Quaternary alkylammonium disinfectants in soil: Accumulation, sorption and its influence on toxicological effects towards bacteria

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A CUMULATIVE DISSERTATION

PRESENTED BY

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List of Abbreviations

QAACs	quaternary alkyl ammonium compounds
USE	ultrasonic extraction
HPLC-MS/MS	high performance liquid chromatography with tandem mass spectrometry
LOD	limits of detection
ATMACs	alkyltrimethylammonium compounds
BACs	benzylalkyldimethylethylammonium compounds
DADMACs	dialkyldimethylammonium compounds
LOQ	limits of quantification
MIC	minimum inhibitory concentrations
QAAV	quartären Alkylammoniumverbindungen
NG	Nachweisgrenzen
ATMAV	Alkyltrimethylammonium-Verbindungen
BAV	Benzylalkyldimethylethylammonium-Verbindungen
DADMAV	Dialkyldimethylammonium-Verbindungen
BG	Bestimmungsgrenzen
MHK	minimale Hemmkonzentrationen
QACs	quaternary ammonium compounds
CMC	critical micelle concentration
K_{OW}	*n*-Octanol / Water Partition Coefficient
TO	tetrahedral and octahedral layer
TOT	tetrahedral, octahedral and tetrahedral layer
CEC	cation exchange capacity
SPE	solid phase extraction
SD	standard deviation
C_{org}	organic substance
p. f. u.	per formula unit

Abstract

The cationic surfactants of the group of quaternary alkyl ammonium compounds (QAACs) are used in numerous industrial and agricultural applications and are therefore released into the environment in large amounts.

If these substances are present in the soil in non-toxic concentrations, soil microorganisms could develop resistance mechanisms that likewise increase their resistance to antibiotics (co-selection of antibiotic resistance). Against this background, this work aims at improving the understanding of the behavior and the ecotoxicological effects of QAACs in soil. Two hypotheses were tested: i) Continuous inputs of QAACs cause a long-term accumulation of these compounds in soils, and ii) Sorption of QAACs to expandable 3-layer clay minerals reduces their ecotoxicological effects.

The first step was to develop methods for extracting QAACs from soils and sewage sludge and for quantifying their concentrations. Ultrasonic extraction (USE) followed by high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) turned out to be the best method for this purpose. Detection limits (LOD) for alkyltrimethylammonium compounds (ATMACs), benzylalkyldimethylethylammonium compounds (BACs) and dialkyldimethylammonium compounds (DADMACs) ranged from 0.1 µg kg^{-1} to 1.3 µg kg^{-1} and limits of quantification (LOQ) were between 0.2 µg kg^{-1} and 2.1 µg kg^{-1}. The developed method allowed the investigation of the concentrations of QAACs in agricultural soils of the Mezquital Valley, Mexico, which were irrigated for different durations (0 to 88 years) with wastewater from Mexico City, to test hypothesis i). The most abundant QAAC homologues in the soils were BACs >ATMACs > DADMACs. Concentrations of QAACs increased linearly and slowly in the first years of irrigation ($\sum$ QAAC: 2 - 23 µg kg^{-1}), but after 40 years of wastewater irrigation, an exponential increase in QAAC concentrations (up to 155 µg kg^{-1}) was observed. In contrast to pharmaceuticals, no apparent steady-state of concentration was reached after decades of wastewater irrigation. The long-term accumulation of QAACs in soil also suggested an effective sorptive preservation of the compounds from biodegradation. The reduction of toxicological effects on microorganisms due to sorption was tested by quantifying minimum inhibitory concentrations (MICs) of QAACs on selected bacteria (*Escherichia coli, Acinetobacter, Enterococcus faecium, Enterococcus faecalis, Pseudomonas fluorescence*) in the presence and absence of the clay minerals smectite and kaolinite. In the absence of smectite and kaolinite, the MICs ranged from 10 µg ml^{-1} to 30 µg ml^{-1} for BAC-C12 and from 1.0 µg ml^{-1} to 3.5 µg ml^{-1} for DADMAC-C10 for all strains except the more sensitive Acinetobacter strain. For all strains tested and both QAACs tested, the presence of smectite apparently increased the MIC values, while kaolinite had no effect on the MICs. Batch sorption experiments with the clay minerals proved that this shift in apparent MICs was related to a reduction of dissolved concentrations of the QAACs due to their sorption. The findings of this thesis thus prove the postulated long-term accumulation of QAACs in soils caused by continuous inputs and the key role of sorption for controlling their fate and ecotoxicological effects.

Zusammenfassung

Die kationischen Tenside aus der Gruppe der quartären Alkylammoniumverbindungen (QAAV) werden in zahlreichen industriellen und landwirtschaftlichen Anwendungen eingesetzt und gelangen daher in großen Mengen in die Umwelt.

Sind diese Substanzen im Boden in nicht-toxischen Konzentrationen vorhanden, könnten Bodenmikroorganismen Resistenzmechanismen entwickeln, die ebenfalls ihre Resistenz gegenüber Antibiotika erhöhen (Ko-Selektion von Antibiotikaresistenzen). Vor diesem Hintergrund zielt diese Arbeit auf ein besseres Verständnis des Verhaltens und der ökotoxikologischen Effekte von QAAVs im Boden ab. Zwei Hypothesen wurden getestet: i) Kontinuierliche Einträge von QAACs führen zu einer langfristigen Akkumulation dieser Verbindungen in Böden, und ii) Sorption von QAACs an aufweitbaren 3-Schicht-Tonmineralen reduziert deren ökotoxikologische Effekte.

Im ersten Schritt wurden Methoden zur Extraktion von QAAVs aus Böden und Klärschlämmen sowie zur Quantifizierung ihrer Konzentrationen entwickelt. Die Ultraschallextraktion mit anschließender Hochleistungsflüssigchromatographie mit Tandem-Massenspektrometrie erwies sich als beste Methode für diesen Zweck. Die Nachweisgrenzen (NG) für Alkyltrimethylammonium-Verbindungen (ATMAV), Benzylalkyldimethylethylammonium-Verbindungen (BAV) und Dialkyldimethylammonium-Verbindungen (DADMAV) lagen im Bereich von 0,1 µg kg^{-1} bis 1,3 µg kg^{-1} und die Bestimmungsgrenzen (BG) lagen zwischen 0,2 µg kg^{-1} und 2,1 µg kg^{-1}. Um Hypothese i) zu testen wurden anschließend die Konzentrationen von QAAVs in landwirtschaftlichen Böden des Mezquital-Tals, Mexiko, untersucht, die für unterschiedliche Zeiträume (0 bis 88 Jahre) mit Abwasser aus Mexiko-Stadt, bewässert wurden. Die am häufigsten vorkommenden QAAV-Homologe in den Böden waren BAV > ATMAV > DADMAV. Die Konzentrationen von QAAV stiegen in den ersten Jahren der Bewässerung linear und langsam an ($\sum$ QAAV: 2 - 23 µg kg^{-1}), nach 40 Jahren der Abwasserbewässerung wurde ein exponentieller Anstieg der QAAV-Konzentrationen (bis zu 155 µg kg^{-1}) beobachtet. Im Gegensatz zu Pharmazeutika wird nach jahrzehntelanger Abwasserbewässerung kein Sorptionsplateau erreicht. Die langfristige Akkumulation von QAAV in Böden wies bereits auf die Bedeutung ihrer Sorption für die Reduzierung ihres biologischen Abbaus in Böden hin. Die Reduktion ökotoxikologischer Effekte auf Bakterien infolge der Sorption wurde durch Quantifizierung von minimalen Hemmkonzentrationen (MHK) von QAAV auf ausgewählte Bakterien (*Escherichia coli, Acinetobacter, Enterococcus faecium, Enterococcus faecalis, Pseudomonas fluorescens*) in Anwesenheit und Abwesenheit der Tonminerale Kaolinit und Smektit geprüft. Die MHK lagen für alle Stämme außer dem empfindlicheren *Acinetobacter*-Stamm in Abwesenheit von Smektit und Kaolinit im Bereich von 10 µg ml^{-1} bis 30 µg ml^{-1} für BAC-C12 und im Bereich 1,0 µg ml^{-1} bis 3,5 µg ml^{-1} DADMAC-C10. Für beide getesteten QAAV erhöhte die Anwesenheit von Smektit die effektiven MHK, während Kaolinit keinen Einfluss auf die MHK hatte. Sorptionsexperimente mit den Tonmineralen bestätigten, dass diese Verschiebung der effektiven MHK auf die Reduktion der gelösten QAAV-Konzentrationen

infolge der Sorption zurückzuführen war. Die Ergebnisse dieser Arbeit belegen die postulierte langfristige Akkumulation von QAAVs in Böden bei einem kontinuierlichen Eintrag und die Schlüsselrolle der Sorption für das Verhalten und die ökotoxikologische Wirkung dieser Verbindungen.

1 Extended summary

1.1 General Introduction

Quaternary alkylammonium compounds (QAACs) are a group of amphiphilic organic compounds that are used worldwide as surfactants, disinfectants and detergents, for example in household or agricultural chemicals, the food and beverage industry and the medical sector. Their use rises as a consequence of the increasing pressure to reduce usage of antibiotics and improving hygiene. Additionally, the recent COVID-19 pandemic heavily increased the use of disinfectants like alkyltrimethylammonium compounds (ATMACs), benzylalkyldimethylammonium compounds (BACs) and dialkyldimethylammonium compounds (DADMACs) for the inactivation of the SARS-CoV-2 Virus (Hora *et al* 2020).

Whether they are used in hospitals, in factories, in agriculture or in private households, the QAACs end up in wastewater treatment plants where they are only incompletely degraded and thus may prevail at subinhibitory concentrations (Tezel and Pavlostathis 2015). Finally, they are applied with sewage sludge or manure as fertilizer to the soils, where they may promote (antibiotic) resistance in soil bacteria (Mulder *et al* 2018).

Weber and Rutala (2006) and Maertens *et al* (2020) described that microorganisms are more tolerant against antibiotics like ciprofloxacin, when they were in contact with QAACs before. The latter in combination with the COVID-19 pandemic and the rising antibiotic resistance of microorganisms all around the world (Levy and Marshall 2004) and not least a small amount of data for QAACs in soil (Mulder *et al* 2018), motivate this work.

The aim of this thesis is to fill the data gap regarding the fate (chap. 3) and ecotoxicological effects (chap. 4) of QAACs fate in soil. The basis for these investigations is the development of an analytical method for the extraction of QAACs from soil and quantification of their concentrations in soil (chap. 2).

