Toxicity mitigation and bioaccessibility of the cationic surfactant cetyltrimethylammonium bromide in a sorbent-modified biodegradation study

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HIGHLIGHTS

- Biodegradation assays are sometimes hampered by inhibitory effects.
- Inert sorbents were successfully used to mitigate these inhibitory effects.
- A stepwise approach is presented to use these mitigating sorbents.
- This approach increases the environmental relevance of biodegradation assays.
- Correct biodegradability assessment contributes to resource preservation.

ARTICLE INFO

ABSTRACT

Biodegradation potential of cationic surfactants may be hampered by inhibition of inoculum at concentrations required to accurately measure inorganic carbon. At >0.3 mg/L cetyltrimethylammonium bromide (CTAB) negatively impacted degradation of the reference compound aniline. We used silicon dioxide (SiO_2) and illite as inorganic sorbents to mitigate toxicity of CTAB by lowering freely dissolved concentrations. In an OECD Headspace Test we tested whether 16.8 mg/L CTAB was readily biodegradable in presence of two concentrations of SiO_2 and illite. SiO_2 adsorbed 85% and 98% CTAB, resulting in concentrations of 2.5 and 0.34 mg/L, mineralized to CO_2 >60% within 16 and 23 d, respectively. With 89% and 99% sorbed to illite, 60% mineralization was reached within 9 and 23 d, respectively. However, higher sorbent concentrations increased time needed to reach >60% mineralization. Thus, desorption kinetics likely decreased bioaccessibility. It is therefore essential to determine appropriate concentrations of mitigating sorbents to render a Headspace Test based on carbon analysis suitable to determine ready biodegradability of compounds which might inhibit inoculum. This would avoid use of expensive radiolabeled compounds. However, high sorbent concentrations can reduce bioaccessibility and limit degradation kinetics, particularly for relatively toxic substances that require strong mitigation.

1. Introduction

Environmental persistence of potentially toxic chemicals is a key property in aquatic and terrestrial environmental risk assessment.

Persistency may cause widespread occurrence and accumulation of concentrations near input streams. Evidence of significant biodegradation in standard test protocols, even without qualifying as readily biodegradable, is therefore of critical importance for routinely applied chemicals, such as ingredients of products for every-day consumer use, or commonly prescribed drugs that enter environmental systems via (treated) waste water effluent. Use in applications such as corrosion inhibitors or antifouling coatings may also generate fairly continuous environmental input. There are various types of standardized assays to determine biodegradability

of a chemical by microbial communities, such as those present in activated sludge, sediment, or soil (OECD, 1981, 1992a, b, 2001, 2008, 2014). Ideally, these tests reproducibly determine the time needed to mineralize organic contaminants into CO_2. CO_2 production due to biodegradation is routinely measured using inorganic carbon (IC) analysis, which is often restricted to limits of quantification (LOQ) around 0.5 mg/L, because of background respiration in the inoculum (van Ginkel et al., 2008). As a result, recommended test concentrations in biodegradation assays that depend on carbon analysis are in the range of 10—20 mg organic carbon per liter (mg C/L). Potential issues arise when these concentrations exceed solubility limits or toxic effect levels to critical micro-organisms in the inoculum (Czechowska et al., 2013; Kowalczyk et al., 2015). Dean-Raymond and Alexander (1977), tested ultimate degradation of ten cationic surfactants; eight were found to "resist attack by microorganisms" (Dean-Raymond and Alexander, 1977). Only decyltrimethylammonium bromide and hexadecyltrimethylammonium bromide (cetyltrimethylammonium bromide; CTAB) were degraded by soil and sewage inocula, although CTAB was not degraded at the highest of three tested concentrations (100 mg/L), suggesting inhibitory effects prevented biodegradation for most compounds/concentrations.

The OECD 310 Guideline (OECD, 2014) describes how to measure aerobic mineralization of a chemical to CO_2 in inoculated medium, including stringent requirements to label a chemical as 'readily biodegradable' (>60% of maximum attainable theoretical IC production (ThIC) within 28 d of incubation, which has to be reached in a 10-d window that starts when biodegradation reaches 10%). The Guideline refers to a ring-test of five surfactants, including CTAB (OECD, 2014). The addition of silica gel (SiO_2) is specifically mentioned to "neutralize toxicity" of CTAB, referring to a 2003 study (Painter et al., 2003). It is hypothesized that CTAB inhibits inoculum at the required concentration, although no references were provided. Adsorption of CTAB to silica gel reduced freely dissolved CTAB concentration to below inhibitory levels, resulting in $75 \pm 13\%$ biodegradation (11 participating laboratories). CTAB adsorbed to SiO_2 thus appeared to be bioaccessible, allowing for a potentially toxic concentration of CTAB to be degraded (Painter et al., 2003).

In 2008, another biodegradation study reported the use of 2 g/L SiO_2 for octadecyltrimethylammonium chloride (ODTAC), a cationic surfactant with a longer alkyl chain than CTAB (van Ginkel et al., 2008). ODTAC was toxic to the inoculum, but could be classified as readily biodegradable with addition of SiO_2. In this study, other sorbents that enhanced biodegradation were humic acid and lignosulphonic acid, although biodegradation of ODTAC was fastest with SiO_2 as mitigating adsorbent. A more recent paper by Sweetlove et al. provides some refinement of this work, aiming at high throughput screening (Sweetlove et al., 2016). The studies mentioned above provide rough guidelines on the use of adsorbents (Painter et al., 2003; van Ginkel et al., 2008; Sweetlove et al., 2016), but we argue that ideally a more systematic approach is required to determine the balance between toxicity, bioavailability and bioaccessibility of cationic surfactants in such critical biodegradation studies.

