

Sorbent-modified biodegradation studies of the biocidal cationic surfactant cetylpyridinium chloride

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ABSTRACT

Biodegradability studies for the cationic surfactant cetylpyridinium chloride (CPC) are hampered by inhibitory effects on inoculum at prescribed test concentrations (10–20 mg organic carbon/L). In this study, we used ¹⁴C labeled CPC in the 28 d Headspace Test (OECD 310) and demonstrated that CPC was readily biodegradable (10– > 60% mineralization within a 10 day window) at test concentrations 0.006–0.3 mg/L with CPC as single substrate. Biodegradation efficiency was comparable over this concentration range. CPC inhibited degradation at 1 mg/L and completely suppressed inoculum activity at 3 mg/L. In an extensive sorbent modified biodegradation study we evaluated the balance between CPC bioaccessibility and toxicity. A non-inhibitory concentration of 0.1 mg/L CPC was readily biodegradable with 83% sorbed to SiO₂, while biodegradation was slower when 96% was sorbed. SiO₂ mitigated inhibitory effects of 1 mg/L CPC, reaching > 60% biodegradation within 28 d; inhibitory effects were also mitigated by addition of commercial clay powder (illite) but this was primarily reflected by a reduced lag phase. At 10 mg/L CPC SiO₂ was still able to mitigate inhibitory effects, but bioaccessibility seemed limited as only 20% biodegradation was reached. Illite limited bioaccessibility more strongly and was not able to sustain biodegradation at 10 mg/L CPC.

1. Introduction

Environmental risk assessment compares likely exposure scenarios with associated hazards. Assessment of the overall hazard profile of a chemical includes information on persistency, bioaccumulation potential and toxicity, combined with available information on actual environmental exposure levels. For most chemicals, persistency depends largely on microbial biodegradation rates ultimately resulting in full mineralization to inorganic carbon (IC) (Boethling et al., 2009). For many chemicals biodegradation by microbial communities present in sewage sludge is the major process to reduce environmental exposure levels. Measuring biodegradation is complicated for chemicals that are toxic to the inoculum at required testing levels (Prado et al., 2010), and for highly sorptive chemicals with insufficient bioaccessibility (Wszolek and Alexander, 1979; Alexander, 2000). Certain cationic surfactants combine these properties: relatively toxic to microorganisms, with several being used as biocides or antiseptics, and adsorptive properties due to favorable electrostatic and hydrophobic

interactions with sorbents (García et al., 1999, 2004; Garcia et al., 2016; Kaczerawska et al., 2018; Li and Brownawell, 2010; Brownawell et al., 1990). Specific microbial strains capable of metabolizing these surfactants have been identified (van Ginkel et al., 1992).

Metabolites resulting from the primary degradation of most cationic surfactants can be identified using liquid chromatography/mass spectrometry on aqueous samples from a biodegradation study, but formation of metabolites provides limited evidence for complete mineralization potential. Therefore, most standardized biodegradation test methods aim to measure complete mineralization using e.g. standard IC analysis (OECD, 2014). However, to generate IC levels significantly above inoculum background IC production, recommended test concentrations are 10–20 mg organic carbon/L (mg_C/L) (OECD, 2014; OECD, 1992). For ¹⁴C-radiolabeled substrates, mineralization to ¹⁴C-CO₂ can be detected at much lower (< 1 µg/L) and potentially non-inhibitory test concentrations, but labeled compounds are expensive and not readily available.

Abbreviations: CEC, cation exchange capacity; CPC, cetylpyridinium chloride; CTAB, cetyltrimethylammonium bromide; DSB, diffusion-sorption-biodegradation; IC, inorganic carbon; mg_C/L, mg carbon per liter; NIEC-STP, non-inhibitory effect concentration for sewage treatment plant inoculum; STP, sewage treatment plant.

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Recently, we evaluated biodegradability of the cationic surfactant cetyltrimethylammonium bromide (CTAB), spiking potentially inhibitory concentrations with introduction of mitigating adsorbents silicon dioxide (SiO₂) and illite (Timmer et al., 2019). The concept of toxicity mitigation of CTAB while maintaining adequate bioaccessibility was also demonstrated in the OECD 310 ring test and other studies (OECD, 2014; Painter et al., 2003; van Ginkel et al., 2008). However, in our recent study there were indications that higher adsorbent doses limited bioaccessibility, reducing biodegradation efficiency (Timmer et al., 2019). This is in agreement with studies suggesting ultimate biodegradability of surfactants and polycyclic aromatic hydrocarbons is determined by desorption rates (Van Ginkel et al., 2003; Cornelissen et al., 1998). In the current study, we aimed to further elucidate the balance between toxicity, bioavailability and bioaccessibility using cetylpyridinium chloride (CPC). CPC is a common antiseptic, and is also used in various organoclay processes (Zhu et al., 2009; Lee and Tiwari, 2012; de Paiva et al., 2008; Ganguly et al., 2011), and as a key component of ionic liquids (Pendleton and Gilmore, 2015; Tang et al., 2017); and detectable levels are present in the environment (Shrivastava and Wu, 2007). CPC has been widely studied: a search for literature with 'cetylpyridinium' as keyword in Scopus resulted in 4385 hits (March 2019). However, no studies on ready biodegradability of CPC were identified, although in a recent study Nguyen & Oh explored the removal by and impact on activated sludge of environmentally relevant levels of CPC (Nguyen and Oh, 2019). Ready biodegradability of the structurally related surfactant benzalkonium chloride received more attention (Garcia et al., 2016; Ertekin et al., 2016; Geerts et al., 2015).