1.2 Properties of QAACs

1.2.1 Chemical properties

The group of QACs consist of three main groups (a) linear alkylammonium compounds (b) imidazole-derived compounds and (c) pyridin-derived compounds (Figure 1.1). QAACs consist of a positive charged nitrogen which is accountable for the good water solubility of QAACs molecules and at least one hydrophobic alkyl chain, which provides their hydrophobicity. These two properties make them widely used surfactants. QAACs form a specific sub-group of quaternary ammonium compounds (QACs) and are characterized by their linear alkyl chains. The permanently positively charged nitrogen surrounded by either (a) three methyl substituents and one alkyl group (ATAMCs), or (b) two methyl groups and two alkyl groups (DADMACs), or (c) two methyl groups, a benzyl

group and an alkyl chain of different length (BACs). Since the groups of QACs and QAACs are very heterogeneous and the inconsistency of their nomenclature in the literature, it is challenging to get an exact overview of their chemical properties, the production volumes and their occurrence and applications from recent publications. Moreover, there are up to 82 synonyms for individual QAACs homologues (Mulder *et al* 2018). This dissertation is focused on linear alkylammonium compounds.

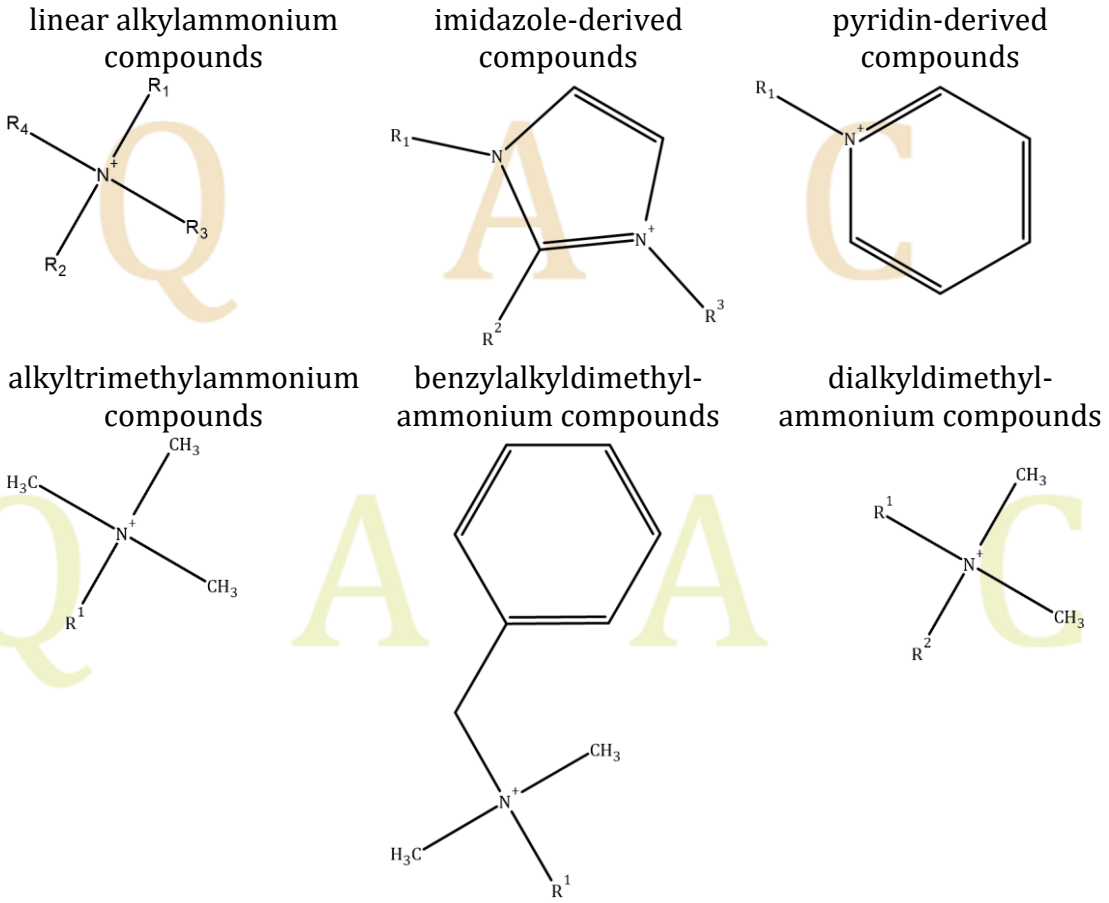

Figure 1.1 Overview of the three main groups of quaternary ammonium compounds (QACs) - Linear alkylammonium compounds, imidazole-derived compounds, pyridin-derived compounds and the three main groups of quaternary alkylammonium compounds (QAACs) - alkyltrimethylammonium compounds (ATMACs), benzylalkyldimethylammonium compounds (BACs), dialkyldimethylammonium compounds (DADMACs).

DADMACs have two alkyl chains (Figure 1.1) and are therefore more hydrophobic than BACs and ATMACs. While ATMACs only have a linear alkyl group, BACs have not only a linear alkyl group but also an additional benzyl group (PubChem 2021). As a result, BACs are more hydrophobic than ATMACs resulting in the following relationship for compounds of equal alkyl chain length regarding hydrophobicity:

ATMACs < BACs < DADMACs.

At lower concentration, QAACs exist as monomers in aqueous solutions. Above a specific concentration, QAACs begin to form micelles. This critical concentration is termed critical micelle concentration (CMC). This parameter is characteristic for surface active agents and marks the range where important properties such as e.g. solubilisation and surface

tension change abruptly (Tadros 2005). In aqueous media, the cationic parts of QAACs are arranged outwards and the hydrophobic part is enclosed in the inner part of the (spherical) micelle. Thus, above the CMC, QAACs are technically not dissolved in the aqueous solution but rather form a second phase similar to an emulsion.

Parameters like the *n*-Octanol/Water Partition Coefficient (K_{OW}) and water solubility are often used to describe the ecological effect of substances (Bliefert 2002). K_{OW} is the key parameter to describe sorption (and bioaccumulation) of chemicals in the environment and the water solubility is a key parameter of hydrophobic substances regarding their retention or leaching potential from soil due to soil water flow (Schwarzenbach and Westall 1981, Razzaque and Grathwohl 2008). The sorption of QAACs to biosolids is driven by ionic and hydrophobic interaction. Therefore, Ismail *et al* (2010) and Mulder *et al* (2020) propose that the CMC is a suitable parameter for the description of the effects and the fate of QAACs in the environment and that K_{OW} is of limited applicability/not applicable for characterizing the behavior of QAACs in the environment.

Due to their remarkable surfactant and disinfectant properties, QAACs have seemingly ubiquitous fields of applications: ATMACs with chain length C-16 (also called cetrimonium chloride), C-18 (also called steartrimonium chloride) and C-22 (also called behentrimonium chloride) are commonly used in hair care products to make the hair softer, easy to comb and antistatic (SCCS 2007, Lara-Martín *et al* 2010). DADMACs were used in fabric softeners (Gerike *et al* 1994) but were replaced by esterquats due to ecological risk concerns mostly (Mishra and Tyagi 2007). DADMACs are often used with various DADMACs as mixtures containing up to > 90 % DADMAC C-10. In the EU it is mostly used for the protection of ornamental plants (EURL 2016) but we also found containers of detergents used for cleaning the stables containing DADMACs at local hog farms where we took soil and manure samples. BACs are used as disinfectants since 1935 (Domagk 1935, Frankl 1941). Today they are, often referred to as benzalkonium chloride, used as antiseptic agent in a wide range of pharmaceuticals for example eye drops and nasal spray (Aronson 2015). The antiseptic effect arises from its potential to denaturize proteins and the lysis of cytoplasmic membranes (Ittoop *et al* 2015). BACs do also enhance the drug delivery into human bodies (Burstein and Klyce 1977), which is another reason for the pharmaceutical use.

1.2.2 Interaction of QAACs soil and soil minerals

Due to their permanent positive charge, QAACs tend to interact with negatively charged surfaces in soils (Boethling and Lynch 1992). They accumulate at solids rather than in the aqueous phase (Suter *et al* 1997, Martínez-Carballo *et al* 2007).

The sequestration of sarafloxacin and difloxacin (antibiotics) to soil leads to non-bioaccessable fraction of these substances due to strong and rapid sorption. Thus, neither nitrification nor denitrification was affected by difloxacin in experiments with realistic soil concentrations (Rosendahl *et al* 2012). Similar was described by Förster *et al* (2009),

they concluded that Sulfadiazine may persist in soil for years because it is not totally bioaccessible due to sequestration in soils.

Clay minerals are known to retain QAACs, which is used for the application of QAACs in organoclays for barrier applications (Zanini *et al* 2013, Zhao *et al* 2017). Further important soil substances and possible sorption sites and sinks are the organic matter, ferric oxides and the interior of soil microaggregates. It is known, as described in chapter 1.1, that there are several pathways for QAACs to enter agricultural soils. Nevertheless, soil concentration data is rare and consequently the knowledge about the long-term fate of QAACs in soil is currently lacking as well (Mulder *et al* 2018). We only found an old soil study from Gerike *et al* (1994) who found 15 mg kg^{-1} as maximum concentration of DADMACs in soil to which sewage sludge had been applied.

The data scarcity on QAACs in soils is further evidenced by own findings in a recent literature survey using Web of Science. A search for "QAC" resulted in 671 references, "QAAC" resulted in 9 and "QAAC OR QAC" refined by "soil" showed 28 results, but the small amount of studies is also affected by the numbers of synonyms in use as described before. In contrast, there are many published data on the fate of QAACs in aquatic ecosystems, wastewater and in particular bound to sediments (Zhang *et al* 2015).

To explore the effect of different clay minerals towards the toxicity of QAACs it is important to have an idea of the sorption process of QAACs to different clay minerals. There are many different clay minerals, which predominate the < 2 µm fraction in soils. These group of phyllosilicates can be divided in three major categories, which are (i) 1:1 layer minerals, (ii) 2:1 layer minerals and (iii) 2:1:1 layer minerals (Barton 2002). In this dissertation the focus was on the first two categories: 1:1 layer minerals consisting of a tetrahedral and an octahedral layer (TO) and, 2:1 layer minerals consisting of two tetrahedral and one octahedral layer between them (TOT).

Due to isomorphic substitution for Si^{4+} by Al^{3+} in the layers, the net charge of tetrahedral layers is negative while the net charge of octahedral layers is positive. Net layer charge is expressed as atom per unit (p. f. u.) (Guggenheim *et al* 2006). Since smectites consist of two tetrahedral layers, each with negative net charge and one octahedral layer (positive net charge) the net negative charge of smectite clay minerals (TOT) is x = 0.2 – 0.6 p. f. u. (per formula unit) Kaolinite (TO) consists of one tetrahedral layer and one octahedral layer resulting in x = 0 p. f. u. (Theng 2012). Kaolinite which is a typical representative of the TO minerals is not expandable because the layers are connected via hydrogen bonding. In contrast, smectites layers are attracted to each other by electrostatic forces arising from exchangeable cations in the interlayers. When the cations are exchanged by water, the interlayers of the minerals expand.