Our goal was to run a stepwise series of tests as a combined protocol to determine the biodegradation potential of CTAB in a sorbent-modified Headspace Test (OECD, 2014). In previous studies it was identified that SiO_2 formed a gel-like structure, hindering the use of magnetic stirring to aid gas exchange and increase interaction between sorbent and inoculated medium (van Ginkel et al., 2008) Cationic surfactants readily sorb to mineral surfaces such as clay minerals (Droge and Goss, 2013; Martinez-Costa and Leyva-Ramos, 2017; Ramprasad and Philip, 2017), and can cause analytical difficulties as they tend to sorb significantly to glassware and

laboratory equipment (Timmer et al., 2018). Since clay minerals with a higher cation exchange capacity than SiO_2 provide more adsorption sites, less sorbent material should be needed to mitigate toxicity. In addition, clay minerals do not form a viscous gel layer and might therefore offer faster desorption kinetics, enhancing bioaccessibility. We tested SiO_2 and illite as sorbents unlikely to influence background respiration, since they contain no organic material. Previously, illite was used in a systematic evaluation of the sorption affinity of organic cations (Droge and Goss, 2013). This allows for close comparison between the sorption study by Droge and Goss and the conditions in the current biodegradation study (Droge and Goss, 2013). Furthermore, illite is a 2:1 non-expanding clay mineral with only siloxane surfaces and therefore only has outer surfaces to adsorb organic cations, which should facilitate bioaccessibility through desorption. Compared to another non-expanding clay mineral such as kaolinite, illite has a relatively high cation-exchange capacity (~200 meq/g and ~20 meq/g, respectively) (Essington, 2015).

First, we determined CTAB concentrations that maintain full inoculum activity, by determining the no observed effect concentration of CTAB at which biodegradation of a reference compound is inhibited. Degradation of aniline was followed for 14 d using the manometric respirometry method with the OxiTop respirometer system. Secondly, sorption isotherms with SiO_2 and illite were determined to calculate required sorbent loadings to achieve CTAB concentrations below the adverse effect level with a spiked CTAB concentration of 10 mg C/L (= 16.8 mg/L). The selected sorbents easily settled upon centrifugation, allowing for analysis of dissolved concentrations in supernatant and calculation of sorbed concentrations using a mass balance approach. Thirdly, we tested bioaccessibility of adsorbed CTAB, and whether employing these sorbents during a 28 d ready biodegradability study would serve to classify CTAB as readily biodegradable. Biodegradability is an intrinsic property of a chemical compound and has significant implications in environmental risk assessment. It is therefore highly relevant to pursue methodology preventing compounds from being incorrectly classified with respect to their biodegradability. We used radiolabeled [^{14}C]CTAB to study biodegradation at a concentration of 16.8 mg/L, using the two mitigating sorbents at two different sorbent loadings that would amount to initially adsorbed fractions of 90% and 99%.

2. Materials and methods

2.1. Chemicals and sorbents

CTAB (analytical grade), sodium benzoate (analytical grade), aniline (analytical grade), and silicon dioxide (Davisil Grade 633, particle size 35—75 μm, surface area 480 m^2/g), and all solvents (analytical grade) were obtained from Sigma-Aldrich (Gillingham, Dorset, UK). Illite clay was purchased as a fine powder and stems from Argiletz (France). [^{14}C]CTAB (>98% by HPLC-PMT), was obtained from American Radiolabeled Chemicals Inc. (Saint Louis, MO, USA). [^{14}C]CTAB was mixed with CTAB; production of radiolabeled CO_2 was a proxy for total biodegradation. A stock solution of [^{14}C]CTAB was prepared in acetone and the exact concentration was determined using liquid scintillation counting (LSC), after which unlabeled CTAB was added to reach the desired ratio of labelled to unlabeled material. The stock solution was then kept at $4 \pm 2\,°C$ until use.

Salts used to prepare buffers and mineral media, potassium hydroxide (KOH), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), and trifluoroacetic acid were all analytical grade (Sigma-Aldrich). Scintillation cocktails (Hionic-Fluor, Pico-Fluor Plus and Permafluor E+), Carbosorb and combustion cones for the sample oxidizer were

purchased from PerkinElmer (Seer Green, Beaconsfield, UK). Each batch of mineral salts medium was prepared one hour before use with concentrated stock solutions and ultrapure water (PURELAB flex, ELGA LabWater, High Wycombe, UK), as described in the Guideline (OECD, 2014).

2.2. Inoculum

Inoculum was extracted from activated sludge obtained from Broadholme Sewage Treatment Works (BSTW; Ditchford Road, Wellingborough & Irthling, UK), which treats sewage from approximately 80,000 households; <15% of the organic load is attributable to industrial discharge (van Egmond et al., 2013). Sludge was sparged with CO_2 free air overnight at room temperature ($21 \pm 1\,^{\circ}C$) to remove inorganic carbon. The sparged suspension was then transferred to a flow-breaking beaker, and homogenized at 12,000 rpm for 5 min using a Polytron PT 3000 dispersion unit (Kinematica AG, Littau, Switzerland). The resulting suspension was centrifuged at $800\,g$ for 10 min, after which remaining suspended solids were removed by slowly pouring through a funnel filled with laboratory grade glass wool (Sigma-Aldrich). Subsequently, the filtered inoculum was added to mineral salts medium (final inoculum concentration: 10 mL/L). Concentration of suspended solids in the activated sludge was generally 5 ± 1 g/L, but it should be noted that only the filtered supernatant after centrifugation was used for inoculation.

2.3. CTAB toxicity test

A 14 d manometric respirometry test (OECD, 1992a) was used to determine the no observed effect concentration in activated sludge (NOEC-STP) of CTAB. Five CTAB concentrations were prepared in triplicate (0.3, 1.0, 3.0, 8.0 and 24 mg/L). All vessels were spiked with 9 mg/L aniline (theoretical oxygen demand (ThOD): 24 mg/L). Three control vessels containing only aniline and three blanks were included. Unlabeled CTAB stock solution in ultrapure water was added to 80 mL of medium, followed by 80 mL of medium containing 20 mL/L inoculum. Rubber inserts containing KOH were lodged into the vessels' opening. Manometric measuring heads (OxiTop, WTW, VWR, East Grinstead, UK) were tightly screwed onto each bottle to start the measurement. Vessels were maintained at $22 \pm 1\,^{\circ}C$ in HT Multitron stackable incubators (Infors AG, Bottmingen, Switzerland), programmed for orbital shaking at 100 rpm. Measuring heads recorded pressure at 360 time points in equally spaced intervals for 14 d, which was automatically converted into oxygen consumption. Comparing oxygen consumption to the ThOD yields a percentage degradation. The NOEC-STP of CTAB was considered to be the highest concentration at which $\geq 60\%$ aniline was biodegraded within 14 d.