The first aim was to determine whether ¹⁴C-labeled CPC (¹⁴C-CPC) was readily biodegradable (definition in Supporting Information (SI)) by sewage treatment plant (STP) inoculum in a standard 28 d Headspace Test (OECD, 2014). Since ready biodegradability experiments are very stringent, compounds that pass will likely be degraded rapidly under environmental conditions. Hence, it is desirable for high production volume chemicals to be classified as readily biodegradable, as they will not be persistent. The second aim was to determine whether biodegradation efficiency with CPC as a single source of carbon had a threshold level in the non-inhibitory µg/L range (Egli, 2010; Fenner et al., 2013). With the knowledge that CPC was toxic at concentrations below the recommended 10 mg_C/L spiking level, we then explored biodegradation efficiency enhancement of a mixture of labeled and non-labeled surfactant through addition of sorbent. Based on the premise that increased sorbent levels reduce bioavailable concentrations, we also aimed to define under which conditions bioaccessibility could become rate limiting. Systematic series of biodegradation studies were performed with addition of two inert inorganic sorbents (SiO₂ and illite) at different surfactant spiking levels (non-inhibitory, inhibitory, ~10 mg_C/L). This should clarify whether a biodegradation study following a stepwise sorbent addition approach (determine inhibitory threshold concentration, sorption studies, perform sorbent modified biodegradation test) has applicability boundaries or is applicable to multiple potentially inhibitory cationics. It should be noted that the methods proposed in this manuscript were specifically designed to remain within the boundaries of OECD Guidelines. Ultimately, this should lead to a defensible decision regarding biodegradability of surfactants under more realistic field conditions where sorptive substrates are available, e.g. sand and sludge itself in a STP, contaminated sediment, or soil on which sewage sludge is applied. In addition, it would be more relevant to employ test item concentrations close to the predicted environmental concentration; recommended test concentrations are generally unrealistically high and proposed matrices lack sorptive substrates.

2. Materials and methods

2.1. Chemicals and sorbents

CPC (M_w 340 g/mol), sodium benzoate (reference substrate), silicon dioxide (SiO₂; Davisil Grade 633, particle size 35–75 µm, surface area

480 m²/g), and buffer salts were all analytical grade and obtained from Sigma-Aldrich (Gillingham, Dorset, UK). Illite (fine powder; Argiletz, France), was comparable as in related sorption studies with surfactants (Droge et al., 2009; Droge and Goss, 2013a). Potassium hydroxide (KOH), sodium hydroxide (NaOH), sulfuric acid (H₂SO₄) and trifluoroacetic acid (TFA) were analytical grade (Sigma-Aldrich). Scintillation cocktails (Hionic-Fluor, Pico-Fluor Plus, Permafluor E+), Carbosorb, and combustion cones for the sample oxidizer (Packard Model 307) were purchased from PerkinElmer (Seer Green, Beaconsfield, UK).

¹⁴C-CPC was obtained from Quotient BioResearch Ltd. (Cardiff, UK); the molecule contained two ¹⁴C-atoms within the pyridinium, which is likely the last moiety to be biodegraded. Radiochemical purity was 99.8% (Radio-HPLC). A 20.62 mg/L ¹⁴C-CPC stock solution was prepared in acetone; concentration was confirmed by LSC (TriCarb 2810 TR, PerkinElmer). Higher concentrations were prepared by supplementing with unlabeled CPC; formation of ¹⁴C-CO₂ is a proxy for total mineralization.

2.2. Inoculum

Activated sludge was obtained from Broadholme Sewage Treatment Works (BSTW; Ditchford Road, Wellingborough & Irthingborough, UK). The BSTW treats sewage from approximately 80,000 households and < 15% of the organic load is attributable to industrial discharge (van Egmond et al., 2013). Sludge was treated as described previously (Timmer et al., 2019). Suspended solids concentration of activated sludge was 5 ± 1 g/L; filtered supernatant after centrifugation was used as inoculum.

2.3. Sorption test

Three doses of illite and SiO₂ (0.80, 4.0 and 20 g/L) were exposed to three concentrations of ¹⁴C-CPC (13.5 mg/L, 1.35 mg/L, and 135 µg/L) in 20 mL borosilicate vials (PerkinElmer), and placed overnight on a roller mixer at 30 rpm (Thermo Fisher Scientific, Waltham, MA, USA), at room temperature. Control vials without sorbent were also prepared. All vials were centrifuged at 2400 g for 30 min, followed by visual inspection for absence of suspended particles and sampling of three 2 mL aliquots of supernatant. Kerr et al. successfully applied centrifugation combined with LSC to determine sorption coefficients using radiolabeled cationic surfactants of comparable alkyl chain length (CTAB), sorbed to activated sludge (Kerr et al., 2000). Concentration of ¹⁴C-CPC was determined by LSC, followed by calculation of sorbed amounts by assuming 100% - supernatant = sorbed amount; possible sorption to glassware was disregarded as affinity for sorbent is theoretically much higher and the aim was to provide an estimated sorption coefficient. Measurements were used to fit linear sorption isotherms and calculate sorption coefficients.

2.4. Preparation of Headspace Test

The OECD 310 Guideline was used to design the biodegradation experiments performed (OECD, 2014), as outlined in more detail in the Supporting Information. In brief, ¹⁴C-CPC was added to 50 mL mineral medium in 125 mL borosilicate glass serum bottles containing appropriate amounts of sorbent. Vessels were left standing for 2 h to allow CPC to equilibrate between medium and sorbent. Thereafter, 50 mL of mineral medium containing 20 mL/L of inoculum was added to all test vessels, resulting in a 64 mL headspace and an inoculum concentration of 10 mL/L. Vessels were incubated under orbital shaking (100 rpm) in HT Multitron incubators (Infors AG, Bottmingen, Switzerland) at 22.0 ± 1.0 °C.