The resulting negative surface charge of smectites can attract the cationic QAACs more than kaolinites and additionally QAACs can be trapped in mineral interlayer spaces. Different working groups have shown, that the adsorption of QAACs in the interlayer space occurs in different arrangements. Zanini et al. (2013) described that QAACs adsorb

to clay surfaces in monolayers and bilayers in flat arrangement when the QAACs concentration is around the cation exchange capacity (CEC). At concentrations above CEC, the molecules are arranged orthogonally at the surfaces with the hydrophobic chain pointing away from the clay surface. Presumably due to hydrophobic interaction of the alkyl chain, a second layer of QAACs can interact and adsorb to the QAACs. Bergaya 2006, Paiva *et al* 2008 and Zhao *et al* 2017 described the adsorption of QAACs into the interlayer space. Depending on the QAACs concentration this occurs either in monolayers (Figure 1.2 a), or bilayers (Figure 1.2 b). At even higher concentration the QAACs are arranged in paraffin like structures (Figure 1.2 c). This effect only occurs in TOT clay minerals.

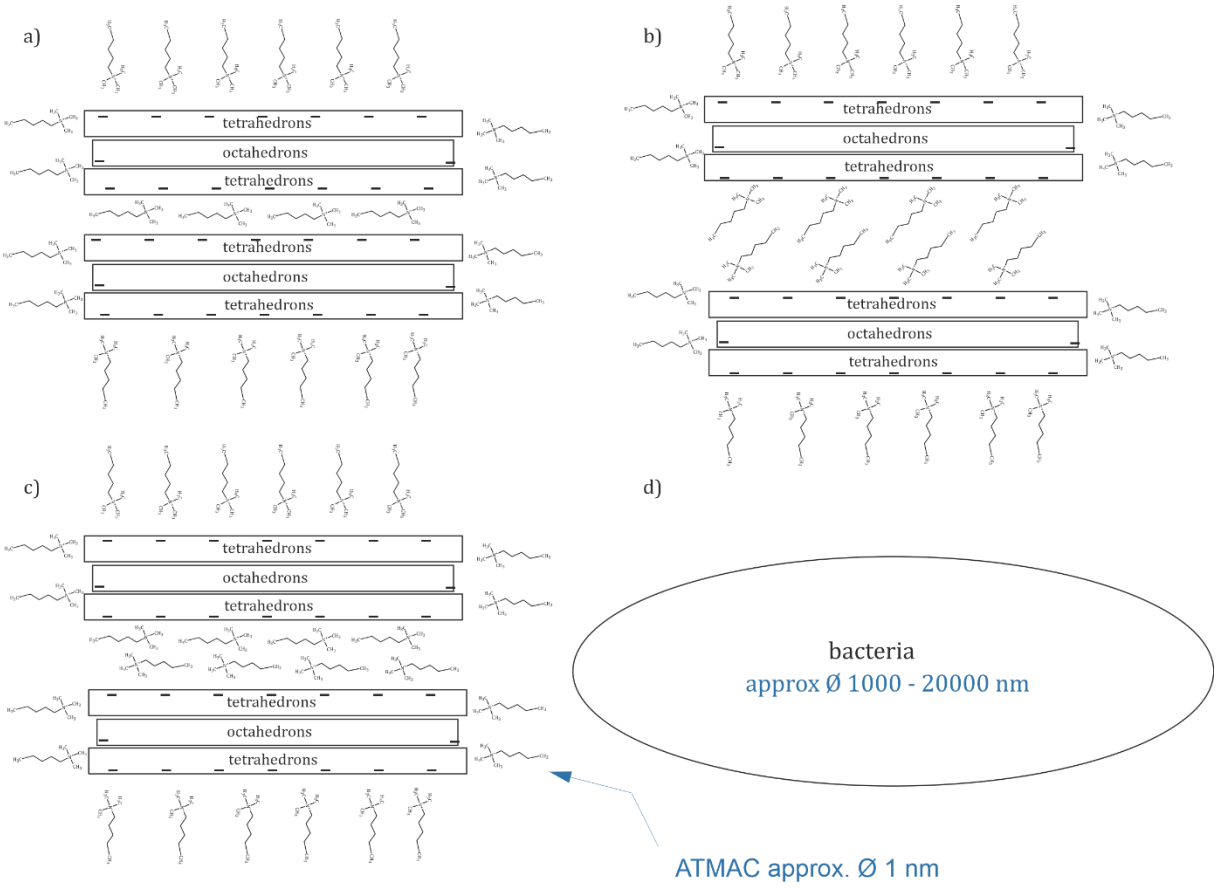

Figure 1.2 Adsorption of ATMAC to 2:1 clay minerals like smectite and the size comparison with bacteria (d; not drawn to scale). Sorption can occur in monolayers (a) bilayers (b) or paraffin-like structure (c). (modified from (Bergaya 2006, Paiva *et al* 2008, Zhao *et al* 2017). True-to-scale representation for size comparison of bacteria (d) and the interlayer space.

Soil bacteria and clay minerals are almost the same size and both larger by a factor of 1000 compared to ATMACs and the magnitude of basal spacing in clay minerals (*Figure 1.3*). Besides binding energies and other bonding properties, the mere size of bacteria may hinder them to reach the molecules bound in interlayer spaces (cf. *Figure 1.2* d).

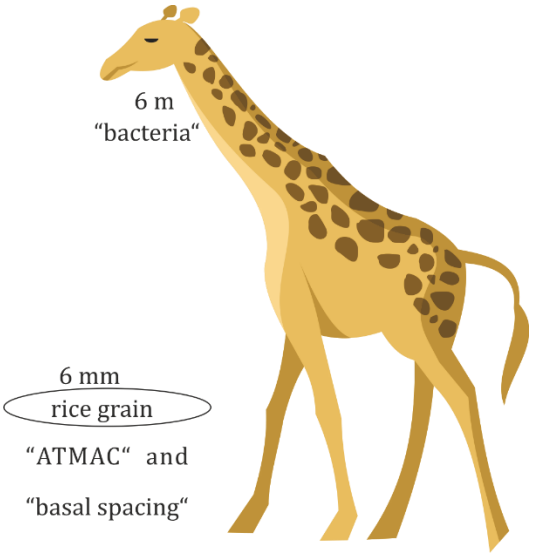

Figure 1.3 Scale comparison of an ATMAC molecule or the basal spacing of clay minerals (approx. 1 nm) and bacteria or clay minerals (approx. 1 µm). Giraffe picture from vectorportal.com with CC license.

1.2.3 Ecotoxicological effects of QAACs

On the one hand, microorganisms can degrade QAACs (Games *et al* 1982, Clara *et al* 2007) and on the other hand QAACs have a toxic effect towards bacteria, which is known since 1935 (Domagk 1935).

The biocidal effect of QAACs towards microorganisms is mainly based on the positively charged nitrogen, which is interacting with the negatively charged parts of acidic phospholipids in the cell membranes (Buffet-Bataillon *et al* 2012). The modeled integration of QAACs in cell membranes is shown in Figure 1.4. Alkhalifa *et al* (2020) showed, that a QAACs molecule is integrated in the cell wall with one alkyl chain after another and ultimately it disturbs the integrity of the structure.

Bragg *et al* (2014) described the toxic effect of QAACs smilarly. The toxicity arises from the ability to cause leaking and eventually destruction of cell membranes of bacteria, which leads to a release of the inner cellstructures from the cytoplasma and death of the cell.

Gravel *et al* (2017) described a worrying effect of BACs towards gram-negative bacteria. They hypothesized that the cell membrane integrity of bacteria is disrupted by BACs in two steps – outer membrane permeabilization and inner membrane permeabilization. The first step occurs below their designated MIC value of 45 µg L^{-1}. Consequently, a sublethal concentration leads to stress adaption in bacteria cells and should be avoided.

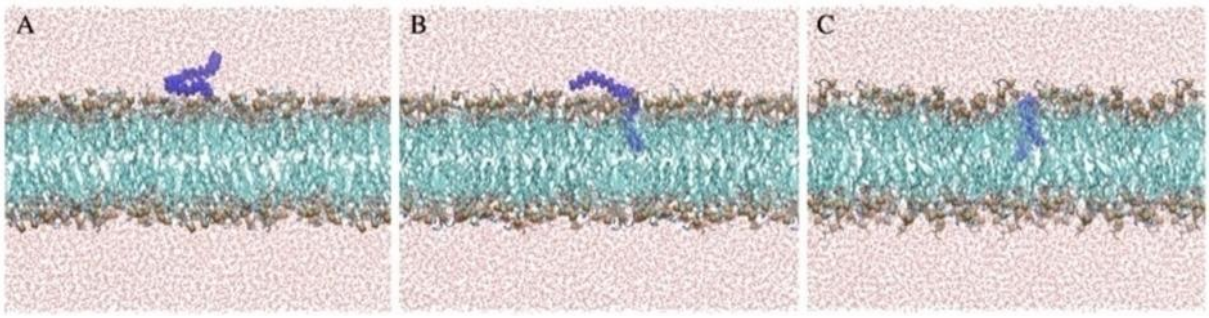

Figure 1.4 Modeled BAC (A) approach, (B) integration into the lipid bilayer of *E.coli* and (C) destruction of the cell structure (Alkhalifa *et al* 2020).

Toxicity data for water organisms are described by many authors (Sandbacka *et al* 2000, García *et al* 2001, Kreuzinger *et al* 2007, Zhu *et al* 2010). Effect concentrations for aquatic biota are summarized by Zhang *et al* (2015) and are between 0.021 mg L^{-1} for *P. subcapitata* (BAC-C12) and 40.53 mg L^{-1} for rainbow trouts *Oncorhynchus mykiss*. In contrast, the ecotoxicological effects of the compounds in soils are hardly described. Sarkar *et al* (2010) described that ATMAC-C16 is more toxic to nitrification activities in soil than ATMAC-C18. Bioaccumulation in earthworms has been described but there is no data about its effects regarding the toxicity to the worms (Sarkar *et al* 2013).

Due to the positively charged nitrogen atom in their structure, it is indicated that QAACs get into the interlayers of 2:1 clay minerals in natural soils (Zanini *et al* 2013). Slow desorption from the interlayers could lead to sub-inhibitory QAACs concentrations in the soil solution, potentially leading to co-selection antibiotic resistance genes of microorganisms (Mulder *et al* 2018). Weber and Rutala (2006) and Kim *et al* (2018) described that microorganisms show in laboratory experiments a lower susceptibility to antibiotics when they were in contact with disinfectants before. However, they mentioned restrictively that the resistance was not clinically relevant because they studied either not human pathogens or the used antibiotics were not used to treat the pathogens in clinical practice.