2.4. CTAB sorption test

SiO_2 and illite clay form suspensions that readily settle upon standing still. Using 20 mL borosilicate scintillation vials (PerkinElmer), three CTAB concentrations (0.1, 1.0 and 10 mg/L) were exposed to three concentrations of sorbent (0.80, 4.0 and 20 g/L) in triplicate. Control vials without sorbent were also prepared to correct for sorption to the borosilicate surface. Vials were placed on a roller mixer (Thermo Fisher Scientific, Waltham, MA, USA), set at 30 rpm and $20 \pm 1\,^{\circ}C$ overnight, centrifuged at 2,400 g for 30 min, after which three 4 mL aliquots of the supernatant were analyzed by LSC (Tri-Carb 2710 TR, PerkinElmer). Sorbed amounts were calculated using a mass balance approach; a linear sorption isotherm was fitted to calculate average sorption coefficients. After determining the sorbent-water sorption coefficient ($K_{sorbent-water}$) it

is possible to calculate how much sorbent ($V_{sorbent}$) to add to a certain amount of toxicant ($A_{toxicant}$), in order to decrease the freely dissolved concentration to levels sufficiently below the NOEC-STP ($C_{aq,safe}$):

$$A_{toxicant,\, safe} = C_{aq,\, safe} \times V_{water} + C_{aq,\, safe} \times K_{sorbent-water} \\ \times V_{sorbent} \qquad (1)$$

thus

$$V_{sorbent} = \frac{A_{toxicant,\, safe} - C_{aq,\, safe} \times V_{water}}{C_{aq,\, safe} \times K_{sorbent-water}} \qquad (2)$$

2.5. CTAB biodegradability test

Biodegradability of a CTAB concentration exceeding the NOEC-STP was studied under variable sorbent additions during 35 d, using the design of the OECD Guideline (OECD, 2014). Based on the obtained sorption coefficients sorbent concentrations were chosen to yield sorbed CTAB fractions of 0% (no sorbent), ~90% and ~99%. The spiked CTAB concentration of 16.8 mg/L in these three treatments was selected to be both in the inhibitory range determined in the toxicity test (8–24 mg/L) as well as comparable to spiking levels recommended for using IC analysis (i.e. 10–20 mg C/L; approximately 16–32 mg/L CTAB). Due to using [^{14}C]CTAB relatively low freely dissolved concentrations of CTAB and CO_2 could still be determined accurately, while the mass balance could also be monitored, even in systems with high sorbent concentrations (see below).

For each sorbent-dosed CTAB treatment, triplicates were prepared for 10 measuring points in a 35 d test period. Six duplicates without sorbent were prepared to monitor inhibition levels each week. Each 125 mL borosilicate serum vessel (Wheaton, New Jersey, USA) was filled with a total of 100 mL medium, resulting in a 64 mL headspace. Vessels were first spiked with [^{14}C]CTAB acetone stock solution, loosely covered and left standing overnight at room temperature to allow acetone to evaporate. Gentle flushing with nitrogen gas removed residual acetone vapors. Dry sorbent was added, and a Perimatic Premier precision dispensing pump with a re-circulation loop (Jencons Scientific Ltd, VWR, East Grinstead, UK) delivered 50 mL mineral salts medium without inoculum. At least 2 h was allowed for interaction between dissolved CTAB and sorbent. Abiotic control vessels were filled with a second 50 mL aliquot of medium without inoculum. A 50 mL aliquot of medium with 20 mL/L of inoculum was added to all other vessels. Vessels were then tightly capped using 20 mm butyl rubber stoppers and aluminum crimp caps (Sigma-Aldrich), and incubated in HT Multitron stackable incubators at $22 \pm 1\,^{\circ}C$ and an orbital shaking rate of 100 rpm.

Sodium benzoate was the reference compound in the CTAB biodegradability test. Benzoate is a more common reference than aniline and should be fully biodegraded within 14 d; usually, ~90% biodegradation is reached within 7 d of incubation. Blanks should yield an IC content of <3 mg/L after 28 d. Supplementary controls with benzoate and sorbent (20 g/L SiO_2 or 4 g/L illite clay) were incorporated to rule out effects of sorbent on inoculum performance. These control vessels were all incubated and treated with 7 M NaOH as described below, but after opening the contents of each vessel was divided over three 20 mL vials compatible with a Shimadzu ASI-V autosampler (Shimadzu Ltd., Milton Keynes, UK). Vials were closed immediately after pouring in order to prevent uptake of atmospheric CO_2. Within 24 h after initial sampling, IC content was determined using a Shimadzu TOC-V. IC production in

the control vessels was corrected for background IC production in the blanks. Biodegradation was then quantified based on ThIC of the added benzoate.

2.6. CTAB detection, analysis and mass balance

Before sampling, vessels were injected with 1 mL of 7 M NaOH, to solubilize all inorganic carbon, and returned to the incubator for 60 min. After this time, suspended sorbent particles were allowed to settle if needed. Immediately after opening, three 3 mL aliquots were sampled to analyze the total carbon content (TC). Three additional 3 mL aliquots were acidified using 1 mL of 4 M H_2SO_4 to convert HCO_3^- to gaseous CO_2, which was vented off by leaving samples uncapped overnight. Acidified samples therefore only contain organic carbon (TOC), and the difference between alkaline (TC) and acidified (TOC) samples represents mineralization i.e. the amount of IC (CO_2 and carbonates) formed. After adding 16 mL of Hionic-Fluor followed by rigorous shaking for 5 s, samples were analyzed by LSC on a Tri-Carb 2710 TR. Percentage biodegradation was then calculated by comparing the amount of radiolabeled CO_2 formed with the total applied dose of radioactivity. All biodegradation data was fitted using the '[Agonist] vs. response − Variable slope (four parameters)' model as included with GraphPad Prism 7.04 for Windows.

