume of aquatic phase (about 54 mL) at the end of the experiment. Nevertheless,
326 due to complex nature of the degradation experiments with FeS nanoparticles (Li et al., 2016), the
327 mass balance must include also the amounts of α -HCH, 1,2,4-TCB and 1,2-DCB removed from
328 the degradation bottles by sampling. The number of moles removed from the bottles by sampling
329 were: $0.7508 \text{ }\mu\text{mol}$ for α -HCH, $0.0360 \text{ }\mu\text{mol}$ for 1,2,4-TCB and $0.0050 \text{ }\mu\text{mol}$ for 1,2-DCB, and
330 these values were calculated individually for every compound as sum of numbers of moles of
331 removed in every sampling point (with the volume of 14 mL), taking into account that 1,2,4-TCB
332 and 1,2-DCB were measured only in the last seven, respectively five sampling point (out of eight).
333 The total sum of these values at the end of experiment 1 was $2.42 \text{ }\mu\text{mol}$ which means a recovery

334 of 70.4 % in the mass balance, comparing with the initially spiked amount of 3.44 μmol of $\alpha\text{-HCH}$
335 (the spiked concentration of $\alpha\text{-HCH}$ was 20.7 μM at an initial volume of 166 mL). This slightly
336 difference between the numbers of moles at the beginning and at the end of experiment 1 is
337 probably due to transformation of $\alpha\text{-HCH}$ in the intermediates PCCH and TCCH, as well to the
338 more volatile nature of 1,2,4-TCB and 1,2-DCB.

339 *3.2. Carbon isotope fractionation in the course of $\alpha\text{-HCH}$ dehalogenation by FeS*

340 In the first degradation experiment performed at pHs 8.1 and 11.8, the carbon isotope composition
341 of $\alpha\text{-HCH}$ changed during dehalogenation. Since a pH value of 8.1 is of more relevance for real
342 environmental conditions (compared to pH of 11.8 where hydrolysis is fast), the isotope
343 fractionation of $\alpha\text{-HCH}$ was assessed only at pH value of 8.1. Note that Ren et al (2005) obtained
344 a half-life time of 74 days for hydrolysis of $\alpha\text{-HCH}$ at pH 8.3. The $\delta^{13}\text{C}$ values of $\delta^{13}\text{C}$ of the
345 internal standard HCB stayed constant. The carbon isotope composition of $\alpha\text{-HCH}$ increased from
346 $-25.8 \pm 0.6 \text{‰}$ to $-23.1 \pm 0.4 \text{‰}$, whereas the $\alpha\text{-HCH}$ concentrations decreased from $9.0 \pm 0.4 \mu\text{M}$
347 to $5.6 \pm 0.1 \mu\text{M}$ (Fig. 3A). 1,2,4-trichlorobenzene (1,2,4-TCB) and 1,2-dichlorobenzene (1,2-DCB)
348 were detected after one and respectively 6 days of degradation, in concentrations of 0.014 ± 0.001
349 μM for 1,2,4-TCB and $0.019 \mu\text{M}$ for 1,2-DCB, respectively. At the end of the experiment (after 22
350 days), the concentrations increased to $0.844 \pm 0.045 \mu\text{M}$ (1,2,4-TCB) and to 0.115 ± 0.098 (1,2-
351 DCB) (Fig. 3B), while $\beta\text{-PCCH}$ was detected only as traces. For this experiment, the concentrations
352 of 1,2,4-TCB, 1,2-DCB and $\beta\text{-PCCH}$ were smaller than the linearity range of the GC-C-IRMS
353 instrument and thus their isotope compositions could not be determined. Nevertheless, in the 2nd
354 experiment, the evolution of concentration of 1,2,4-TCB in the bottle with the pH of 11.8, allowed
355 a determination of isotope signature of 1,2,4-TCB during its formation, as well the elucidation of
356 trends of 1,2,4-TCB, 1,2-DCB and benzene concentrations during dehalogenation of $\alpha\text{-HCH}$ by
357 FeS performed at more alkaline pHs. In the bottle with the pH of 11.8, the first changes in the
358 concentrations of 1,2,4-TCB, and 1,2-DCB and benzene appeared after about 40 minutes, when

359 the 1st sample was taken (Fig. 4A and 4B), due to the fast dehalogenation reaction. After about 40
360 minutes, the recorded concentration of 1,2,4-TCB was 8.5 μM (the first day of the experiment) and
361 increased to a maximum of 15.7 μM after 3 days of degradation. Afterwards its concentration
362 decreased to 7.9 μM at the end of the experiment, whereas the isotope composition of 1,2,4-TCB
363 decreased from $-31.2 \pm 0.3 \text{ ‰}$ after 3 days of degradation (lighter than the initial isotope
364 composition of α -HCH) to $-25.2 \pm 0.1 \text{ ‰}$ after 32 days of degradation (Fig. 4A).