As described above, QAACs are toxic for microorganisms but they can also develop a tolerance towards QAACs. There are various resistance mechanisms discussed in the literature, for example enhanced biofilm development to hinder the disinfectant to come in contact with most of the bacteria individuals (Bridier *et al* 2011, Andersson and Hughes 2014) and a modification of the cell membrane to make it less vulnerable towards membrane disruption (Tezel and Pavlostathis 2015). The main resistance mechanism is driven by plasmid-borne genes. Bjorland *et al* 2003 found the gene *qacJ* in three Staphylococcus species involved in an efflux-mediated resistance. Additionally, *qacA* and *qacB* coding for putative transport and efflux-proteins also seem to be involved in such resistance mechanisms (Paulsen *et al* 1996). Further, *qacG* and *qacH* are responsible for QAACs resistance development (Wassenaar *et al* 2015, Worthing *et al* 2018). For *qacC* Fuentes *et al* 2005 described that the expressed proteins lead to beta-lactam antibiotic resistance in gram-positive and -negative bacteria. This cross-resistance could lead potentially to antibiotic resistance of soil bacteria, even if those bacteria did never face

antibiotics but QAACs, which alone gives rise to concern. Wassenaar *et al* 2015 suggested that *qacA* genes have even been spread across bacterial taxa. Furthermore, *qac* Genes are located on class 1 integrons (Gillings *et al* 2009, Stalder *et al* 2012) that express gene cassettes with resistance genes for almost any antibiotic family (Stalder *et al* 2012, Mulder *et al* 2018). An integron is a genetic mechanism in bacteria that has a site to catch and release small pieces of DNA which are called gene cassettes (Bennett 1999). These gene cassettes are small mobile genetic units that can move passively as free molecules (Collis and Hall 1995, White *et al* 2001). They are horizontally transmissible (Rao *et al* 2020) and only transcribed when caught by integrons.

1.3 Objectives

The overarching hypotheses of this dissertation are:

i) Continuous inputs lead to a long-term accumulation of QAACs in soils.

ii) The sorption of QAACs in the interlayer spaces of clay minerals leads to a reduction of their bio-accessibility for microorganisms and reduces their ecotoxicological effect towards microorganisms.

To address these hypotheses the work of this thesis was split in three work packages:

(a) Establishing a method for the extraction and quantification of QAACs in environmental samples.
(b) Soil concentrations of QAACs and their fate in agricultural soils.
(c) Exploring the effect of different types of clay minerals towards the toxicity of QAACs for microorganisms.

1.4 Approaches and Methods

1.4.1 Extraction and quantification of QAACs

In order to address the analytical challenges and to establish a stable QAAC analyzing and extraction method (work package (a)), the following steps in method development were necessary:

- Adaptation of a suitable extraction method from the literature and performance of recovery rate tests.
- Identification of a suitable analytical method and the transfer of this method to QAACs and our HPLC MS/MS system
- Determine and possibly reduce background concentrations from laboratory equipment such as vials and the HPLC-MS/MS as well as solvents.

Extraction

The existing extraction methods for surfactants from environmental samples range from supercritical fluid extraction (Fernández *et al* 1996) over solid/liquid extraction followed by liquid/liquid extraction (Levsen *et al* 1993, Gerike *et al* 1994) and accelerated solvent

extraction (Ferrer and Furlong 2002) to USE (Xiang *et al* 2015, Kang and Shin 2016). We also tested (USE) methods, which were developed for the extraction of different pollutants for example pharmaceuticals (Dalkmann *et al* 2012).

Chromatography and Mass spectrometry

The analysis of QAACs consists of two parts, their chromatographic separation via HPLC and their detection in the MS/MS system. In general, there are various analytical column types available for the separation of substances. The common ones use one of three main substance characteristics, which are polarity (1), electrical charge (2) and molecular size (3). With regards to the separation of various QAACs homologues we tested three different columns with different mechanisms. All were reverse phase columns, which means that the stationary phase is non-polar while the mobile phase is polar. A Zorbax RX C8 column that was described in a QAAC analyzis study by Ruan *et al* (2014) and a Nucleodur π^2 whose separation mechanism is based on hydrophobic interaction (and π-π interaction) were tested. Furthermore, we tested an XSelect CSH Phenyl Hexyl column which has a positive charged surface that could lead to faster retention times, since QAACs have a permanent positive charge at the nitrogen atom, leading to repulsion effects.

Only a few gradient programs for chromatographic separation were found in the literature (Li and Brownawell 2009, Chen *et al* 2013, Ruan *et al* 2014). Chen et al. (2013) used an isocratic method with acidified methanol (A) and water (B). The method was developed based on Ruan *et al* (2014) and Li and Bownawell (2009). Both working groups used either acidified acetonitrile or acidified methanol (A) and water (B) as well as 2-propanol (C). The goal was to reduce the number of solvents in order to reduce preparation times and the error-proneness, both on the users and the instruments side.

MS tunes for parameters like cone voltage, collision energy and system temperatures for QAACs were performed with a direct injection system. Thus, the parameters were optimized for each of the QAACs (ATMAC C-8, ATMAC C-10, ATMAC C-12, ATMAC C-14, ATMAC C-16, BAC C-8, BAC C-10, BAC C-12, BAC C-14, BAC C-16, BAC C-18, DADMAC C-8, DADMAC C-10, DADMAC C-12, DADMAC C-14, DADMAC C-16). Ion transitions were taken from Ruan et al. (2014). The results for the MS/MS optimization are shown in chapter 2.

Background concentrations

As described before, QAACs are widely used for cleaning almost everything and laboratory equipment is no exception. We also assumed that some QAACs may be used as lubricants or as some kind of separating agent during the manufacturing process of HPLC equipment. To minimize the background signals in blank measurements, we tested various glassware pretreatments like rinsing the whole equipment with acetonitrile before use as well as rinsing it with Decon 90 as recommended by Bassarab *et al* (2011). Decon 90 is originally a cationic cleaning agent, which was in our case intended to occupy the sorption sites in glassware to prevent analytical substance loss during the measurement. Vials of different manufactures (amber glass and clear glass) and their cap

material (Polytetrafluoroethylene or rubber) were also tested with regard to QAACs loss or release during analysis.

1.4.2 Soil concentrations and fate of QAACs in agricultural soils

For work package b (c. f. 1.3) we took samples from three sets of samples: A first sample set consisted of samples from soils that were fertilized with sewage sludge from the exact same wastewater treatment plant (I) and a second set of samples that was collected from soils that have only been fertilized with liquid manure (II). The manure and sewage sludge were sampled as well. A third sample set was taken in the Mezquital Valley, Mexico City (III). Sample set (I) was taken from basaltic loamy sand acres for corn cultivation fertilized with sewage sludge and one without sewage sludge as control field. Mixed samples from both acres were taken before sewage sludge fertilization and in time intervals of 3 d, 8 d and15 d afterwards. The second sample set (II) was taken on two acres (loess loam) for corn and wheat cultivations. Both were fertilized with liquid manure and one of them additionally with sewage sludge. Here again, a time sequence was taken - from before spreading the sewage sludge and afterwards (2 d, 9 d, 17 d).

Samples from a manure fertilized millet field with a clayey soil (Figure 1.5) as well as from an unfertilized field were taken again before the first yearly fertilization and four times after that (4 d, 8 d, 14 d, 28 d). The manure stemmed from pig husbandry and was collected and thus mixed with grey water in tanks next to the stables. We could confirm with the farmer and the detergents in use that DADMAC C-12 was employed for cleaning of the pigpens. Sample sets I and II were all from Hesse and taken in winter and spring 2017.

Figure 1.5 Sampling the frozen soil and the manure tank in January 2017.

The sample set III consists of samples from two sampling events. The first samples were taken in 2011 by the working group for the Dalkmann *et al* (2012) study and the second samples were taken in 2018 by Jan Siemens and Christina Siebe. Both samplings represent a chronosequence of wastewater irrigation (0, 1, 3, 6, 8, 13.5, 35, 85 years).

The Mezquital Valley in Mexico in the north of Mexico City is one of the largest wastewater irrigated area is worldwide (900 ha) and it is still expanding (Siebe and Cifuentes 1995, Broszat *et al* 2014, Contreras *et al* 2017) as a consequence of the expansion of the city.

Due to the wide field of application of QAACs and as a result of their presence in the domestic, municipal as well as industrial wastewater, we hypothesized that QAACs are applied with wastewater used for irrigation to the soils of the agricultural land. The main soil types in the region are Leptosols, Phaeozems and Vertisols according to the World Reference Base for Soil Resources, which are rich in clay minerals (Siebe 1998).

On the one hand, irrigation with wastewater provides not only water, but also nutrients like nitrogen and phosphorus to crops increasing their yield. On the other hand, the wastewater is also a source of contamination for pollutants such as heavy metals, pharmaceuticals and probably disinfectants that accumulate in those soils (Gutiérrez-Ruiz *et al* 1995, Dalkmann *et al* 2012). Concerning are the findings of Jechalke *et al* (2015) that the abundance of all antibiotic resistance genes tested increased significantly during wastewater irrigation in the Mezquital Valley area.

We performed QAACs analysis with HPLC-MS/MS and additionally determined organic carbon content, $CaCO_3$ content, pH-value, soil texture and trace metal content. With these values, a correlation analysis was performed in order to investigate the influence of soil parameters on the QAACs content. Furthermore, these data provided comparative insight on the fate of QAACs and metals in soil. Eventually, the fate of QAACs and pharmaceuticals can be compared, since Dalkmann *et al* (2012) performed pharmaceutical analytics of the exact same soils (sample set from 2012).

1.4.3 Exploring the effect of different types of clay minerals towards the toxicity of QAACs for microorganisms.

We assume that the apparent MIC of QAACs is higher in an environment with smectites compared to kaolinites. The MIC describes lowest concentration of an antimicrobial substance that inhibits the visible growth of microorganisms after a night of incubation (Andrews 2001). The knowledge about the different MICs of soil bacteria plays a central role in order to understand QAACs tolerance of bacteria, and furthermore, the spread of antibiotic genes in soils.

Taken together, the toxicity of QAACs, the ability of smectites to sequestrate QAACs and slowly releasing them and the adaptability of bacteria towards QAACs, which eventually can lead to antibiotic resistance, demonstrate the importance of this work package.