After sampling, vessels were emptied and sorbent was removed by rinsing the vessels with 10 mL MilliQ. To extract CTAB sorbed to the glass wall, 5 mL washing solution (90% (v/v) methanol with 0.1% (v/v) trifluoroacetic acid in MilliQ) was added to each vessel and left for 2.5 h at 30 rpm on a roller mixer (Thermo Fisher Scientific). The washing solution was then poured into a 20 mL liquid scintillation vial and mixed with 16 mL Hionic-Fluor for LSC.

On Day 22 the contents of the vessels were filtered under vacuum using a 100 mm Whatman GF/C glass fiber filter. If needed, vessels were flushed twice with 6 mL of MilliQ to collect all sorbent. Filters were left to dry overnight, and stored at room temperature until further analysis. The residue on each filter was divided over multiple cellulose PerkinElmer combustion cones for use in a Packard Model 307 sample oxidizer (PerkinElmer). Emptied filters were cut in halves, and stuck into separate combustion cones. Before introduction into the sample oxidizer, combustion cones were enriched with 100 μL analytical grade hexadecane to increase the combustion temperature in order to volatilize any remaining [^{14}C]CTAB. [^{14}C]CO_2 formed during combustion was trapped in approximately 5 mL of Carbo-Sorb E, which was mixed with 7 mL of Permafluor E+. Combustion samples were then topped up with 8 mL of Pico-Fluor to decrease quenching, primarily caused by small soot particles from the combustion process. After LSC, measurements of glass extracts, combusted and alkaline samples were used to determine a mass balance, with abiotic control vessels as a 100% reference.

3. Results and discussion

3.1. CTAB no observed effect concentration

A manometric respirometry assay was used to determine the approximate concentration at which CTAB inhibits microbial degradation of aniline. The positive control containing only aniline showed close overlap between triplicate vials, and blanks showed no significant background oxygen consumption (SI Fig. S1). A lag phase of approximately 4 d can be observed (Fig. 1), and after 10 d 60% of ThOD is reached. Results obtained were suitable to estimate the no observed effect concentration in activated sludge (NOEC-STP). The CTAB treatment of 0.3 mg/L showed a biodegradation profile comparable with the positive control. At the highest CTAB

concentration of 24 mg/L no biodegradation was recorded at all, indicating complete inoculum knockout. At 8.0 mg/L CTAB a prolonged lag phase was observed, in combination with significantly reduced biodegradation after 14 d, which was also observed at 3.0 mg/L. At 1.0 and 0.3 mg/L the lag phase was comparable with the positive control, but biodegradation at 1.0 mg/L reached only 50% after 14 d. These results indicate that inhibitory effects of CTAB on inoculum activity are likely to become significant above 1.0 mg/L, and stronger effects can be expected between 8.0 and 24 mg/L. A freely dissolved CTAB concentration of <1 mg/L through addition of sorbent should then be sufficient to mitigate inhibitory effects on the inoculum. The NOEC-STP for CTAB is up to two orders of magnitude lower than the recommended CTAB test concentration of 16–32 mg/L. Graphs showing mean raw data of all 360 measurement points per series can be found in the SI, Fig. S7.

3.2. CTAB sorption affinity to SiO_2 and illite clay

For optimal toxicity mitigation it is essential to accurately predict how addition of sorbent will influence freely dissolved concentrations of the inhibitory compound. CTAB sorption isotherms obtained for SiO_2 and illite are presented in Fig. 2. For SiO_2 the isotherm is linear (fitted slope of 1) over the concentration range 2.2 μg/L to 2.4 mg/L, adequately covering concentrations exceeding the NOEC-STP (0.3 mg/L). Two independently performed series show good agreement; a second experiment was performed since the first measurement series shows signs of leveling off at its respective highest concentration (100 nM). Fit of the combined data results in a $K_{sorbent-water}$ of 1820 L/kg SiO_2. For illite the isotherm covered concentrations from 0.9 μg/L to 0.2 mg/L, ending slightly below the NOEC-STP. Obtaining a detailed sorption isotherm was beyond the scope of our study. Since the data acquired allowed for a reasonably realistic estimate of the sorption affinity, we therefore decided not to perform a second measurement series. The data indicate a $K_{sorbent-water}$ of 8130 L/kg, about 4.5 times higher than for SiO_2. Interestingly, the calculated sorption coefficient of CTAB to illite is in agreement with predicted $K_{illite-water}$ based on a cation exchange model proposed by Droge & Goss
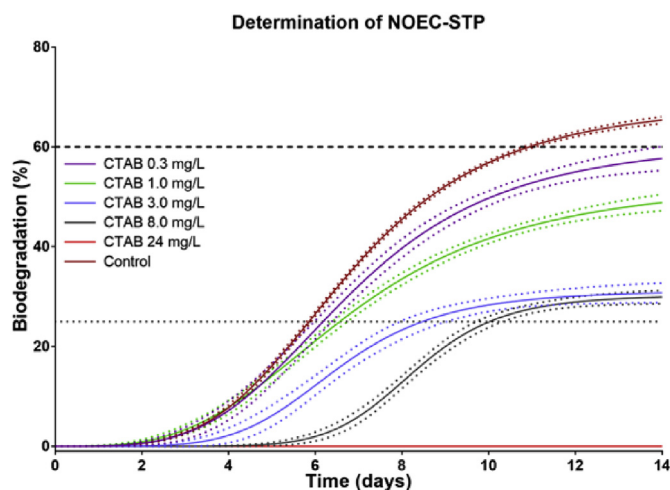

Fig. 1. Biodegradation of aniline by itself and in combination with different concentrations of CTAB, measured using the automated OxiTop respirometer assay, to determine the no observed effect concentration of CTAB. Fitted curves based on 360 measurements; dotted lines represent the 95% confidence bands for each curve, fitted using a four parameter variable slope agonist/response model; the dotted line at 60% indicates the required level to satisfy one of the conditions for ready biodegradability. The curve of CTAB 24 mg/L lies on the X-axis as no mineralization to CO_2 was measured.

in 2013 (Droge and Goss, 2013). Applying eq. (2) for (i) the recommended concentration of 16.8 mg/L in the OECD Headspace test [$A_{toxicant,\ safe}$], (ii) the NOEC-STP of 0.3 mg/L [$C_{aq,safe}$], in (iii) the volume of medium applied (0.1 L) [V_{water}], and (iv) the derived sorption coefficients [$K_{sorbent-water}$], suggests required sorbent concentrations of 31.1 g SiO_2/L and 7 g illite/L.