365 The first recorded concentration of 1,2-DCB was 0.31 μM at 40 minutes (in the 1st day of the
366 experiment), then it increased to a maximum of 0.45 μM after 5 days of degradation, and afterwards
367 decreased to 0.24 μM , after 32 days of degradation (Fig. 4B). The concentration of benzene
368 increased from 0.08 μM after 3 days of degradation to 0.12 μM , after 32 days of degradation (data
369 not shown). These changes in concentration of α -HCH degradation products showed that reaction
370 rates of 1,2-DCB and respectively benzene are slower comparing with the initial reaction of α -
371 HCH to 1,2,4-TCB. Here too, the concentrations of 1,2-DCB and PCCH and benzene were smaller
372 than the linearity range of the GC-C-IRMS instrument and thus their isotope compositions could
373 not be determined.

374 3.3. Quantitative assessment of isotope fractionation during dehalogenation of α -HCH by *FeS*

375 The enrichment factor (ϵ_C) of α -HCH dechlorination in the 2nd experiment was calculated using
376 according to the Rayleigh equation (equation 2) and gave a value of $\epsilon_C = -4.7 \pm 1.3 \text{ ‰}$, while the
377 correlation coefficients (R^2) was 0.94 (Fig. 5). This ϵ_C is higher (in absolute value) than the values
378 of $-1.6 \pm 0.3 \text{ ‰}$ and $-1.0 \pm 0.2 \text{ ‰}$ recorded for *S. indicum strain B90A* and *S. japonicum strain*
379 *UT26*, respectively, for the dehydrochlorination pathways recorded by (Bashir et al., 2013) during
380 aerobic biodegradation of bulk α -HCH, but lower than the ϵ_C of $-7.6 \pm 0.4 \text{ ‰}$ recorded by (Zhang
381 et al., 2014) during dehydrochlorination of bulk α -HCH induced by alkaline hydrolysis (pH 9.78).
382 As demonstrated by (Zhang et al., 2014), the carbon isotope enrichment factors of individual
383 enantiomers ($\epsilon_{C(+)}$, $\epsilon_{C(-)}$) of α -HCH upon chemical transformation were statistically identical with

384 each other and they fall within the respective ϵ_C of bulk α -HCH within a 95% confidence interval,
385 therefore an enantioselective stable isotope analysis (ESIA) of α -HCH was not investigated in this
386 paper.

387 Considering dehydrohalogenation as the dominant degradation pathway of α -HCH by FeS, then
388 the recorded values of $\epsilon_C = -4.7 \pm 1.3 \text{ ‰}$ from this study is also lower than the enrichment factors
389 (ϵ_C) of γ -HCH recorded recently (Schilling et al., 2019) during dehydrohalogenation of γ -HCH by
390 lindane dehydrochlorinases enzymes LinA1 ($-8.1 \pm 0.3 \text{ ‰}$) and LinA2 ($-8.3 \pm 0.2 \text{ ‰}$).
391 Nevertheless, if the vicinal dihaloelimination is taken into consideration as possible degradation
392 pathway of α -HCH by FeS (albeit a minor one), then the recorded ϵ_C is similar with the ϵ_C of -4.9
393 $\pm 0.1 \text{ ‰}$ previously reported by (Zhang et al., 2014) during dihaloelimination of α -HCH by Fe
394 nanoparticles and with the ϵ_C of $-3.7 \pm 0.8 \text{ ‰}$ reported by (Badea et al., 2011) during
395 dihaloelimination pathway during anaerobic biodegradation of α -HCH by *C. pasteurianum*. The
396 recorded $\epsilon_C = -4.7 \pm 1.3 \text{ ‰}$ from this study is also similar to the ϵ_C values of $-4.2 \pm 0.4 \text{ ‰}$
397 and $-3.6 \pm 0.4 \text{ ‰}$ recorded by (Liu et al., 2020b) for α -HCH and γ -HCH, respectively, during
398 anaerobic biodegradation by an enrichment culture. Comparing with the ϵ_C from this study,
399 using the same enrichment culture, (Liu et al., 2020b) recorded a lower ϵ_C of $-1.9 \pm 0.3 \text{ ‰}$ for
400 β -HCH, but a higher one of $6.4 \pm 0.7 \text{ ‰}$ for δ -HCH. Regarding the isotope fractionation appeared
401 during the abiotic degradation of organohalides by FeS, (Koster van Groos et al., 2018) recorded
402 an isotope enrichment factor of $-19.3 \pm 2.4 \text{ ‰}$ during reductive debromination of the 1,2-
403 dibromoethane by FeS.