Sodium benzoate (C₇H₅O₂.Na) was used as reference substrate at a concentration of 17 mg/L, corresponding with ~10 mg_C/L. After incubation bottles were injected with 1 mL 7 M NaOH and shaken for 60 min, after which contents of each bottle were divided over three

20 mL vials. Benzoate biodegradation was quantified by triplicate IC measurements on a TOC-V total organic carbon analyzer (Shimadzu Ltd., Milton Keynes, UK). IC in blanks was subtracted from IC in test vessels to control for background IC production. Biodegradation was determined by comparing IC production with organic carbon in the spiked concentration.

2.5. Biodegradation studies without sorbent

Six ^{14}C -CPC concentrations (6.0–3000 $\mu\text{g/L}$) were tested without sorbent to assess influence of freely dissolved concentration (C_{free}) on biodegradability. This also allowed determination of a non-inhibitory effect concentration for STP inoculum (NIEC-STP). Three concentrations (6, 25, and 300 $\mu\text{g/L}$) were sampled at seven time points (see Table S1), which was sufficient to assess kinetics and presence of inhibitory effects. Ten and eight time points (see Table S1) were prepared in duplicate for 100 $\mu\text{g/L}$ and 1.0 mg/L, respectively. These concentrations were tested in more detail since they were the nominal concentrations to be added in the experiments with sorbent. Based on inconclusive replicates at high test concentrations in previous work (Timmer et al., 2019) and the expectation of full inoculum knockout at 3 mg/L, twelve replicates were prepared to be sampled on Day 28. Upon sacrificial sampling, vessels were injected with 1 mL of 7 M NaOH and further treated as described in section 2.7.

2.6. Biodegradation studies with sorbent

For sorbent modified biodegradation series two different sorbents were used (SiO_2 and illite) at doses of 0.8, 4.0 and 20 g/L. As SiO_2 was the chosen sorbent in literature (Painter et al., 2003; van Ginkel et al., 2008), SiO_2 was also employed at 100 $\mu\text{g/L}$ CPC. For the 1 mg/L CPC treatment both sorbents were applied to assess suitability of illite and to allow comparison with SiO_2 at this inhibitory concentration. At 10 mg/L CPC only the highest sorbent dose was tested, based on sorption coefficients and expected NIEC-STP. Before sampling, designated vessels were injected with 1 mL of 7 M NaOH and further treated as described in section 2.7. Benzoate controls were incubated at three concentrations of SiO_2 to evaluate sorbent effects on inoculum activity; due to a significant IC content (1.7%) illite was not suitable for use with standard IC analysis and could not be tested with benzoate.

2.7. Detection and analysis of ^{14}C -CPC

After shaking with NaOH (60 min), vessels were left standing for 5 min to settle suspended sorbent. Six 3 mL aliquots were sampled; 1 mL of 4 M H_2SO_4 (sulfuric acid) was added to three aliquots. These acidified samples were left uncapped in a fume hood overnight to vent off CO_2 . Mineralization, calculated from the difference between alkaline (containing $^{14}\text{CO}_2$) and acidified (without $^{14}\text{CO}_2$) samples, was then compared with applied radioactivity to calculate biodegradation percentages. ^{14}C -CPC potentially sorbed to the glass surface was extracted as follows. Vessels were emptied after sampling, while shaken vigorously if necessary to re-suspend and pour out residual sorbent. Vessels still containing visible amounts of sorbent were quickly rinsed twice with 5 mL MilliQ. A 5 mL aliquot of washing solution (0.1% TFA in 90/10 MeOH/MilliQ v/v) was added, after which vessels were capped and put on a roller mixer (Thermo Fisher Scientific) at 30 rpm for 150 min. This extract was collected in a 20 mL liquid scintillation vial. All samples were prepared for LSC by adding 16 mL of Hionic-Fluor followed by forceful shaking for 5 s.

2.8. Mass balance

The contents of all Day 7 vessels without sorbent (0.1, 1.0 and 10 mg/L CPC) were filtered under vacuum using 100 mm Whatman GF/C filters. This was repeated for vessels with and without sorbent

sampled on Day 28. Sorbent was flushed onto the filter by rinsing vessels twice with 5 mL MilliQ. Filters were carefully placed on marked pieces of aluminum foil, which were left overnight in a fume hood to dry and were subsequently analyzed by combustion. Filter residue was distributed over multiple cellulose combustion cones, after which the emptied filters were divided over two combustion cones. Shortly before combustion the contents of every combustion cone were enriched with 100 μL analytical grade hexadecane to increase combustion temperature and efficiency. CO_2 formed during combustion was trapped in ~ 5 mL Carbo-Sorb E, which was mixed with 7 mL Permafluor E+ and 8 mL Pico-Fluor to prevent quenching, which was observed during a trial run and was likely caused by soot particles in the samples. LSC measurements of alkaline samples, glass extracts, and combusted samples were used to determine the mass balance; control vessels without inoculum served as 100% reference.

2.9. Statistics

Curves presenting biodegradation results were fitted using the '[Agonist] vs. response – Variable slope (four parameters)' model included with GraphPad Prism 7.04 for Windows.

3. Results and discussion

3.1. Biodegradation studies without sorbent

BSTW inoculum was shown to be of adequate activity using a positive control with benzoate. Results after 4 d and 7 d of incubation indicate > 80% biodegradation in all vessels (SI Fig. 1). This confirms that SiO_2 has no negative impact on inoculum activity. Rapid biodegradation without apparent lag phase was observed for the three lowest CPC concentrations (Fig. 1), reaching > 30% biodegradation after 4 d, > 60% biodegradation within 10 d, and a maximum biodegradation of 70–80%, satisfying the criteria for ready biodegradability. Biodegradation at 300 $\mu\text{g/L}$ was slightly slower and failed to meet the criteria for ready biodegradability, although > 60% biodegradation was reached after 18 d. Larger differences in biodegradation efficiency became apparent at 1 mg/L, with a three-week lag phase and some inconsistency between replicates in further measurements, especially on Day 21. At 1 mg/L, an average biodegradation of 40% was reached after 25 d. Only two of the twelve 3 mg/L replicates showed > 5% biodegradation after 28 d. This not only reflects the strong inhibitory effect of CPC at this concentration, but simultaneously illustrates that results obtained in this kind of biodegradability experiments can be variable. Even at inhibitory test item concentrations significant biodegradation can occasionally occur in individual replicates.