3.3. CTAB biodegradation in Headspace Test without sorbent

Abiotic controls showed stable (<2% variation) concentrations of [^{14}C]CTAB throughout the 35 d incubation period, providing a mass balance reference. Adequate inoculum activity was demonstrated in the benzoate controls (see Supporting Information (SI) Fig. S2), also in the presence of sorbents; 80—90% biodegradation within 7 d for controls without sorbent and with SiO_2. Results for illite, however, surprisingly reached 250% biodegradation. We suspect IC release from clay particles might be the cause for this, as clay minerals are known to sorb CO_2 and carbonates (Gomez et al., 2007), and illite used in this experiment had not undergone any pretreatment. This suspicion was confirmed by total carbon analysis of commercially available illite, which had a carbon content of approximately 20 mg/g. Sorbent controls were included to rule out inhibitory effects of added sorbent, which can be considered successful. For the biodegradation test with [^{14}C]CTAB, possible release of IC from illite should have no impact, since [^{14}C]CO_2 will be analyzed specifically.

The high rate of biodegradation at 16.8 mg/L CTAB (SI Fig. S3, also visible in Figs. 3 and 4) was unexpected in comparison with the results of the NOEC-STP determination (Fig. 1). After at least a 7-d lag phase, measurements from Day 14 onwards show biodegradation of ~60%. This suggests CTAB was actually not inhibitory in the current experiment, whereas previously 8.0 mg/L strongly reduced biodegradation of aniline, and 24 mg/L caused full inoculum knockout (Fig. 1). These differences might be partially explained by several factors. Firstly, while CTAB impacted microorganisms that degrade aniline in the toxicity tests, it may be that microorganisms able to convert CTAB are different species, less sensitive to CTAB. Secondly, inoculum in the toxicity test was obtained from the same source (BSTW), but sampled at a different timepoint, and activated sludge samples are known to lack in reproducibility (Goodhead et al., 2014). Thirdly, different vessels and volumes were used, which may result in different fractions

sorbed to glass surfaces, although the mass-balance (see Fig. 5) indicates only a minute fraction of [^{14}C]CTAB was actually sorbed to the glass surface on Day 22. Fourthly, the manometric respirometry method can be a less direct assessment of biodegradation, since CO_2 might be present in the aqueous phase as carbonate while gaseous CO_2 does not react with the KOH instantaneously. Nonetheless, the CTAB concentration that would inhibit microbial activity was underestimated, so full toxicity mitigation of CTAB cannot be investigated with the current data. However, the sorbent–modified biodegradation studies focus on lag phase delay instead and are informative on the effect of bioaccessibility.

3.4. CTAB biodegradation in Headspace Test with additions of SiO_2 or illite clay
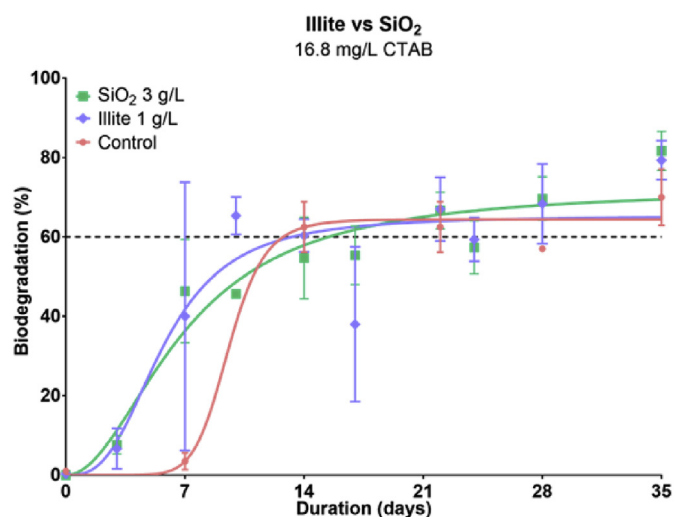
Sorbent levels were chosen to yield freely dissolved CTAB