404 For a more comprehensive elucidation of the reaction pathway, the apparent kinetic isotope effect
405 ($AKIE_C$) (Elsner et al., 2005) was assessed assuming an elimination reaction with concerted bond
406 cleavage at one axial H/Cl pair of α -HCH to form pentachlorocyclohexene (PCCH). In this case,
407 two carbons with axial chlorine hydrogen pairs ($x = 2$) with two indistinguishable positions ($z = 2$)
408 were considered in the calculation of $AKIE_C$, while α -HCH has 6 carbon atoms ($n=6$). The resulting

409 $AKIE_C$, 1.029 ± 0.008 , is slightly lower compared to the $AKIE_C$ value of 1.035 ± 0.004 calculated
410 by (Liu et al., 2019) for LinA1 but higher to the $AKIE_C$ value of 1.011 ± 0.001 calculated for
411 LinA2, during a study involving dehydrochlorination of α -HCH by *Sphingobium indicum* Strain
412 B90A and by corresponding enzymes. The resulting $AKIE_C$, 1.029 ± 0.008 , is also lower than the
413 value of 1.048 ± 0.003 recorded by (Zhang et al., 2014) during alkaline hydrolysis of α -HCH.
414 However, if we assume that two C-Cl bonds are stepwise and reductively cleaved during the
415 reaction of α -HCH to TCCH, we have to consider that a two-electron transfers to the α -HCH
416 molecule for dichloroelimination can take place stepwise or in a concerted mode (Tobiszewski and
417 Namieśnik, 2012). The calculated $AKIE_C$ values of 1.029 ± 0.008 (for a stepwise electron transfer)
418 and of 1.014 ± 0.004 (for a two-electron transfer) are similar to the values of 1.026 ± 0.003 (for a
419 stepwise electron transfer) and of 1.013 ± 0.001 recorded by (Liu et al., 2020b) during anaerobic
420 biodegradation of α -HCH by an enrichment culture. The recorded $AKIE_C$ values from this study
421 are also similar to the values of 1.030 ± 0.0006 (for a stepwise electron transfer) and of
422 1.015 ± 0.0003 (for a two-electron transfer) recorded by (Zhang et al., 2014), during
423 dihaloelimination of α -HCH by Fe nanoparticles. As suggested by (Butler and Hayes, 1998), the
424 predominance of dehydrochlorination in the degradation of α -HCH by FeS from this study, instead
425 of vicinal dihaloelimination, might be attributable to the FeS surface hydrolysis and this creates
426 heterogeneously vicinal species (that can be either protonated or deprotonated depending on pH).
427 The heterogeneously vicinal species might favor the dehydrochlorination (elimination of HCl)
428 instead of vicinal dihaloelimination (elimination of two Cl atoms).

429 **4. Conclusions**

430 The results from this study clearly showed that carbon isotope fractionation occurs during
431 dechlorination of α -HCH by iron sulfide nanoparticles. Therefore, beside the biotic isotope
432 fractionation of HCH isomers induced by sulfate-reducing bacteria, the abiotic isotope
433 fractionation by biotic-formed FeS must be considered in anoxic sediments and aquifers

434 contaminated with HCH isomers, especially if the FeS is present in significant concentrations in
435 those areas. Nevertheless, the degradation of HCH isomers has been studied only with abiotic FeS
436 and therefore the extent of HCHs degradation in the field by biotic FeS is yet to be elucidated.
437 These degradation products identified by GC-MS demonstrated that the dehydrochlorination is the
438 main degradation pathway of the α -HCH by FeS, followed by formation of 1,2,4-TCB and by a
439 novel degradation pathway which involves a to further reductive dechlorination of 1,2,4-TCB to
440 1,2-DCB and finally to benzene, without accumulation of monochlorobenzene. The results of the
441 three degradation experiments clearly showed that the apparent first-order rate constants during
442 dehalogenation of α -HCH by FeS increased with pH, which confirms the previous studies
443 performed on the degradation of γ -HCH by FeS nanoparticles. Taking into account the novel
444 reaction mechanism proposed in this study, the isotope enrichment factor obtained can be used as
445 reference to assess and quantify the chemical and biological α -HCH transformation in the anoxic
446 environments. The relevance of various dehalogenation reaction mechanisms of α -HCH by FeS
447 are yet to be investigated using multi-element CSIA (i.e. hydrogen vs. carbon vs. chlorine) This
448 approach can provide new perspectives to assess also the environmental fate of all HCH isomers,
449 as well of other contaminants of emerging concern (CECs).

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578

579 **Tab. 1.** The values of the apparent rate constants for the dehalogenation of α -HCH calculated at
580 different pH values. The concentration of FeS was 9 g/L in all experiments.