Overall, results indicate CPC caused inoculum inhibition at 1 mg/L with partial recovery in the fourth week. There is complete inoculum knock-out at 3 mg/L, while signs of inhibition were already apparent at 300 $\mu\text{g/L}$. The NIEC-STP of CPC was 100 $\mu\text{g/L}$, at least 100 times lower than recommended concentrations in most ready biodegradability studies (OECD, 2014; OECD, 1992). Inconsistent replicates in 1 and 3 mg/L groups are likely caused by inoculum heterogeneity and variability in developmental rate of micro-organisms capable of degrading CPC. Optimal toxicity mitigation can be achieved by assessing the NIEC-STP, as this allows estimating the amount of sorbent needed to reduce freely dissolved concentrations (C_{free}) to non-inhibitory levels, especially if data on sorbent affinity is available or determined experimentally.

3.2. Sorption coefficients

Isotherms for sorption of CPC to SiO_2 and illite were plotted with a fixed slope of 1 (Fig. 2), as sorption is expected to be linear at concentrations well below the cation-exchange capacity (CEC) (Droge and Goss, 2013a; Jolin et al., 2016, 2017). Two independent experiments using SiO_2 were in good agreement; the fitted curve indicates a $\log K_D$

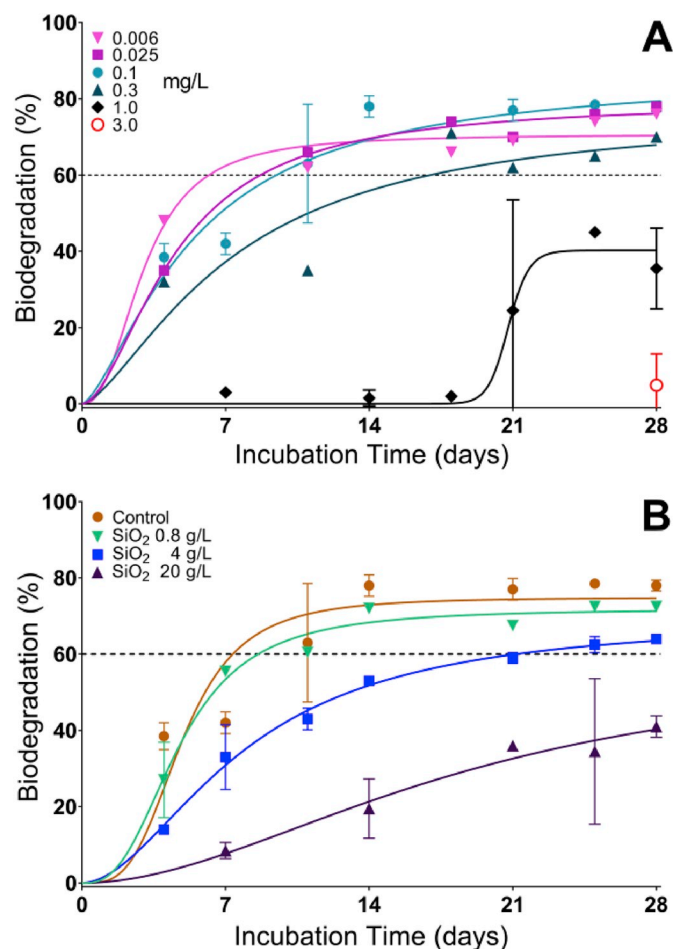


Fig. 1. (A) CPC biodegradation without sorbent. Single replicates for 6, 25, and 300 $\mu\text{g/L}$. Duplicates for 0.1 and 1 mg/L (averages + error bars). Twelve 3 mg/L replicates were sampled after 28 d. (B) Influence of SiO_2 on biodegradation at non-inhibitory CPC concentration (0.1 mg/L).

of 3.5 and spans two orders of magnitude, with highest concentration (47 $\mu\text{g/L}$) close to the NIEC-STP (100 $\mu\text{g/L}$). Although used concentrations were sometimes above this concentration range the K_D can still be used to predict C_{free} , as previous work with a surfactant of comparable alkyl chain length (CTAB) demonstrated linear sorption up to 1 mg/L, using the same batch of SiO_2 (log K_D 3.3) (Timmer et al., 2019).

The isotherm for illite covered a 20-fold concentration range, with highest concentration a factor 20 below the NIEC-STP and ~500 times lower than the reported CEC of 288 mmol/kg (Pleysier and Juo, 1980). The calculated log K_D of 4.5 was used as a representative sorption affinity; establishing a sorption isotherm encompassing all test concentrations was not within the scope of our work. Furthermore, this experimentally determined K_D (mineral medium; pH 7.4; divalent cation concentration ~0.1 mM Mg^{2+}) was in agreement with predicted K_D (log 4.18) based on a cation-exchange model for $\text{C}_x\text{H}_y\text{N}^+$ amines as derived by Droge & Goss (pH 7; medium with 5 mM Ca^{2+}) (Droge and Goss, 2013b). Measurements of actual C_{free} on Day 0 were used to estimate initially sorbed fractions in the biodegradation study. SI Fig. 2 compares measurements with predictions based on experimentally determined K_D , and shows these are in good agreement for 1 mg/L groups and at the two highest sorbent dosages in particular.