We investigated the difference of the growth dynamics and MIC values for eight different bacteria taxa in presence of TOT and TO clay minerals. BAC C-12 and DADMAC-C10 were selected as representative of the most used QAACs.

1.5 Summary of results

1.5.1 A method for the extraction and quantification of QAACs in environmental samples.

The Zorbax RX C8 column showed the lowest signals. The Nucleodur π^2 and the XSelect CSH Phenyl Hexyl column showed similar signals but the later formed narrower peaks and has a wider pH stability (1 – 11). The extracts that were cleaned up with a solid phase extraction (SPE) had pH values < 0.3 since they were eluted with methanol/HCl. Thus, we decided to use the XSelect column which is more stable at the lower pH values.

For the measurement of only one or two homologues e.g. sorption experiments I developed a fast HPLC method. This method is an isocratic method with two eluents (A) 15 % MQ-water and 50 mM formic acid and 10 mM ammonium formate and (B) 85% acetonitrile. This method is faster than a gradient program because there is no equilibration time needed after each run and also the retention times were shorter mainly because the portion of acetonitrile is higher compared to a gradient program that starts usually with the aqueous phase. For the gradient program that was developed for the analyzes of soil samples we tried to reduce the eluents from three as used by Ruan *et al* (2014) for sewage sludge to two because it is less prone to errors in terms of solvent preparation or pumping issues. Methanol was exchanged with acetonitrile because we observed faster elution and less tailing with acetonitrile. Additionally, the pressure experienced by the column is lower when acetonitrile was used, which solved the problem with high pressures in the system for us.

Pressurized solvent extraction, microwave extraction and supercritical fluid extraction resulted in poor recoveries which were mostly below the limits of detection (LOD). Ultrasonic extraction turned out as the best method. Recovery rates for the non-deuterated analytes were between 47 % and 57 % with at the same time good precision (≤ 3 % SD). Another advantage of USE is its simplicity (compared to the other tested ones), which makes the method usable also in small laboratories without special equipment. Extraction with acetonitrile with HCl worked best for (spiked) soils. We observed almost no matrix effect when comparing analyte recovery in pure solvent compared to spiked soil extraction. But nevertheless we decided to add deuterated internal standards to compensate possible matrix effects and instrumental drift. Soil extracts were transparent without floating particles. In contrast, sewage sludge extracts were not transparent and they also consisted of two phases: one aqueous and a second phase that appeared dark and oily (Figure 1.6). Therefore, a cleanup step with SPE was needed. After SPE the pH-values were < 0.5. We raised the pH in the extracts afterwards with NaOH (1 mol), but it was tedious and in certain samples the amount of NaOH was exceedingly high and led to a visible precipitation in the samples. The crystalline precipitation was NaCl, which would not disturb the measurement but in the course of this reaction, QAAC could also be precipitated. Hence, the pH-value was no longer raised. In combination with the little

injection volume of 20 µL and the buffer ability of the solvents, this pH values did not harm the instruments.

Figure 1.6 Extracts of sewage sludge with visible two phases (left) and extracts in SPE cartridges (right).

We measured calibrations for all analytes in each run and also determined LOD and limits of quantification (LOQ). Nevertheless, we calculated LOD and LOQ over three years of routine analyzes and chose the median of those measurements as robust LOQ and LOD. LOQ for the different ATMACs homologues were between 0.3 µg kg^{-1} (ATMAC C-8) and 1.1 µg kg^{-1} (ATMAC C-16), for BACs between 0.3 µg kg^{-1} (BAC C-8) and 0.9 µg kg^{-1} (BAC-C16) and for DADMACs between 0.5 µg kg^{-1} and 2.1 µg kg^{-1}. The LOQ and LOD were all lower and some even much lower than those of previous studies (Merino *et al* 2003, Martínez-Carballo *et al* 2007, Ruan *et al* 2014, Bergé *et al* 2016). In general, the recovery rates of DADAMCs were relatively low and the LOQ were relatively high. Both could be due to the two alkyl chains which they comprise of. On the one hand, this leads to more hydrophobic interaction between DADMACs and hydrophobic soil particles like soil organic matter and thus lower extraction efficiency. On the other hand the high LOD of DADMACs could be due to incomplete elution from the analytical column, since the used column separates based on hydrophobic interaction. Higher background contamination due to the extraction equipment could be another reason.

In order to tackle the problem of background concentrations, the extraction equipment was washed with acetonitrile in advance of each experiment. With regards to the vials used for the measurement we found no difference between clear glass vials and amber glass vials, neither in blank values nor with analyte solution. The same applies to the vial treatment with DECON 90. However, the material of the caps seemed to have an influence towards BAC-C12 recovery. Predictably, Polytetrafluoroethylene caps did not influence the recovery of BAC-C12, quite the opposite of the rubber caps that absorbed > 40 % of a 100 µg L^{-1} BAC-C12 solution.

1.5.2 Soil concentrations of QAACs and their fate in agricultural soils after longtime wastewater irrigation compared to pharmaceuticals, metals and soil properties.

The pH-value of the soils decreased slightly with the duration of irrigation of the Mexican soils in both sample sets (2012, 2018). These results have also been shown in other studies on wastewater irrigation (Siebe Grabach 1994, Xu *et al* 2010, Broszat *et al* 2014). The decrease could be due to proton production by nitrification which is stronger due to the input of organic nitrogen (Hernández-Martínez *et al* 2018). Additionally the oxidation of organic matter (C_{org}), which is imported with the wastewater, leads to higher CO_2 concentrations and eventually to higher H_2CO_3 concentrations in soil resulting in lower pH values (Amelung *et al* 2018).

The carbonate content remained stable at low values in all samples (approx. 1 %). As expected, the organic carbon content was highest after long-term irrigation (34 a and 85 a). The clay content remained stable between 39 % and 54 % and was apparently independent from irrigation time, because the amount of fine material transported by wastewater was too small to increase the clay content of the soils.

The chromium values were remarkably high (50 mg kg^{-1}) even in the rain fed soils. This may be due to the volcanic origin of the parent material of the soils in the valley. The concentration of cadmium, chromium, copper, nickel, phosphor, sulfur and zinc were higher the longer the wastewater irrigation. While this was to be expected and has previously been demonstrated by Siebe and Cifuentes (1995) it supports the selection of the soils as a suitable choice because it shows the accumulation of deposited substance in soils due to wastewater irrigation.

All samples contained at least one QAACs homologue. The group of BACs were detected most frequently with up to 131.2 µg kg^{-1} followed by ATMACs with 12.7 µg kg^{-1} and DADMACs with 11.3 µg kg^{-1}. BACs are the most common used QAACs in households and thus also in the wastewater from a city. The most QAACs in total were detected in soils with 88 years of irrigation. As described before, we had samples from 2011 and 2018.

The concentration of the sum of QAACs correlated strongly with irrigation time ($r = 0.88$). The same was observed for sum of ATMACs and BACs ($r = 0.89$). DADMAC had a weaker correlation with the time of irrigation ($r = 0.60$), which I suggest is due to lower input from wastewater compared with other inputs (c. f. 1.2). DADMACs are often used directly at the agricultural land or farm for cleaning purposes or plant protection, while BACs and ATMACs are more often used in households or the medical sector. In general the recovery rates of DADMACs are lower compared to other QAACs especially DADMACs with alkyl chains longer than 12 C atoms (c. f. 1.4.1.). Multiple regression showed, that the time of irrigation explained the observed increase of QAACs concentrations in soil ($p = 0.044$ in 2018; $p = 0.013$ in 2001) better than pH and clay content.

For ATMACs and BACs we found a biphasic accumulation with increasing time of irrigation. This was also observed for DADMACs concentrations in samples from 2011. More precisely, the accumulation in the first 40 years of irrigation was linear. The accumulation in the following years (> 40 a) rose exponentially. This biphasic fate is neither comparable to the fate of heavy metals in soil as described by Siebe and Cifuentes (1995), nor similar to the fate of pharmaceuticals as described by Dalkmann *et al* (2012). It is obvious that the fate is different (Figure 1.7). In contrast to QAACs concentrations, concentrations of pharmaceuticals reach a plateau with longer irrigation time, marking a steady-state of input and dissipation of pharmaceuticals (Figure 1.7). There are three possible reasons for the different behavior of QAACs compared to pharmaceuticals, (i) QAACs were used in excessive amounts after their invention around the 1940s and their use then declined to a lower level or (ii) QAACs are stronger bound to soil particles after long-term irrigation which leads to reduced biodegradation, or (iii) the plateau is not yet reached after 80 years of irrigation and the QAACs still open up new sorption places due to hydrophobic interaction with each other. It is also conceivable that a mixture of all three mechanisms is responsible for the exponential increase in QAACs concentrations in soil over irrigation. But another limitation should be mentioned, the amount of samples and consequently data points is much larger for the first 40 years (c. f. 1.3.).

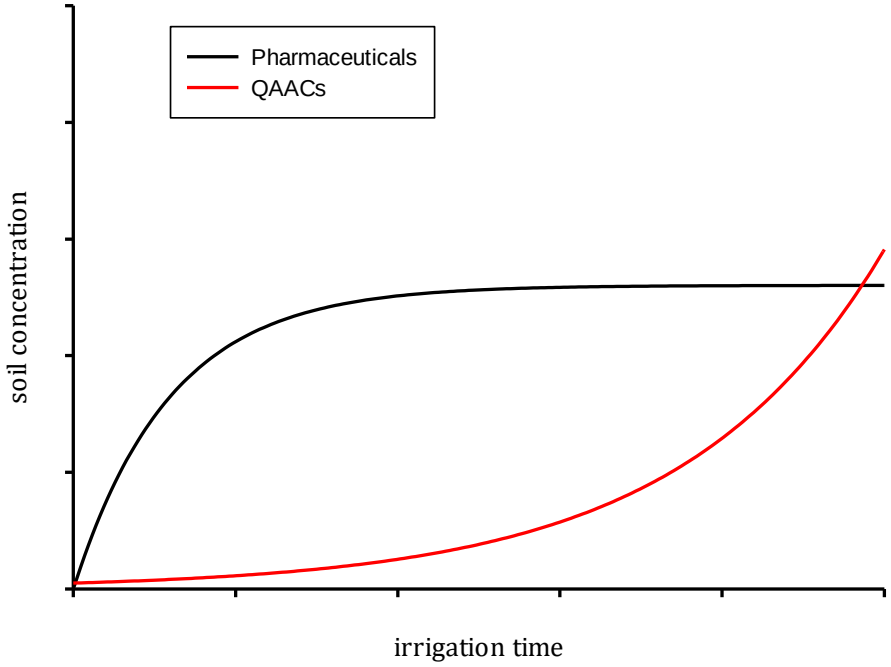

Figure 1.7 Schematic representation of the sorption of QAACs (red) and pharmaceuticals (black) towards soil particles over time of wastewater irrigation.