Crt. No.	pH value	Apparent rate constant (d⁻¹)	Experiment
1	2.4	0.009	2 nd
2	5.3	0.014	2 nd
3	8.1	0.018 ± 0.002	1 st
4	9.9	0.253	3 rd
5	11.8	1.098	2 nd

581

582

583 **Tab. 2.** The concentration of α -HCH and of its major degradation products (1,2,4-TCB and 1,2-DCB) in aquatic samples and FeS slurry at the end of
 584 the 1st experiment (after 22 days) (pH=11.8).

Theoretical spiked α -HCH concentration (μ M)	Final α -HCH aquatic concentration (μ M)	Final α -HCH concentration in FeS slurry (μ mol/g)	Final 1,2,4-TCB aquatic concentration (μ M)	Final 1,2-DCB aquatic concentration (μ M)	Aquatic ratio $C_{1,2,4\text{-TCB}}/C_{1,2\text{-DCB}}$	Final 1,2,4-TCB concentration in FeS slurry (μ mol/g)	Final 1,2-DCB concentration in FeS slurry (μ mol/g)	Final sum of α -HCH (μ mol)	Final sum of 1,2,4-TCB (μ mol)	Final sum of 1,2-DCB (μ mol)
20.7	5.6 ± 0.1	0.840 ± 0.008	0.844 ± 0.045	0.115 ± 0.098	7.3	0.008 ± 0.003	0.0009 ± 0.0004	2.3132	0.0935	0.0125