3.3. Bioaccessibility and biodegradability with sorbent

At 0.1 mg/L CPC was readily biodegradable without sorbent, as well as with the lowest sorbent dose at which ~83% was initially adsorbed (Fig. 1B). Interestingly, biodegradation rates seemed to decrease with increasing sorbent concentration. At 4 g/L (sorbed fraction ~88%) biodegradation was slower and reached 60% after 21 d. At 20 g/L (sorbed fraction ~96%; C_{free} ~4 $\mu\text{g/L}$) biodegradation slowed down further and total mineralization after 28 d was half of control, while C_{free} in these vessels was comparable to the lowest concentration without sorbent, which showed rapid and extensive biodegradation (Fig. 1A). However, nominal amount of CPC in these vessels was 17 times higher. This supports the notion of bioaccessibility limitations, with highest SiO_2 dose associated with lowest bioaccessibility. Availability of carbon (in the form of CPC) is apparently sufficient to maintain degrading microorganisms, but might be insufficient to allow for the exponential growth necessary to degrade all CPC within 28 d.

Results observed with 1 mg/L CPC were different than those obtained with 0.1 mg/L CPC. As discussed in section 3.1, controls without sorbent showed a three-week lag phase (Figs. 1A, 3A and 3B), indicating inhibitory effects. This lag phase was strongly reduced with SiO_2 as mitigating sorbent. All three SiO_2 doses show comparable results during the last two weeks of the experiment (Fig. 3A), with 60–70% of CPC mineralized. Apparently, at these concentrations CPC was sufficiently bioaccessible to allow exponential growth of CPC-degrading microorganisms. The delayed onset of biodegradation at 20 g/L SiO_2 is indicative of lower bioaccessibility initially impairing exponential growth. During the first three weeks a number of replicates at 0.8 g/L showed lower biodegradation. This suggests inhibitory effects

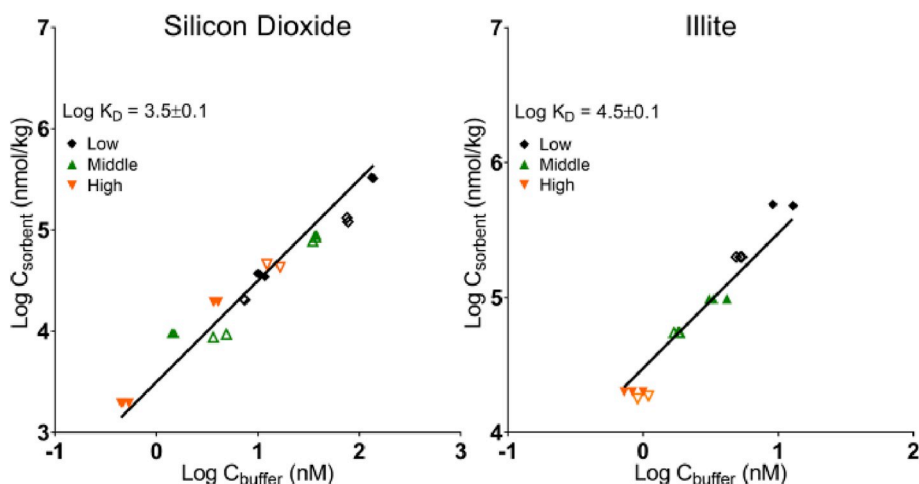


Fig. 2. Isotherms for CPC sorption to SiO_2 (left) and illite (right). Symbols correspond with associated sorbent dosages and distinguish between a first (open symbols) and second (closed symbols) measurement series.