Fig. 3. CTAB biodegradation profile with sorbent concentrations resulting in sorbed fractions of ~90% (3 g/L SiO_2 - squares; 1 g/L illite −diamonds), compared to controls without sorbent (control − circles); the dotted line at 60% indicates the required level to satisfy one of the conditions for ready biodegradability; biodegradation (%) is calculated by comparing radioactive CO_2 with total applied radioactivity, and was fitted using a four parameter variable slope agonist/response model.
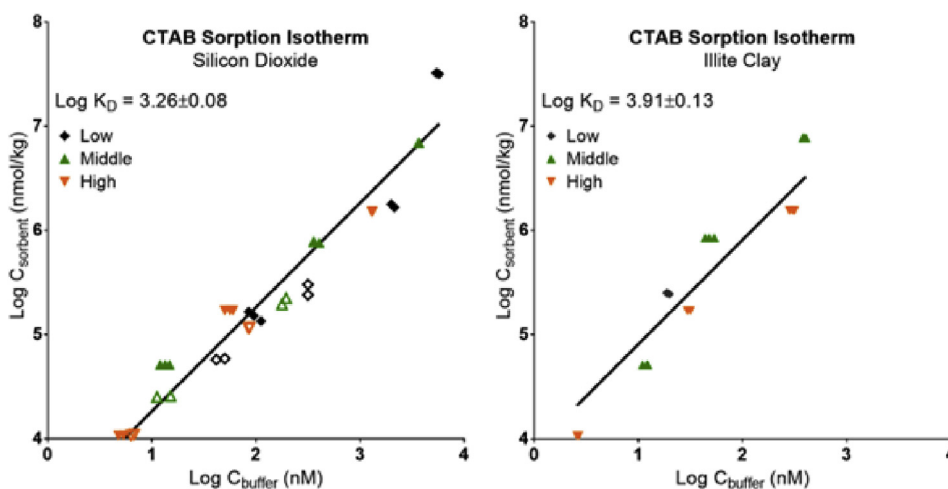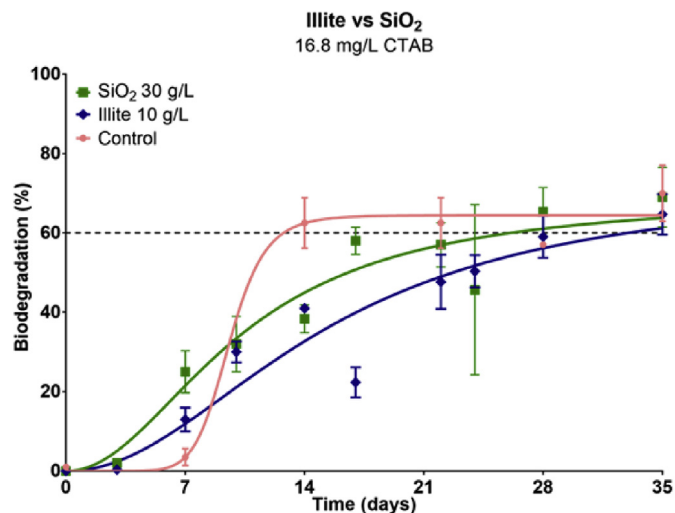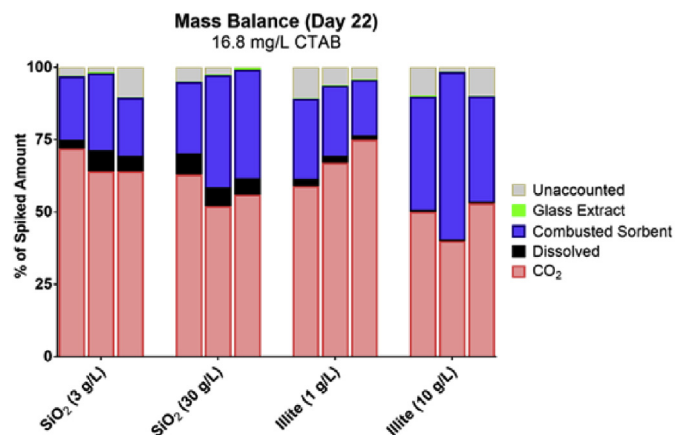

Fig. 2. CTAB isotherms for sorption to SiO_2 (left) and illite clay (right); symbols have been color coded to relate them with associated sorbent concentrations; open symbols for SiO_2 are to distinguish between the first (open symbols) and second (closed symbols) series. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Illite vs SiO₂
16.8 mg/L CTAB

Fig. 4. CTAB biodegradation profile with sorbent concentrations resulting in sorbed fractions of ~99% (30 g/L SiO_2 - squares; 10 g/L illite −diamonds), compared to controls without sorbent (control −circles); the dotted line at 60% indicates the required level to satisfy one of the conditions for ready biodegradability; biodegradation (%) is calculated by comparing radioactive CO_2 with total applied radioactivity, and was fitted using a four parameter variable slope agonist/response model.

concentrations slightly below and somewhat above the NOEC-STP of 0.3 mg/L. Based on the obtained $K_{sorbent-water}$ values, the 3 and 30 g/L SiO_2 or 1 and 10 g/L illite applied in the Headspace Test currently performed resulted in respective sorbed fractions of 84.5% and 98.2% for SiO_2 and 89.0% and 98.8% for illite, and respective freely dissolved CTAB concentrations of 2.6 and 0.3 mg/L for SiO_2, and 1.9 and 0.2 mg/L for illite.

Fig. 3 shows the biodegradation profile of CTAB with low concentrations of the two sorbents. Although it was expected this would still result in moderately inhibitory effects based on the toxicity test with aniline, biodegradation was already initiated on Day 3, and was ~40% within 7 d. On Day 7 IC production in controls without sorbent was <5%, indicating that sorbent additions shortened the lag phase and had a positive effect on inoculum activity. During the last three weeks no differences between controls and sorbent treated groups were observed. This would suggest that the freely dissolved CTAB concentrations in the low sorbent concentration treatments were non-inhibitory to the inoculum fraction responsible for degradation of CTAB.

Mass Balance (Day 22)
16.8 mg/L CTAB

Fig. 5. Day 22 mass balance, determined based on mean of three samples. Glass extract accounts for <0.6% of total.

Results of the high sorbent concentration (30 g/L SiO_2 and 10 g/L illite) are presented in Fig. 4. Dissolved CTAB concentrations below the NOEC-STP were expected. Just as with the lower sorbent concentration treatments, the lag phase was shorter in the sorbent groups, with an average biodegradation after 7 d of 25% for SiO_2 and 14% for illite, compared to <5% in the controls. However, whereas the lower sorbent concentrations reached approximately 60% between Day 7 and 10, the higher sorbent concentration treatments showed a slower biodegradation rate between Day 7 and 21. For both sorbent treatments, CTAB biodegradation leveled off at 60%. Graphs showing all measurement points can be found in the SI, Figs. S8 and S9.

The shorter lag phase in all sorbent groups is indicative of mitigation of moderately inhibitory effects of the applied CTAB concentration. The slower increase of biodegradation in high concentration sorbent treatments compared to lower concentration treatments (See also SI Figs. S4 and S5) could be explained by larger sorbed fractions that need to fully desorb in order to reach the inoculum and be biodegraded, which would make bioaccessibility rate-limiting. In high concentration sorbent groups freely dissolved fractions were considerably smaller (1.8% with 30 g/L SiO_2 and 1.2% with 10 g/L illite clay) than in the low concentration sorbent treatments, requiring a higher flux of CTAB from sorbent to medium in order to maintain a constant freely dissolved concentration assuming a fixed biodegradation rate. Note that the amount of CTAB freely dissolved in the high concentration sorbent treatments was <2% of that available in the control group without sorbent, although the freely dissolved concentration could be maintained more rapidly at higher sorbent loadings.