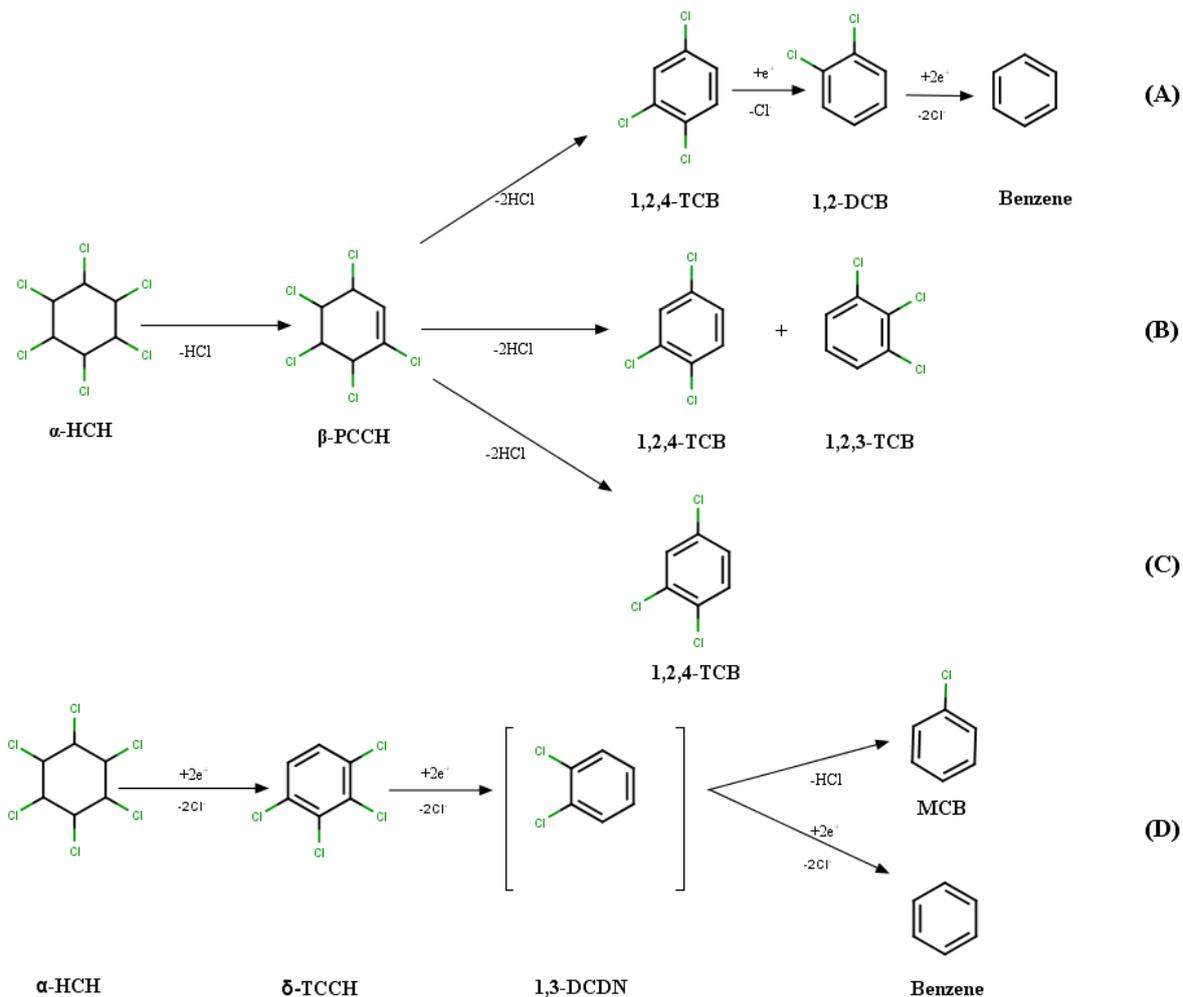
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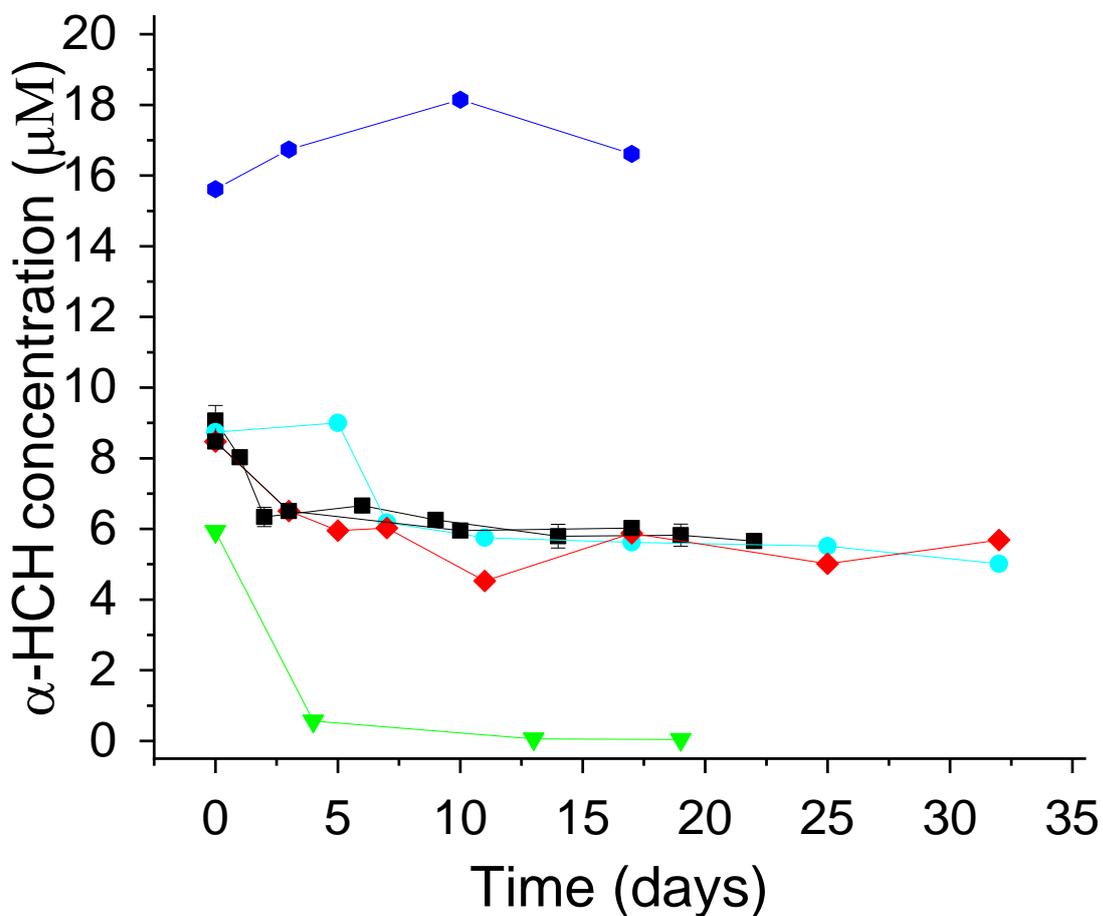
591 **Fig. 1.** The proposed pathway for the reductive dehalogenation of α -HCH with FeS nanoparticles

592 from this study (A); Hydrolysis pathway proposed by (Kannath et al., 2019) (B); Biotic

593 dehydrochlorination degradation pathway proposed by (Suar et al., 2005) (C); Biotic vicinal

594 dihaloelimination pathway proposed by (Badea et al., 2011) (D).