Overall, we determined higher concentrations in samples from 2018 than from 2011, which could be caused by aging effects of the QAAC in the soil and biodegradation by bacteria. The concentration of trace metals were different.

Although both sample sets were stored at -32 °C, the samples from 2018 were processed almost immediately while the samples from 2011 were stored for seven years. But, even though some working groups described an aging effect of QAACs due to the association to stronger sorption sites (Fernández *et al* 1996, Li and Brownawell 2009), Doherty (2013) found that ATMACs and DADMACs did not degrade in environmental samples under anoxic conditions and QAACs were extracted in the same amount as from fresh samples after one year. Since the samples were stored in a freezer (-32 °C) oxic degradation by bacteria is unlikely. A possible reason is that the samples from 2011 were not taken in order to perform QAACs analyzes but pharmaceuticals and thus they were not handled QAACs specific. Additionally they might have been defrosted more often for other experiments.

Additionally to the samples from Mexico, soil samples from the Wetterau (Hesse; Germany) were measured. The original plan to examine five different sample sites could not be carried out in the context of the present dissertation. For technical reasons, in the form of a defective HPLC pump, only the samples from Mexico and a small number of samples from Germany could be examined so far. This also kept me from measuring QAACs in the samples in triplicates. Nevertheless, I found first evidence that the samples from the Wetterau had no elevated QAACs concentrations directly after sewage sludge application. However, in samples taken eight days after application QAACs have been detected. I assume, that a lack of turbation and selective sampling of soil (without visibly still differentiable applied dried sewage sludge on top of the soil on the first day after application) may be the reason for this phenomenon.

1.5.3 The effect of different types of clay minerals towards the toxicity of QAACs for microorganisms.

The MIC values in absence of clay mineral particles were in the range between 5 and 30 mg L^{-1} for BAC-C12 and 1.0 to 3-5 mg L^{-1} for DADMAC-C10 for most tested bacteria. We have seen different growth kinetics for the bacterial taxa. Those kinetic data revealed, that even with the same endpoint MIC, bacteria showed different response to QAACs. Concentrations of QAACs smaller than the MIC did extend the lag phase (Figure 1.8) or changed growth rates. This suggested that the risk assessment of QAACs should not only focus on the MICs as endpoint of the assessment but take into account bacterial growth kinetics. Some bacteria showed different growth behavior within the same species and even more so among replicates (*P. fluorescens*), which is a good indication that bacteria adapt spontaneously. Nishihara *et al* (2000) have shown a similar behavior for *P. fluorescens*. It has been proposed that the expression of QAAC-efflux pump genes and changes in the cell membrane´s fatty acids are possible spontaneous adaptions (Guérin-Méchin *et al* 1999, Jennings *et al* 2015).

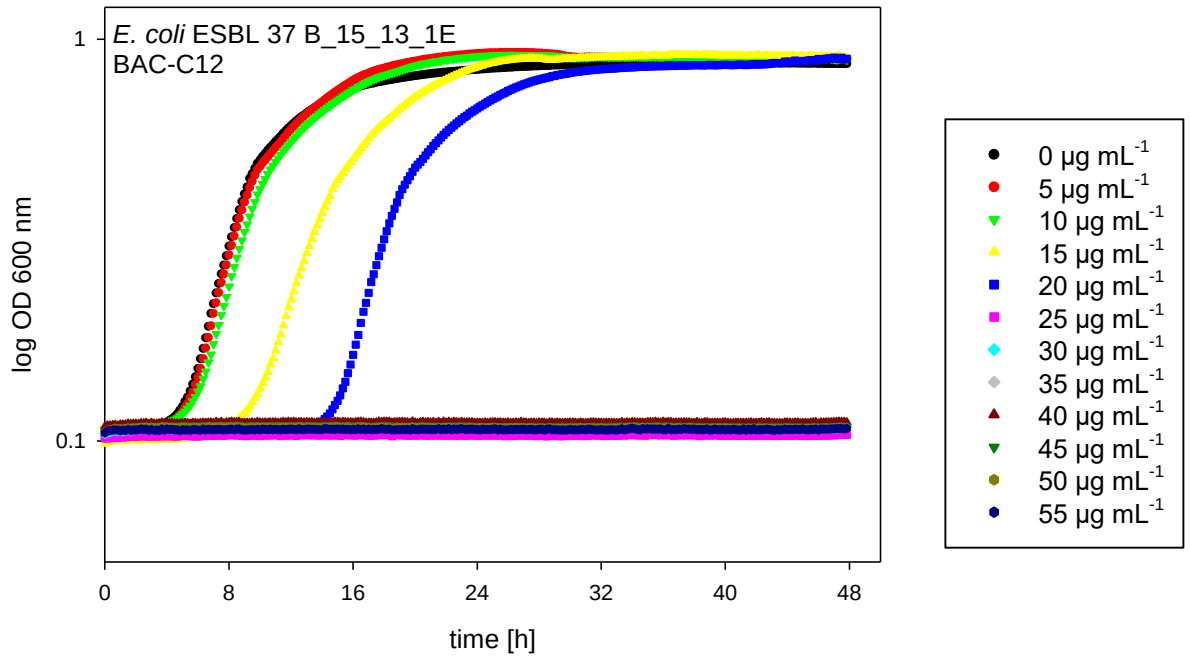

Figure 1.8 Optical density (log OD), marking the growth of *E. coli,* over time with the addition of BAC-C12. At concentrations of 15 and 20 µg mL^{-1} a shift towards delayed growth is apparent.

The addition of the 2:1 layer clay mineral smectite led to higher apparent MIC values for all tested strains (Table 1.1). This confirmed parts of our overarching hypothesis that smectite minerals buffer the toxicity of the selected QAACs. In contrast, the effect of kaolinite minerals compared to the control without mineral added was negligible. Possibly a significant fraction of the QAACs was sorbed in the expandable interlayer spaces of the smectite clay minerals, which is not accessible for bacteria (see Figure 1.2).

Table 1.1 Shift in apparent MIC [µg mL^{-1}] of four tested strains with BAC-C12 and DADMAC-C10 while adding smectite or kaolinite compared to the control with no addition of clay.

Test strain	Clay mineral	MIC BAC-C12 [µg mL^{-1}]	MIC DADMAC-C10 [µg mL^{-1}]
E. coli ESBL 37 B15_13_1E	none	12.5	2.5
	kaolinite	< 6.25 / 12.5[a]	2.5
	smectite	25	20
E. coli ConF4	none	< 6.25	1.25
	kaolinite	< 6.25	1.25
	smectite	12.5 / 25 [a]	10
P. fluorescens DSM 50090^{T}	none	12.5	2.5
	kaolinite	12.5	2.5
	smectite	25	10 / 20[a]
E. faecalis DSM 20478^{T}	none	> 6.25	1.25
	kaolinite	> 6.25	1.25
	smectite	12.5 / 25 [a]	10

[a] two values due to differences in duplicates

Additionally, we observed that the MIC values shift after addition of smectites was more strongly pronounced for DADMAC-C10 as compared to BAC-C12. Thus, a compound specific interaction occurred and DADMAC-C10 appeared to be sequestered more selectively than BAC-C12. Hydrophobic interaction of DADMACs takes place on via two alkyl chains, while BAC-C12 can only interact with a single alkyl chain. Ismail *et al* (2010) found that BACs sorption to sewage sludge was positively correlated with their hydrophobicity, which confirms the findings.

The sorption isotherms showed that sorption occurred below and above the CEC in both, kaolinite and smectite experiments (Figure 1.9). Thus, cation exchange alone is not the

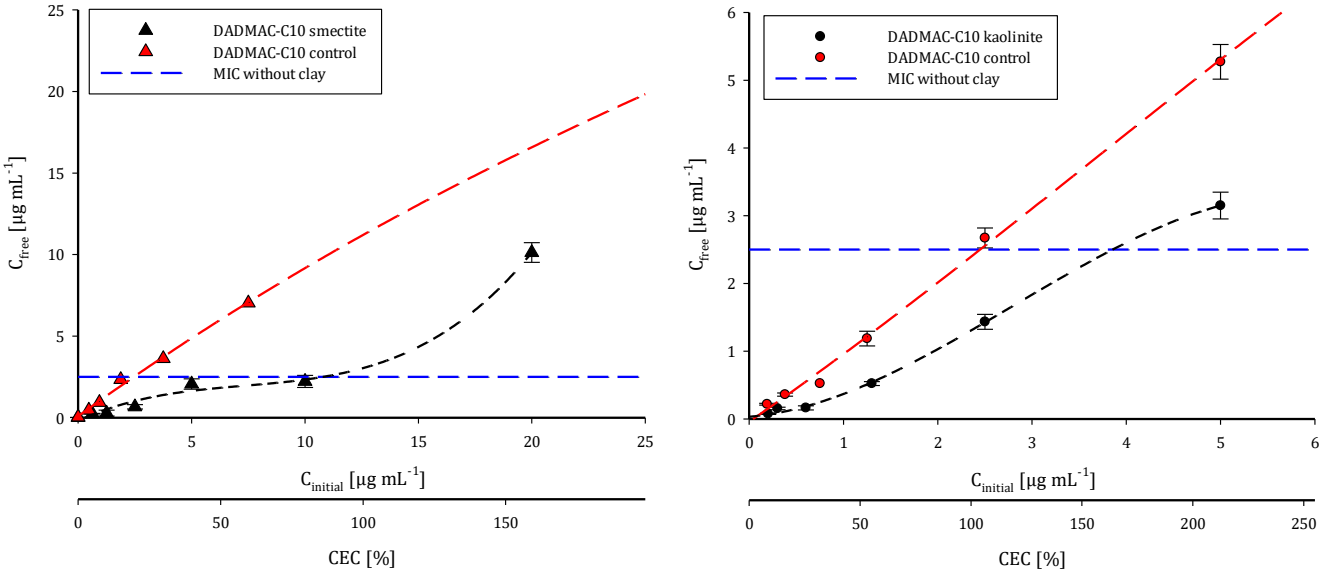

Figure 1.9 Dissolved concentration [C_{free}] versus initial concentration [$C_{initial}$] of DADMAC-C10 and smectite (left) and DADMAC-C10 and kaolinite (right) and MICs shown as blue long dash lines for *E. coli* ESBL37B15_13_1E and *P. fuorescens* DSM 50090T. The according controls without clay minerals are shown as black triangles and circles.

only responsible mechanism. I propose that after all sorption places at the clay minerals surface and interlayer spaces are occupied (marking the CEC), hydrophobic interaction of the QAACs with other QAAC molecule hydrophobic moieties took place. Most likely this occurred not only on the outer clay surface but also within the interlayer spaces. QAACs are known to form bilayer and multilayer structures in the mineral interlayer spaces (Zhu *et al* 2003), which leads to an increase in hydrophobic interaction capacity for smectites compared to kaolinites. The expansion of the interlayer space in the smectite experiments with QAACs was confirmed by TEM analyzes that made an expansion of the interlayer spaces directly visible.