Comparing concentrations of both sorbents, it becomes clear that bioaccessibility of CTAB is mainly shaped by sorption affinity of CTAB, not by nature or morphological structure of these sorbents. Orbital shaking sufficiently suspends illite particles, maximizing interaction between inoculated mineral medium and sorbent particles, while SiO_2 can form a gel-like precipitate. Although this gel is hydrated, interaction with the inoculum suspended in the mineral medium is presumably smaller than for fully suspended illite. However, both sorbent types show similar kinetics at comparable sorbed fractions, indicating that in SiO_2 treatments inoculum activity may have been concentrated on the settled particles.

3.5. CTAB mass balance in the Headspace Test

Sorbent containing vessels sampled on Day 22 were used to determine a mass balance (see Fig. 5). Average mass balance was >90% for all treatment conditions, although two of the twelve vessels sampled scored slightly lower (89.6% and 89.2%). Since complete oxidation of samples containing biomass and sorbent was not expected, this was considered to be an excellent mass balance. Measured freely dissolved CTAB in the low concentration sorbent treatments was in the same order of magnitude as calculated using sorption coefficients, which was also true for the high concentration of illite. However, for unknown reasons the high concentration of SiO_2 showed freely dissolved CTAB comparable to the low concentration of SiO_2. This might be due to the formation of silica gel at high sorbent loading, while at low SiO_2 concentration a larger fraction of sorbent remained in suspension.

Amounts extracted from inside glass surfaces of vessels with sorbent were generally <1% (see also Table S1 in SI). However, this fraction is significant when compared with the freely dissolved concentration, with which it should theoretically be in equilibrium. For vessels with SiO_2, the average amount of CTAB extracted from glass vessels accounted for approximately 5% of the freely dissolved amount. These percentages were 8% and 39% for 1 g/L and 10 g/L illite clay, respectively. However, freely dissolved concentrations

were a factor 3 and 10 lower, respectively, while absolute amounts of CTAB extracted from glass vessels were comparable. Overall, glass binding was lower than what was expected based on previous work (Timmer et al., 2018), although results are within the same order of magnitude.

Combustions of the sorbent indicated that significant portions of CTAB were still sorbed on Day 22 (~23% for both low sorbent concentrations, ~34% for 30 g/L SiO_2 and ~44%% for 10 g/L illite), while for low sorbent concentrations ~67% was already biodegraded. This raises the question if this sorbed fraction is bioaccessible or if it represents a strongly or irreversibly sorbed, or perhaps a microbially incorporated fraction of the radiolabeled carbon (Urstadt et al., 1995; Brillet et al., 2018). Combustion of sorbent samples was difficult and it is therefore plausible that actual sorbed fraction (and therefore the mass balance) would turn out higher if a more thorough extraction method would have been available. Interestingly, for one 10 g/L vessel sorbent was divided in smaller aliquots over a larger number of combustion cones. This sample showed ~8% higher recovery than its replicate samples and the highest recovery from sorbent overall (~58%), indicating that more extensive subsampling could increase recovery of sorbent combustion and improve mass balance. We have provided a comparison between mass balance on Day 22 with sorbent combustion and on Day 35 without sorbent combustion in the SI (Fig. S6), illustrating the likelihood of a >90% mass balance at the end of the experiment.

4. Conclusion

CTAB met the criteria for ready biodegradability without sorbent, which signifies the relevance of reducing the lag phase through addition of inert sorbent. The most important element of this study was verifying that with a balanced amount of sorbent, unlabeled CTAB and carbon analysis could have been used to test biodegradability at freely dissolved concentrations below the NOEC-STP. It has been clearly demonstrated that CTAB inhibits inoculum activity by different metrics, such as impact on biodegradation of the reference substrate aniline, a significant lag phase in the current Headspace Test (which decreased after addition of sorbent), and absence of biodegradation in literature (Dean-Raymond and Alexander, 1977). Findings presented in this manuscript clearly illustrate that addition of inorganic sorbents can potentially mitigate toxicity of a broad range of well-adsorbing compounds in the Headspace Test. A stepwise approach was followed where NOEC-STP and sorption affinity of the test compound were determined first, in order to calculate the amount of sorbent needed to keep a specific concentration of test chemical below the NOEC-STP. Results suggest that 1 g/L illite clay, resulting in an initially sorbed fraction of 89% and freely dissolved CTAB concentrations a factor 2 above the NOEC-STP, provided adequate bioaccessibility for a successful ready biodegradability classification. However, sorbent dosing resulting in initially sorbed fractions of ~99%, and corresponding bioavailable concentrations below the NOEC-STP, may have resulted in retardation of biodegradation rate by decreasing bioaccessibility. Future research might benefit from more closely elucidating the desorption kinetics, apparent bioaccessibility, and biodegradation kinetics at various sorbent and substrate concentrations. Using [^{14}C]CTAB we confirmed mass balance >90% after 3 weeks of exposure with mitigating sorbents, and identified that 20—60% of [^{14}C]CTAB-related 'material' was still sorbed to sorbent after 22 d, while 40—70% was already fully mineralized. Minor freely dissolved fractions and negligible sorption to glass surfaces were confirmed. Table 1 summarizes the biodegradability classification of CTAB under different conditions in the current study. Addition of an amount of sorbent sufficient to

Table 1
Classification of biodegradability of CTAB (tested at 16.8 mg/L) based on results obtained in the OECD 310 Headspace Test presently performed.

Conditions	Classification	Remarks
no sorbent	Readily biodegradable	Lag phase >7 d; 14 d to reach 60%
SiO_2 3 g/L	Inherently biodegradable	Lag phase <3 d; 16 d to reach 60%
SiO_2 30 g/L	Inherently biodegradable	Lag phase <3 d; 23 d to reach 60%
illite 1 g/L	Readily biodegradable	Lag phase <3 d; 9 d to reach 60%
illite 10 g/L	Inherently biodegradable	Lag phase 3—7 d; 23 d to reach 60%

reduce inhibitory effects of CTAB to the inoculum had a considerable impact on satisfying the 10-d window criterion. This is an important finding, since satisfying the criteria for ready biodegradability has significant consequences for environmental risk assessment and it is therefore important that readily biodegradable compounds are not incorrectly labelled as poorly biodegradable and vice versa.