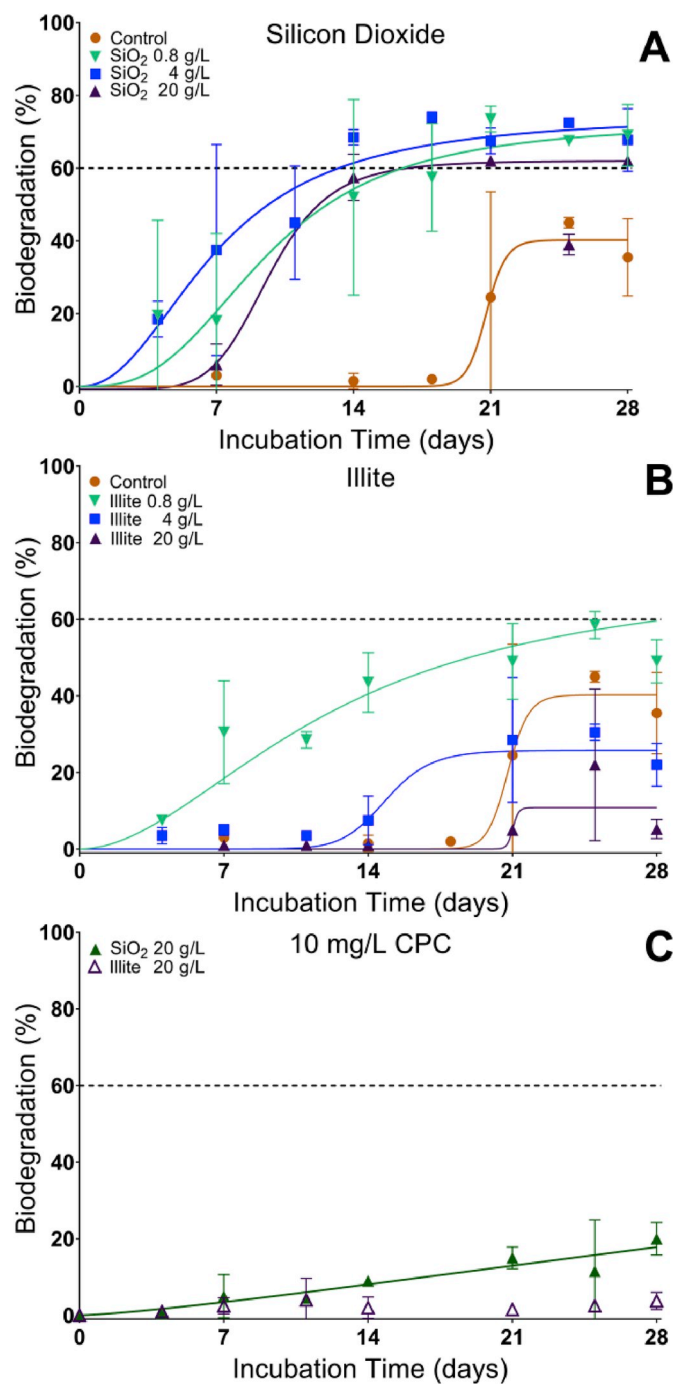


Fig. 3. Influence of SiO₂ (A) and illite (B) on biodegradation at inhibitory CPC concentration (1 mg/L). Influence of both sorbents on biodegradation at 10 mg/L CPC (C); measured C_{free} was < 0.1 mg/L, which should not be inhibitory to the inoculum.

still might occur at relatively high C_{free} associated with the lowest sorbent concentration (sorbed fraction ~85%, reducing C_{free} to 150 μ g/L, a factor 1.5 above the NIEC-STP). Overall, these results support the hypothesis that SiO₂ is a suitable sorbent to mitigate inhibitory effects of CPC, although at a concentration below the 10–20 mg_C/L recommended for IC analysis.

As shown in Fig. 3B, results with illite differed from those obtained for SiO₂. Highest biodegradation levels were observed at the lowest sorbent concentration (adsorbed fraction ~93%), with decreasing biodegradation for increasing sorbent concentrations. While equal doses of illite and SiO₂ had been used, C_{free} will be significantly lower because of

the 10 times higher sorption affinity to illite (Fig. 2). However, despite – or perhaps because of – lower bioavailable concentrations in the illite treatments, none of the illite dosed groups reached > 60% biodegradation. Thus, addition of illite did not result in a readily biodegradable classification. On average, the highest illite dose (> 99.6% adsorbed) showed ~10% mineralization after 28 d, six times lower than the respective SiO₂ treatment.

In the 10 mg/L CPC treatment, in line with Guideline recommendations of 10 mg_C/L (OECD, 2014; OECD, 1992), sorbents were tested only at 20 g/L, as this high CPC concentration requires considerable sorptive capacity to decrease C_{free} below the NIEC-STP. Measurements on Day 0 (SI Fig. 2) confirmed that C_{free} was below the NIEC-STP. As shown in Fig. 3C, addition of SiO₂ results in linear biodegradation with a relatively short lag phase, but at a seemingly slow rate compared to the 20 g/L dose at 1 mg/L CPC. However, converted to absolute amount of CPC (SI Fig. 5) approximately four times more has been mineralized at 10 mg/L than at 1 mg/L. Results for illite show virtually no biodegradation over the 28 d period. Since inhibitory effects can be ruled out based on C_{free} of < 100 μ g/L, this supports the bioaccessibility hypothesis.

Limited biodegradation at high CPC concentrations with high adsorbed fractions indicates microbes might not degrade sorbed fractions directly, and require release to the aqueous phase for sustenance and exponential growth (Egli, 2010). It appears that desorption and subsequent diffusion can be rate-limiting, whereby influx of chemical substrate is insufficient to act as the sole carbon source during exponential growth. Similar findings were reported by Scow & Alexander for decreased biodegradation kinetics of phenol by *Pseudomonas* in the presence of spherical kaolinite aggregates, where microbes were considered to have limited access to substrate aggregates (Scow and Alexander, 1992). Such findings could be explained by a diffusion-sorption-biodegradation (DSB) model (Scow and Alexander, 1992; Scow and Hutson, 1992; Chung et al., 1993). Ugochukwu et al. demonstrated limited substrate bioaccessibility caused by strong sorption/limited desorption, using organoclay and other clay additions to influence biodegradation rate of crude oil hydrocarbons (Ugochukwu et al., 2014; Ugochukwu and Fialips, 2017), while Smith et al. found that quinoline sorbed to smectite was degraded 30 times slower than dissolved quinoline (Smith et al., 1992). Future studies could consider accurate desorption or diffusion rate measurements combined with qualitative and quantitative measurements of inoculum microorganism development, in order to fit experimental data to such DSB models.

Based on a lower degradation rate at 10 mg/L CPC compared to 1 mg/L CPC with similar sorbent doses, it can be argued that the flux microorganisms can handle optimally has a (Michaelis-Menten) maximum, a notion supported by Lo et al. in their *in vitro* research using rainbow trout liver enzymes (Lo et al., 2015). Micelle formation can be ruled out, as the highest CPC concentration applied is still a factor 40 below the critical micelle concentration (~370 mg/L) (Safavi and Karimi, 2002). Compared to porous and rapidly settling SiO₂ particles, which were observed to partially form a gel-like precipitate, it was expected that the flat (non-expanding) platelet structure and suspensibility of illite would facilitate desorption, thereby enhancing bioaccessibility. Contrastingly, our findings indicate SiO₂ bioaccessibility outperformed that of illite. We currently do not have clear explanations for this difference, although illite sedimentation was observed at high sorbent loadings. We hypothesize, though, that the porous SiO₂ structure could facilitate microbial growth near pores (Lahlou et al., 2000), providing benefits from nanoscale distances over which desorption allows for acquiring CPC as carbon substrate, although Johnsen et al. suggest a minimum pore size of 200 nm for microbial colonization (Johnsen et al., 2005).