Next to the described effect of size exclusion of the bacteria cells, which is occurring in the interlayer spaces, we observed another effect. When smectite clay minerals came in contact with QAACs, they formed microaggregates. This effect was described before by

Penner and Lagaly (2000). QAACs molecules that are trapped in these aggregates might be inaccessible for bacteria, reducing their ecotoxicological effects even further.

1.6 Conclusion

An extraction method for soils and sewage was developed within this thesis for which only basic laboratory equipment is required, namely an ultrasonic bath. Therefore, the extraction can be performed worldwide with this method. The developed HPLC-MS/MS method is robust and less error prone than previously published methods (e.g. Ruan *et al* 2014) and it is suitable for soil and sewage sludge samples. The method delivers lower LOQ and LOD than other published methods at constant retention times. I am confident that the method can help to expand the sparse data availability regarding to QAACs concentration in soils.

The analyzes of the QAACs concentrations in Mexican soils highlighted that QAACs are widely present in soils. QAACs were present in each sample, both irrigated and non-irrigated. The sum of the QAACs (ATMACs, BACs, DADMACs) concentrations in Mexican soils reached concentrations of up to 155 µg kg^{-1}. This concentration, along with the fact that each of the analyzed QAACs homologue was found and current knowledge on the potential co-selection of antibiotics resistant bacteria, raises concern. To date neither regulated threshold values for QAACs in soil nor ecotoxicity data are available.

To highlight the risk of uncontrolled deposition to soils, we have shown that the acute toxicity of QAACs towards bacteria is reduced by smectite minerals, whereas kaolinites did not have this effect. However, this supposedly positive effect of detoxification can lead to chronic sublethal concentrations to which bacteria can adapt. It also shows that it is not sufficient to determine MIC values for QAACs in the laboratory in axenic cultures without taking into account the effects of sorption and possible biodegradation.

We observed an extended lag phase of bacteria growth below MIC values. These sublethal QAACs concentrations may also lead not only to an adaption of bacteria towards QAACs but also towards antibiotics in real soils, since the resistance mechanism is similar and in some cases located on the same genes.

Since clay mineralogy seems to have a great influence on the fate and the effect of QAACs in soils, further real soils of different texture and clay mineral composition (e. g. with more TOT clay minerals or TO clay minerals) should be analyzed to transfer the laboratory scale MIC experiments and findings to the environmental scale. Soils containing the same clay content but different types of clay and with similar organic content should be compared to each other to illuminate the effect of clay mineralogy towards the fate of QAACs. For example soils of high kaolinite content from the Hunsrück area could be compared to soils from the Vienna Basin which are dominated by smectites. Further research may be performed in order to address the effect of different loads of QAACs at a time.

One limitation of the toxicity work packages was that we mainly performed endpoint experiments (MIC values). To address the effect towards the whole microbial population in soils, metagenomics studies and microbial genome sequencing of the bacterial community in the present of QAACs should be performed. In order to move back from the group to the individual bacteria, changes in the fatty acid composition and other adaption mechanism of bacteria in contact with sublethal QAACs concentration can help to understand the mechanism of adaption. Since a deep search in the Ecotox database (US EPA 2021) did not yield any results further research is needed in the field of ecotoxicological effects of QAACs towards soil life like *Lumbricidae* and crop plants.

This work has shown for the first time that QAACs accumulate in soils: irrigation of agricultural soils with untreated wastewater led to a biphasic accumulation of QAACs in Mexican soils – a linear rise followed by an exponential rise. This accumulation is different to an apparent steady-state accumulation of pharmaceuticals in soils. This thesis also confirmed the hypothesis that clay minerals play an important role for controlling the bioavailability and toxicity of QAACs for microorganisms.

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2 A fast and robust method for the extraction and analysis of quaternary alkyl ammonium compounds from soil and sewage sludge

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Figure 2.1 Extraction equipment as used for the ultrasonic extraction.

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RESEARCH ARTICLE

A fast and robust method for the extraction and analysis of quaternary alkyl ammonium compounds from soil and sewage sludge

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Abstract

Alkyltrimethylammonium compounds (ATMACs), dialkyldimethylammonium compounds (DADMACs) and benzylalkyldimethylethylammonium compounds (BACs) are quaternary alkylammonium compounds (QAAC), which are released into the environment in large quantities after their use in cleaning agents and disinfectants. Despite their potential role as selective agents promoting resistance against QAACs as well as antibiotics, there is a lack of data for QAACs in soil due to the lack of sensitive analytical methods. Therefore, we present a robust and fast method for the extraction and quantification of concentrations of these compounds in soil and sewage sludge. The method is based on ultrasonic extraction (USE) with a mixture of acetonitrile and HCl followed by a solid phase extraction (SPE) cleaning step and a subsequent quantification of concentrations with high performance liquid chromatography with mass spectrometry (HPLC-MS/MS) in multi mass reaction mode (MRM). The proposed method is suitable for the quantification of ATMACs (chain length C-8 to C-16), BACs (C-8 to C-18) and DADMACs (C-8 to C-16). The achieved limits of quantification (LOQ) range from 0.1 μg kg^{-1} to 2.1 μg kg^{-1}. The recovery rates of spiked soil samples for non-deuterated homologues were between 47% and 57%. The analysis of sewage sludge samples and soil samples revealed that BAC-C12 was the most abundant QAAC with concentrations up to 38600 μg kg^{-1} in sewage sludge and up to 81 μg kg^{-1} in a Mexican soil that was irrigated with wastewater. Overall, the presented methods open perspectives for effectively studying fate and effects of QAACs in soils.

Introduction

Quaternary alkylammonium compounds (QAACs) are broadly used as disinfectants and surfactants in numerous applications. Although, QAACs and related resistance genes were found in several sediment and sewage sludge samples, the environmental fate and effects of QAACs are currently not well understood [1], also due to a lack of fast and reliable methods for extracting them from soils and analyzing their concentrations. The linear alkylammonium compounds alkyl trimethylammonium compounds (ATMACs), dialkyldimethylammonium

compounds (DADMACs) and benzylalkyldimethylammonium compounds (BACs) are employed ubiquitously in industrial, hospital, agricultural and household chemicals [1]. All QAACs are made of a covalently bound, and therefore permanently positively charged, cationic nitrogen accounting for their hydrophilic and an alkyl chain for their hydrophobic properties. On the one hand, QAACs are biological degradable under aerobic conditions [2], on the other hand they exhibit biocidal properties depending on homologue. Generally speaking, the longer the alkyl chains and the less oxygen available, the more their biodegradability decreases and their toxicity increases [3, 4]

QAACs first appeared in the year 1935, when they were introduced as disinfectant agents by Domogk [5] and ever since, due to their unique surfactant as well as biocidal properties their production an use steadily increased. Starting in the 1990s they were heavily used as fabric softeners [6], especially QAACs of the DADMAC-type with C16 to C18 carbon chains. The use of these homologues was voluntarily phased out by the industry due to their poor degradability and replaced by more easily degradable esterquats [7]. Other QAAC homologues can be found in fabric softeners even today. Furthermore, QAACs are used as detergent in agriculture to clean machines and stables. The majority of the QAACs used for cleaning livestock buildings ends up in manure, which is used as fertilizer for agricultural fields, which represents one input pathway of QAACs to soils. Waste water treatment plants and sewage sludge represent other important sources of QAACs in the environment [2]. When wastewater is used for irrigation or sewage sludge for fertilization, QAACs are also released into the soil. Soil bacteria can adapt to QAAC, when QAAC concentrations remain sublethal [2, 1]. Since the genes encoding resistance against QAACs are often located on the same mobile genetic elements as antibiotic resistance [8], QAAC in soils can promote antibiotic resistance, which is one of the global challenges for human and animal health [9].

Despite their widespread application to soils with manure, wastewater or sewage sludge, the majority of the data available on QAACs in the environment deals with their concentrations in sewage sludges and wastewater [10, 11, 3]. Some data are available for QAACs in rivers and river sediments [12, 13], but only little information is available for soils. Gerike et al. [14] provided some data for concentrations of DADMACs in soil shortly after it was amended with sewage sludge. Similar to the Gerike et al. whose study focused on DADMACs, most other studies also address only one group of QAACs.

QAACs are analytically challenging due to their strong tendency to adsorb to surfaces. This can lead to a compound loss during the analytical process due to adherence to the equipment surfaces and, which might then also result in significant background concentrations caused by carry-over of the analytes between samples or between standards and samples. Since the chromatographic separation of the QAAC homologues is difficult, MS/MS detection in multiple reaction mode (MRM) is mostly used for differentiation of various homologues [13, 15–18].

Solid environmental samples, comparable to soil, like sediment and sewage sludge have been previously extracted with various methods: Steaming acidic methanol extraction [16, 12], ultrasonic extraction (USE) [19, 10] and accelerated solvent extraction [13]. Since Ruan et al [10] reached the lowest limits of detection (LOD) for sewage sludge samples with USE, this appeared the most promising method from which to develop an extraction method for soil and sewage sludge.

In summary, the surfactant nature of QAACs pose analytical challenges that leads to a lack of data. If included in multicompound-environmental screening methods, concentrations of QAACs tend to be underestimated. The development of a reliable method specific for the extraction and analysis of the group of QAACs from soils will contribute to completing our picture of the distribution of these components in the environment, thus promoting our comprehensive understanding of their environmental fate and effects. Hence, the goal of our work

was to provide a stable, fast and, in terms of the needed extraction method, simple method for the group of QAACs.

Experimental

Reagents

The CAS numbers and suppliers of target analytes ATMAC-C8 to C16, BAC-C8 to C18, DADMAC-C8 to C16, Chlormequat and benzethonium that were used in the calibration and the internal standards BAC-C12-D7 and DADMAC-C10-D6 are shown in Table 1. A stock solution was prepared by mixing the reference substances with acetonitrile (AcN, ≥ 99.9%, HyperSolv, VWR, France) in large scale (100 mL), which was stored deep-frozen at -32˚C. Working solutions, containing each of the 16 QAACs in the same concentration, were prepared in AcN one time before all analyses and stored at -32˚C until analysis. aliquots were thawed at room temperature before analysis. Ultrapure water (MQ-water) that was used as eluent was made with Merck Milli-Q system (Millipore Merck, Darmstadt, Germany), methanol was purchased from VWR (≥ 99.9%, HyperSolv, France) and HCl from Merck (32%, p. A., Merck, Darmstadt, Germany). The extraction solvent containing (v/v) 99.9% AcN (≥ 99.9%, HyperSolv, VWR, France) and 0.1% HCl (Ph Eur, Merck, Darmstadt, Germany) was freshly prepared prior to every extraction.