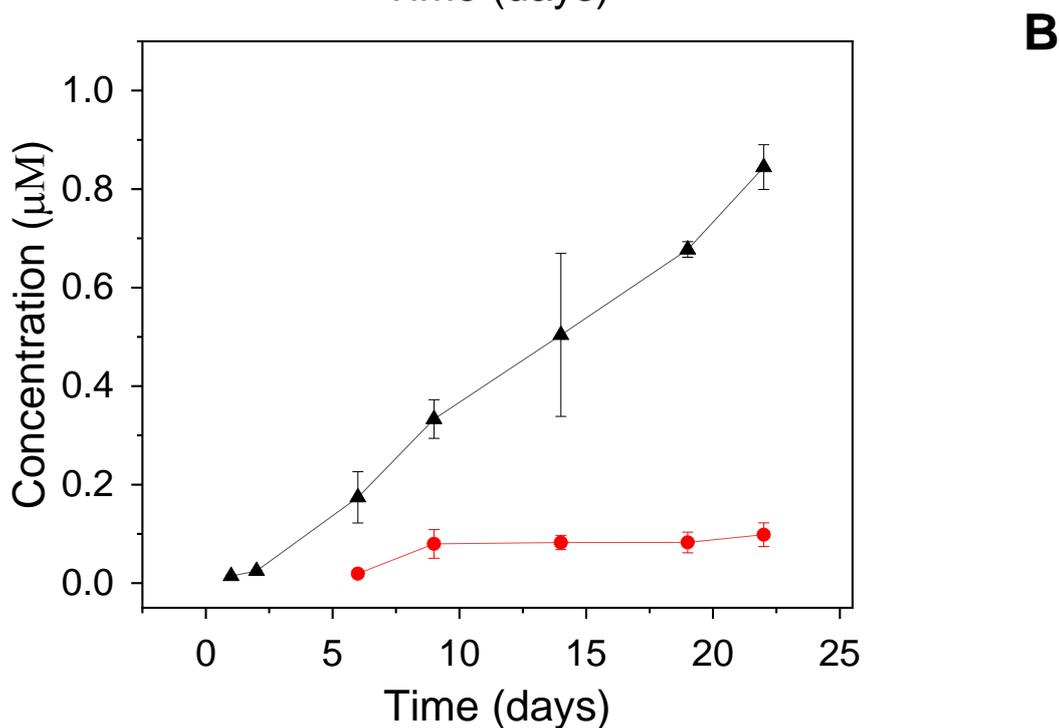
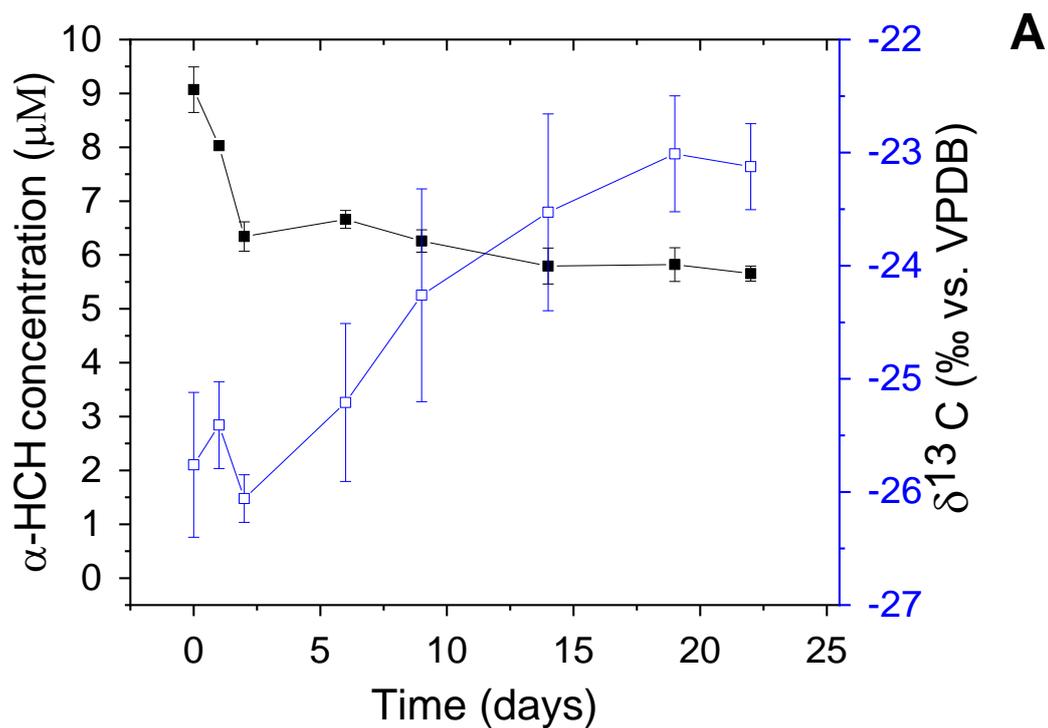
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596

597 **Fig. 2.** The degradation curves of α -HCH by FeS at pH values: 8.1 (■) (experiment 1, black line);
 598 2.4 (♦) and 5.3 (●) (experiment 2, cyan line); and 9.9 (experiment 3, green line) (▼), in three
 599 different degradation experiments, as well the evolution of α -HCH concentration in the control
 600 experiment (●) (experiment 4 without FeS, blue lines) at pH value of 7.0.