3.4. Mass balance

Controls without inoculum were used as 100% reference to calculate a mass balance for Day 7 (SI Fig. 3) and Day 28 (Fig. 4). Mass

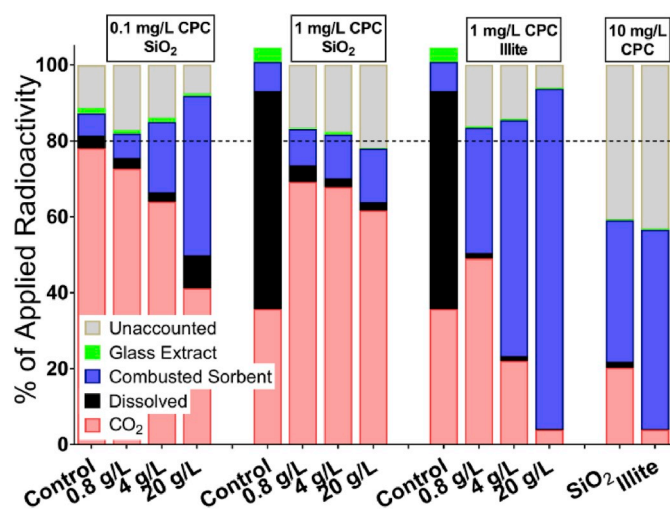


Fig. 4. Day 28 mass balance. 0.1 mg/L CPC at three SiO₂ doses; 1 mg/L CPC at three SiO₂ doses; 1 mg/L CPC at three illite doses; 10 mg/L CPC with 20 g/L of both sorbents. Combusted sorbent fraction might also contain ¹⁴C assimilated by biomass.

balance for 0.1 mg/L CPC on Day 7 is 103%, with 3.5% glass-bound and 42% mineralized. Biodegradation on Day 7 in the 1.0 mg/L treatment is 3.0% and indicative of inoculum inhibition (see Fig. 1B), while a high (> 90%) mass balance was retrieved. Mass balance after 28 d was > 80% (replicate average), except for the 10 mg/L CPC groups (~60%). The poor mass balance in the 10 mg/L group is likely caused by incomplete recovery during sorbent combustion. Fraction of dissolved organic ¹⁴C (CPC and/or metabolites) for sorbent additions ranged between 0.2% (10 mg/L CPC with 20 g/L illite) and 8.8% (0.1 mg/L CPC with 20 g/L SiO₂). At 1.0 mg/L CPC, dissolved fraction decreases with increasing sorbent concentration. The dissolved fraction is 2–8 times lower in illite vessels, demonstrating the 10 times higher sorption coefficient. Glass wall extracts are insignificant, except for vessels without sorbent which have a higher C_{free}.

Combustion of filter residue at 0.1 mg/L CPC retrieved 6–42% of applied radioactivity, which is likely partially due to assimilation of ¹⁴C into biomass. Although this fraction might still contain adsorbed radioactivity, particularly for the 20 g/L dose, this is not necessarily solely unmetabolized ¹⁴C-CPC. Results were more variable within 1 mg/L CPC treatments, especially for filter residues and CO₂ determination. Nevertheless, C_{free} (or rather dissolved organic ¹⁴C) was higher than in the 0.1 mg/L group (8.8–30.1 μg/L vs. 4.4 μg/L). Different vessels with SiO₂ had comparable fractions in filter residue and mineralized to CO₂. This might be an indication that radioactivity in filter residue consists primarily of assimilated ¹⁴C. For vessels with illite, filter residue increased with sorbent dose, and most likely indicates presence of ¹⁴C-CPC and partially metabolized ¹⁴C-CPC. Overall mass balance was 83.8–93.7% for illite treated groups at 1.0 mg/L CPC.

3.5. Toxicity mitigation assessment

Toxicity is caused by exposure of an actual target site to a chemical, which is mechanistically closely related to C_{free} (Escher and Hermens, 2004; Heringa et al., 2004; Hermens et al., 2007). Therefore, the potential for toxicity mitigation is logically expressed by evaluating how sorbents influence C_{free}. During the biodegradation experiments, fractions in acidified samples were closely related to C_{free}, because inorganic ¹⁴C was vented off as CO₂. As shown in SI Fig. 4, there is a clear relationship between C_{free} and sorbent concentration for 0.1 and 1 mg/L CPC treatments. Highest C_{free} was observed in controls, while increasing sorbent concentrations lead to incrementally decreased C_{free}. Lower C_{free} for illite spiked with identical CPC concentration as SiO₂

was supported by the higher sorption coefficient. Comparison between illite and SiO₂ in the 10 mg/L group further confirms this, as C_{free} is consistently lower in vessels with illite.

A distinct influence of incubation time was also observed. C_{free} increases notably for most groups after 4–7 days. Day 0 samples are within range of expectations based on the sorption test, so C_{free} increases by Day 4 may relate to the formation of less sorptive ¹⁴C-CPC metabolites. Following the initially increasing C_{free} a gradual decrease of C_{free} was observed, indicative of ultimate mineralization. Higher amounts of sorbent are more likely to release a continuous flux, which would translate to gradually decreasing C_{free} under the assumption of steady-state biodegradation. The 0.1 mg/L data is a straightforward illustration of this effect, showing rapid decrease in control group, somewhat slower decrease at low sorbent concentration, while C_{free} remains relatively constant at 20 g/L. For 0.1 and 1 mg/L CPC treatments with SiO₂, there is overlap between C_{free} reaching a minimum (SI Fig. 4) and biodegradation reaching plateau (Figs. 1B and 3A). This correlation is lacking for illite, which supports the hypothesis that bioaccessibility is limited by desorption kinetics.