The Argiletz illite, sold as "cosmetic green clay", was available relatively cheap in large volumes in powdered form, making it directly suitable as adsorbent for (labelled) cationic surfactants in biodegradation studies. However, care should be taken with this material when using IC analysis, since the IC signal from the reference substrate reached 250% of theoretical maximum IC production, and subsequent total carbon analysis of illite revealed a carbon content of approximately 20 mg/g. Finally, the use of automated OxiTop respirometer assay in the toxicity tests, where we demonstrated that CTAB affected the biodegradation of the reference substrate aniline, showed to be a promising efficient tool in biodegradability studies. It lacks the need of sacrificial sampling, reducing sample load and associated lab work, and provided detailed time profiles of all vessels tested for each condition. The use of toxicity mitigating adsorbents and such automated respirometer samplers may become even more valuable, expressed in time/cost-efficiency and relevance in biodegradation studies, for cationic surfactants that have a higher intrinsic toxicity to inoculum, and for which no radiolabeled compounds are available. The two sorbents employed during this work resemble sorbing surfaces encountered by sewage when traveling from the point of discharge to the sewage treatment plant, during treatment at the plant itself, and if inadvertently entering the environment. Therefore, the concept of toxicity mitigation is ecologically relevant, can be translated to environmental settings, and should be deemed permissible in biodegradability studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.01.152.

References

Brillet, F., Cregut, M., Durand, M.J., Sweetlove, C., Chenèble, J.C., L'Haridon, J., Thouand, G., 2018. Biodegradability assessment of complex chemical mixtures using a carbon balance approach. Green Chem. 20, 1031—1041.

Czechowska, K., Sentchilo, V., Beggah, S., Rey, S., Seyfried, M., Van Der Meer, J.R., 2013. Examining chemical compound biodegradation at low concentrations through bacterial cell proliferation. Environ. Sci. Technol. 47, 1913—1921.

Dean-Raymond, D., Alexander, M., 1977. Bacterial metabolism of quaternary

ammonium compounds. Appl. Environ. Microbiol. 33, 1037–1041.

Droge, S.T.J., Goss, K.U., 2013. Sorption of organic cations to phyllosilicate clay minerals: CEC-normalization, salt dependency, and the role of electrostatic and hydrophobic effects. Environ. Sci. Technol. 47, 14224–14232.

Essington, M.E., 2015. Soil and Water Chemistry: an Integrative Approach, second ed. CRC Press.

Gomez, M.P., Gazulla, M.F., Zumaquero, E., Orduna, M., 2007. Use of coupled TG-DSC-QMS-FTIR thermal analysis techniques in characterizating clays and ceramic compositions used in ceramic tile manufacture. Quantification of carbon compounds. Bol. Soc. Esp Ceram. V 46, 259–266.

Goodhead, A.K., Head, I.M., Snape, J.R., Davenport, R.J., 2014. Standard inocula preparations reduce the bacterial diversity and reliability of regulatory biodegradation tests. Environ. Sci. Pollut. Control Ser. 21, 9511–9521.

Kowalczyk, A., Martin, T.J., Price, O.R., Snape, J.R., van Egmond, R.A., Finnegan, C.J., Schäfer, H., Davenport, R.J., Bending, G.D., 2015. Refinement of biodegradation tests methodologies and the proposed utility of new microbial ecology techniques. Ecotoxicol. Environ. Saf. 111, 9–22.

Martinez-Costa, J.I., Leyva-Ramos, R., 2017. Effect of surfactant loading and type upon the sorption capacity of organobentonite towards pyrogallol. Colloid. Surface. Physicochem. Eng. Aspect. 520, 676–685.

OECD, 1981. Test No. 302A: Inherent Biodegradability: Modified SCAS Test.

OECD, 1992a. Test No. 301: Ready Biodegradability. OECD Publishing.

OECD, 1992b. Test No. 302B: Inherent Biodegradability. Zahn-Wellens/EVPA Test.

OECD, 2001. Test No. 303: Simulation Test - Aerobic Sewage Treatment – A: Activated Sludge Units; B: Biofilms.

OECD, 2008. Test No. 314: Simulation Tests to Assess the Biodegradability of Chemicals Discharged in Wastewater.

OECD, 2014. Test No. 310: Ready Biodegradability - CO2 in Sealed Vessels (Headspace Test). OECD Publishing.

Painter, H.A., Reynolds, P., Comber, S., 2003. Application of the headspace CO2 method (ISO 14 593) to the assessment of the ultimate biodegradability of surfactants: results of a calibration exercise. Chemosphere 50, 29–38.

Ramprasad, C., Philip, L., 2017. Sorption of surfactants and personal care products in Indian soils. Int. J. Environ. Sci. Technol. 14, 853–866.

Sweetlove, C., Chenèble, J.C., Barthel, Y., Boualam, M., L'Haridon, J., Thouand, G., 2016. Evaluating the ready biodegradability of two poorly water-soluble substances: comparative approach of bioavailability improvement methods (BIMs). Environ. Sci. Pollut. Control Ser. 23, 17592–17602.

Timmer, N., Scherpenisse, P., Hermens, J.L.M., Droge, S.T.J., 2018. Evaluating solid phase (micro-) extraction tools to analyze freely ionizable and permanently charged cationic surfactants. Anal. Chim. Acta 1002, 26–38.

Urstadt, S., Augusta, J., Müller, R.J., Deckwer, W.D., 1995. Calculation of carbon balances for evaluation of the biodegradability of polymers. J. Environ. Polym. Degrad. 3, 121–131.

van Egmond, R., Sparham, C., Hastie, C., Gore, D., Chowdhury, N., 2013. Monitoring and modelling of siloxanes in a sewage treatment plant in the UK. Chemosphere 93, 757–765.

van Ginkel, C.G., Gancet, C., Hirschen, M., Galobardes, M., Lemaire, P., Rosenblom, J., 2008. Improving ready biodegradability testing of fatty amine derivatives. Chemosphere 73, 506–510.