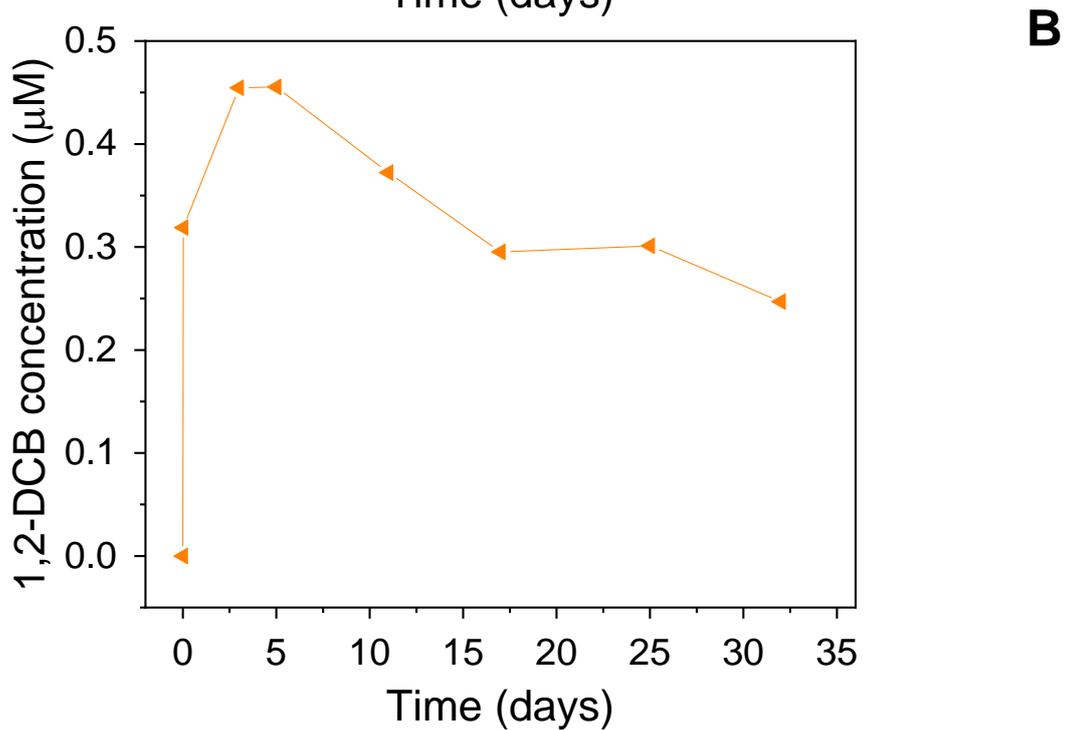
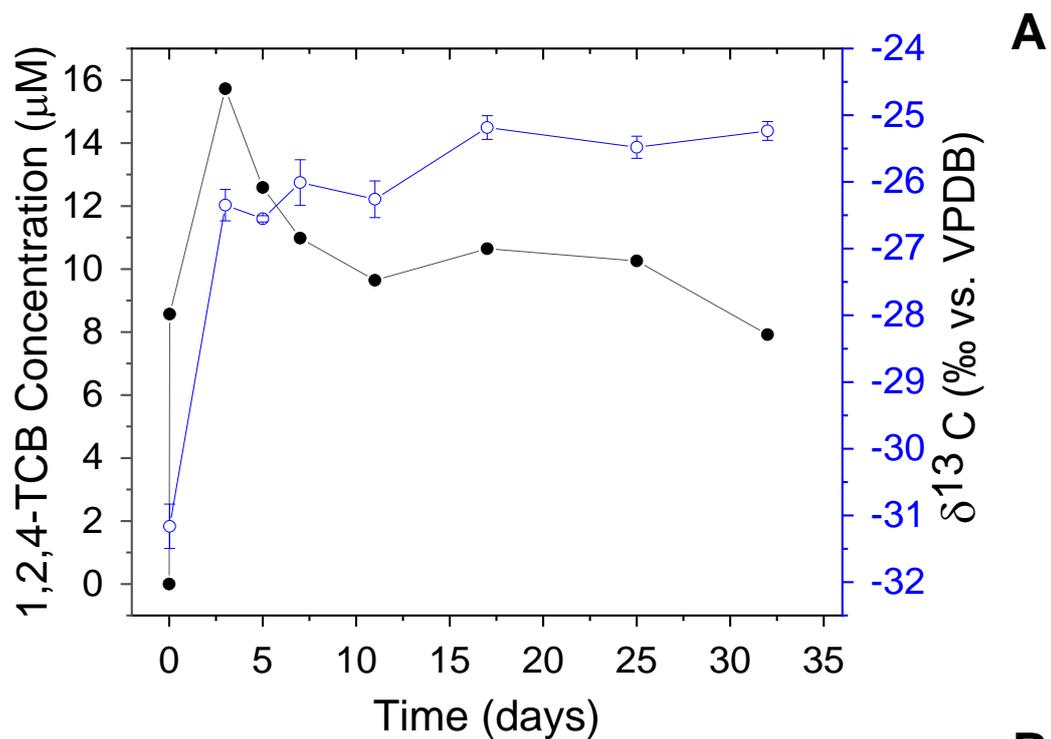
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602