3.6. Classification of CPC biodegradability with different test modifications

No reduction in biodegradation efficiency was observed with CPC as sole carbon source for concentrations as low as 6 μg/L. Table 1 provides an overview of biodegradability results discussed in this section. Inhibitory effect of 1 mg/L CPC could be mitigated by appropriate doses of SiO₂, rendering CPC to satisfy Guideline criteria for ready biodegradability (OECD, 2014). These results carry significant environmental relevance, as CPC and other pyridinium-based surfactants are a key component in various organoclays and ionic liquids (Zhu et al., 2009; Lee and Tiwari, 2012; de Paiva et al., 2008; Ganguly et al., 2011; Pendleton and Gilmore, 2015; Tang et al., 2017), while several authors highlight the limited biodegradability and potential toxicity (Bubalo et al., 2014; Pretti et al., 2011; Wiles et al., 2005).

It was unexpected that, while illite reduced C_{free} from 1 mg/L to below the NIEC-STP, bioaccessibility was apparently too low to reach ready biodegradability. At 3 mg/L, CPC was found to be toxic to STP inoculum, but a nominal CPC concentration of 10 mg/L could be reduced to below NIEC-STP using SiO₂ and illite. Although more CPC was actually mineralized than in the 1 mg/L treatment, the highly sorbed fractions were not adequately bioaccessible within the experimental time frame of 28 days. Lower dosing of a stronger adsorbent is thus not a universal solution to mitigate inhibitory effects when testing biodegradability of non-labeled compounds.

The balance between toxicity mitigation and bioaccessibility would probably need to come from combining a sufficiently high adsorbed fraction with adequate desorption kinetics. Most likely, this can be achieved in the form of a higher dose of a sorbent that would still allow for high mobility of microbes, but sorbs weaker (K_D < 1000 L/kg) than the porous SiO₂ we applied. It should be noted that a concentration of 10 mg/L CPC is unrealistically high from an environmental perspective (Shrivastava and Wu, 2007). The sorbents used resemble sorbing surfaces encountered in the environment, e.g. by sewage en route to the STP, while undergoing processing at the STP, and in sewage sludge itself e.g. when applied as fertilizer. Addition of inorganic sorbents is, therefore, not only a means to mitigate potential inhibitory effects but also serves to decrease C_{free} to predicted environmental levels, increasing relevance of test results. This is supported by findings such as those by Nguyen & Oh, who found approximately 50–80% sorption to activated sludge at environmentally relevant concentrations (Nguyen and Oh, 2019). Related surfactants such as benzalkonium are also known to have strong sorption to activated sludge (Garcia et al., 2006).

Taking into account *in situ* sorption effects, CPC will under most circumstances be degradable by microorganisms at a certain rate. The question remains how this contributes to or counteracts the degree of persistency of cationic surfactants, should desorption kinetics indeed

Table 1
Biodegradability classification of CPC at multiple test and sorbent concentrations.

Sorbent	Sorbent Dose	CPC Concentration	Classification (Boethling et al., 2009)	Remarks
No sorbent	–	0.006 mg/L	Readily biodegradable	No lag phase; < 7 d to reach 60%
	–	0.025 mg/L	Readily biodegradable	No lag phase; < 9 d to reach 60%
	–	0.1 mg/L	Readily biodegradable	No lag phase; 10 d to reach 60%
	–	0.3 mg/L	Inherently biodegradable	No lag phase; 17 d to reach 60%
	–	1.0 mg/L	Inherently biodegradable	Lag phase 18 d; does not reach 60%
	–	3.0 mg/L	Slowly biodegradable	Does not reach > 10%
Silicon dioxide	0.8 g/L	0.1 mg/L	Readily biodegradable	No lag phase; < 9 d to reach 60%
	4.0 g/L		Inherently biodegradable	No lag phase; 21 d to reach 60%
	20 g/L	1.0 mg/L	Inherently biodegradable	Lag phase ~3 d; does not reach 60%
	0.8 g/L		Inherently biodegradable	Lag phase ~2 d; 17 d to reach 60%
	4.0 g/L		Readily biodegradable	No lag phase; 13 d to reach 60%
	20 g/L		Readily biodegradable	Lag phase ~5 d; 17 d to reach 60%
Illite	20 g/L	10 mg/L	Inherently biodegradable	No lag phase; does not reach 60%
	0.8 g/L	1.0 mg/L	Inherently biodegradable	No lag phase; 28 d to reach 60%
	4.0 g/L		Inherently biodegradable	Lag phase 12 d; does not reach 60%
	20 g/L	10 mg/L	Slowly biodegradable	Lag phase 21 d; does not reach 60%
	20 g/L		Slowly biodegradable	Lag phase unclear; does not reach 60%

¹ Classifications ‘readily biodegradable’ and ‘inherently biodegradable’ do not imply Guideline criteria are met definitively, but indicate results provide support for such classification.

hamper rapid biodegradation. Bergero & Lucchesi explored the biodegradability of cationic surfactant sorbed to sterilized activated sludge after addition of specifically selected micro-organisms, and found 90% biodegradation within 24 h (Bergero and Lucchesi, 2018). Furthermore, biodegradability of numerous ammonium-based cationic surfactants has been evaluated in surface water, sediment, sludge, and sludge-amended soil. Findings indicate relatively good degradability of sorbed surfactants, although some types can be persistent under anaerobic conditions (Ying, 2006; Scott and Jones, 2000). Biodegradability classification can have significant environmental and commercial implications, and high amounts of resources are invested in biodegradability experiments. Therefore, we should progress towards more realistic testing methodology to obtain the most environmentally relevant results.

Author contributions

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Appendix A. Supplementary data

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