603 **Fig. 3.** Isotope signatures (\square) vs. concentrations (\blacksquare) of α -HCH during the 1st degradation
 604 experiment by FeS at pH of 8.1 (A). Formation of 1,2,4-TCB (\blacktriangle) and 1,2-DCB (\bullet) in the same
 605 experiment with FeS (B). The error bars shown the standard deviation of the concentration values
 606 between the two replicates, and the standard deviation of the isotope analysis, respectively.

607

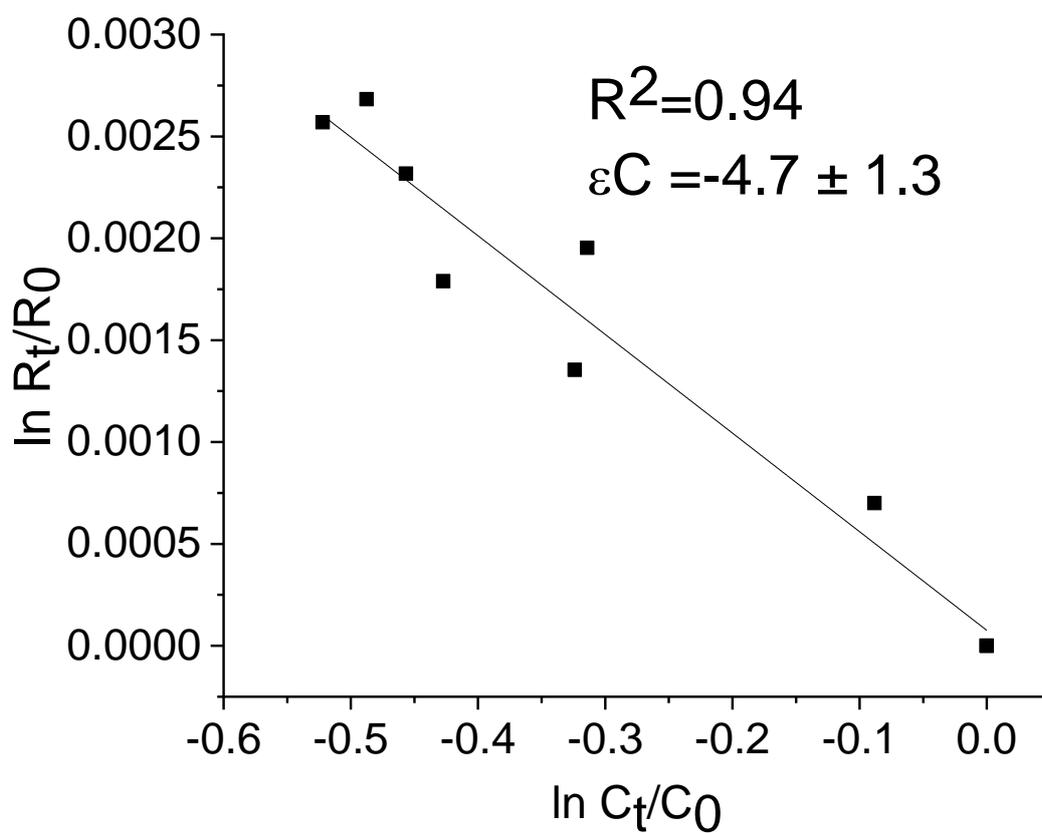


608

609 **Fig. 4.** Isotope signatures (○) vs. concentrations (●) of 1,2,4-TCB during the 2nd degradation

610 experiment by FeS at pH of 11.8 (A). Formation of 1,2-DCB (◄) in the same experiment (B).

611



612

613 **Fig. 5.** Calculation of carbon isotope enrichment factor of α -HCH during dehalogenation by FeS
 614 according to the Rayleigh model in experiment 1 (pH = 8.1).

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616