Table 1. Supplier, CAS numbers and Mol. Masses of the target analytes and the internals standards.

Compound name	Abbreviation	CAS#	Mol. Mass
Alkyltrimethylammonium compounds (ATMACs)			
Octyltrimethylammonium bromide	ATMAC-C8*	2083-68-3	252
Decyltrimethylammonium chloride	ATMAC-C10*	10108-87-9	236
Dodecyltrimethylammonium chloride	ATMAC-C12*	112-00-5	264
Tetradecyltrimethylammonium chloride	ATMAC-C14*	4574-04-3	292
Hexadecyltrimethylammonium chloride	ATMAC-C16*	112-02-7	320
Dialkyldimethylammonium compounds (DADMACs)			
Dioctyldimethylammonium bromide	DADMAC-C8*	3026-69-5	350
Didecyldimethylammonium chloride	DADMAC-C10*	7173-51-5	362
Didodecyldimethylammonium chloride	DADMAC-C12*	3401-74-9	418
Ditetradecyldimethylammonium bromide	DADMAC-C14*	68105-02-2	519
Dihexadecyldimethylammonium bromide	DADMAC-C16*	70755-47-4	575
Didodecyldimethylammonium chloride deuterated	DADMAC-C10-D6†	-	368
Benzylalkyldimethylethylammonium compounds (BACs)			
Octylbenzyldimethylammonium chloride	BAC-C8*	959-55-7	284
Decylbenzyldimethylammonium chloride	BAC-C10*	965-32-2	312
Dodecylbenzyldimethylammonium chloride	BAC-C12*	139-07-1	340
Tetradecylbenzyldimethylammonium chloride	BAC-C14*	139-08-2	368
Hexadecylbenzyldimethylammonium chloride	BAC-C16*	122-18-9	396
Octadecylbenzyldimethylammonium chloride	BAC-C18*	122-19-0	424
Dodecylbenzyldimethylammonium chloride deuterated	BAC-C12-D7†	-	347
Chlormequat chloride	ChlM‡	999-81-5	158
Benzethonium chloride	BenzEth‡	121-54-0	448

*supplied by TCI, Eschborn, Germany

†supplied by HPC Standards (Cunnersdorf, Gemany)

‡supplied by Sigma-Aldrich, Steinheim, Germany.

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For SPE the washing solution contained 1.5 mol L^{-1} HCl (32%, p. A., Merck, Darmstadt, Germany) in water and for column elution the solution was a mix of methanol (≥ 99.8%, VWR, Darmstadt, Germany) and 1.5 mol L^{-1} HCl (4:1, v/v).

The buffer solution for high performance liquid chromatography (HPLC) was prepared with formic acid (≥ 98%, Rotipuran, Carl Roth, Karlsruhe, Germany) and ammonium formiate (99.0%, Acros Organics, Thermo Fisher, Waltham, USA). Pentane (≥ 99%, Rotisolv) for spiking the soil was from Carl Roth (Karlsruhe, Germany).

Instruments and materials

Extraction equipment. The evaporation unit (Synchor Polyvap) the associated 120 mL glass vessels, and the Pressurized Solvent Extractor (PSE) were from Büchi (SpeedExtractor, Flawil, Switzerland) and employed for sample extraction trials. Additionally, and ultrasonic bath (Super RK 225 H) from Sonorex (Berlin, Germany) was used. The following solid phase extraction (SPE) cartridges were tested during optimization: Chromabond CN, Chromabond C18 ec and Chromabond HLB (6 mL, 1000 mg, Machery Nagel, Dueren, Germany), and Oasis HLB (6 mL, 500 mg, Waters, Eschborn, Germany). Twenty mL amber glass extraction vials with PTFE lid were obtained from CS Chromatography (Langerwehe, Germany) and 2 mL safe-lock tubes from Eppendorf (Hamburg, Germany). Fifteen mL polypropylene centrifuge tubes for SPE were purchased from neoLab Migge (Heidelberg, Germany). The Rotanta 460R centrifuge was form Hettich (Tuttlingen, Germany) and the Orbital shaker KS-10 from Bühler (Bodenlshausen, Germany). Prior to the experiments, all of the used analytical flasks and vials were cleaned with AcN.

HPLC-MS/MS analysis. The analyses were performed with a Waters™ alliance 2690 separations module, which was equipped with an autosampler unit, a gradient pump, a column oven and a sample heater. The chromatographic device was connected via Peek capillary (0.18 mm ID) to a Waters™ triple quadrupole mass spectrometer (Micromass Quattro Micro). The final optimized method used a Waters XSelect CSH Phenyl-Hexyl-Column (130 Å, 150 mm length, 2.1 mm ID, 3.5 μm particle size) and a column guard of the same material. Further tested HPLC columns were zorbax RX-C8 (Agilent, 150 mm length 2.1 mm ID, 5 μm particle size) and Nucleodur π^2 (Macherey Nagel, Düren, Germany, 250 mm length 3 mm ID, 5 μm particle size). The 2 mL clear glass vials for the extracts were purchased from neoLab Migge (Heidelberg, Germany).

Procedures

Sample preparation. Luvisol samples were taken from a depth of 0 to 30 cm near Hungen in Northern Hesse, Germany, ten days after the application of sewage sludge for fertilization. A soil that was sampled from an adjacent field with similar soil properties was used for spiking with analytes, but this soil was not fertilized with sewage sludge for a minimum of ten years. Vertisol samples (0 to 30 cm depth) were taken from the Mezquital Valley in central Mexico. Those samples were irrigated with wastewater from Mexico City. Sewage sludge samples from wastewater treatment plants in northern Hesse were freeze-dried prior to extraction.

Soil and sewage sludge samples were collected in aluminum foil and deep-frozen at– 32˚C, freeze-dried for ten days and sieved to < 2 mm afterwards. The sampling tools such as spade and bucket were cleaned with EtOH in between processing different samples.

For the recovery trial, soils that received no sewage sludge or wastewater were spiked with 1 μmol methanolic solutions of ATMAC-C12, ATMAC-C14, BAC-C12-D7, BAC-C16, DADMAC-C12 and DADMAC-C10-D6. To this end, 500 g of freeze-dried and sieved soil were covered overnight with a mixture of pentane and the QAACs solution while shaking continuously in and orbital shaker at 150 rpm with the rest of pentane removed by evaporation the following day. Unspiked control samples were treated in the same way without the addition of QAACs.

Extraction and sample clean up. The final extraction procedure for the target analytes was based on Xiang et al. [20], who used an ultrasonic extraction method for the extraction of three QAACs from vegetables. We placed 5 g of soil or sewage sludge in 20 mL amber glass vials and mixed it with 10 mL of the extraction solution (99.9% AcN / 0.1% HCl v/v). The capped vials were then shaken at 420 rpm for 10 min on an orbital shaker and placed in an ultrasonic bath for 10 min at room temperature. After centrifuging at 870 Relative Centrifugal Force (RCF) for 10 min. the extracts were decanted to evaporator vessels. These steps were repeated three times and the collected extract supernatants were evaporated to $\leq$ 1 mL. and, if necessary, filled up to 1 mL with the extraction solution. The extracts were transferred to safe-lock tubes and centrifuged at 17.000 RCF to remove suspended particles. After transferring the soil extracts to 1.5 mL clear HPLC glass vials they were deep-frozen until HPLC-MS/MS analysis.

Sewage sludge sample extracts required a clean-up step as they contained large amounts of dissolved and particulate organic matter. Therefore, a SPE was implemented according to the manufacturers application guide. Macherey & Nagel CN cartridges were conditioned with 12 mL MQ-water. The 1 ml extract volume from sewage sludge samples were transferred from the safe-lock tubes into 15 mL polypropylene tubes and diluted to 6 mL with MQ-water. Afterwards, the diluted extracts were percolated through the cartridges for 10 minutes. The cartridges were washed afterwards with 6 mL of MQ-water and twice with 1 mL of HCl and dried under vacuum for 30 s. The columns were eluted twice with 1 mL of methanol HCl solution. The samples were evaporated under a constant flow of nitrogen to $\leq$ 1 mL. and, if necessary, filled up to 1 mL with methanol and collected in HPLC vials for the analysis.

Extracts were stored at -32˚C until HPLC-MS/MS analyses. In cases where the detected concentrations exceeded the calibration range, the extracts were diluted with the extraction solution to an expected concentration between 2.5 μg L^{-1} and 300 μg L^{-1}.

Fig 1 summarizes the various extraction procedures and solvent mixtures that were tested for optimal extraction yield. PSE was performed with 0.5 g of soil at 120˚C and 103 bar (3 cycles) and microwave extraction with 0.5 g of soil and 750 W for 30 min after 5 min preheating at 450 W. The centrifuging and evaporating procedures were performed analogously to the final method described above.

High performance liquid chromatography with mass spectrometry (HPLC-MS/MS). For HPLC-MS/MS measurements, 20 μL of the soil or sewage sludge extracts were injected into the system. A buffer solution of formic acid and ammonium formate mixed with AcN were used as liquid phase for the chromatography. As mobile phase (A) a buffer solution containing 50 mM formic acid and 10 mM ammonium formate (90%) mixed with AcN (10%) and as mobile phase (B) a mixture of the buffer solution of formic acid and ammonium formate (10%) and AcN (90%) were used. As shown in Table 2A both eluents were premixed 90% / 10% (v/v) to prevent precipitation of salt in the HPLC system. The flow rate was set to 0.25 mL min^{-1}. After separation, the analytes were detected with tandem mass spectroscopy in MRM mode with argon ($\geq$ 99.999%) as collision gas. Precursor and product ions as described by Ruan et al. [10] were used for ATMACs, BACs and DADMACs, those for chlormequat and benzethonium were determined in our lab. Based on very stable retention times of the analytes (variation $<$ 12 seconds, see below), the specificity of the transition from the precursor ion to the product ion, and our excellent experience with spiked samples regarding the identification of the target analytes, we decided to maximize the sensitivity of our mass spectrometer by detecting 20 product ions instead of 40 ions. Detailed MS/MS settings are presented in Table 2B. Chromatograms of DADMAC-C10, BAC-C12-D7, BAC-C12 and ATMAC-C12 are displayed in Fig 2, chromatograms of all analytes and a total ion current chromatogram are provided in SI (S2 and S3 Figs).

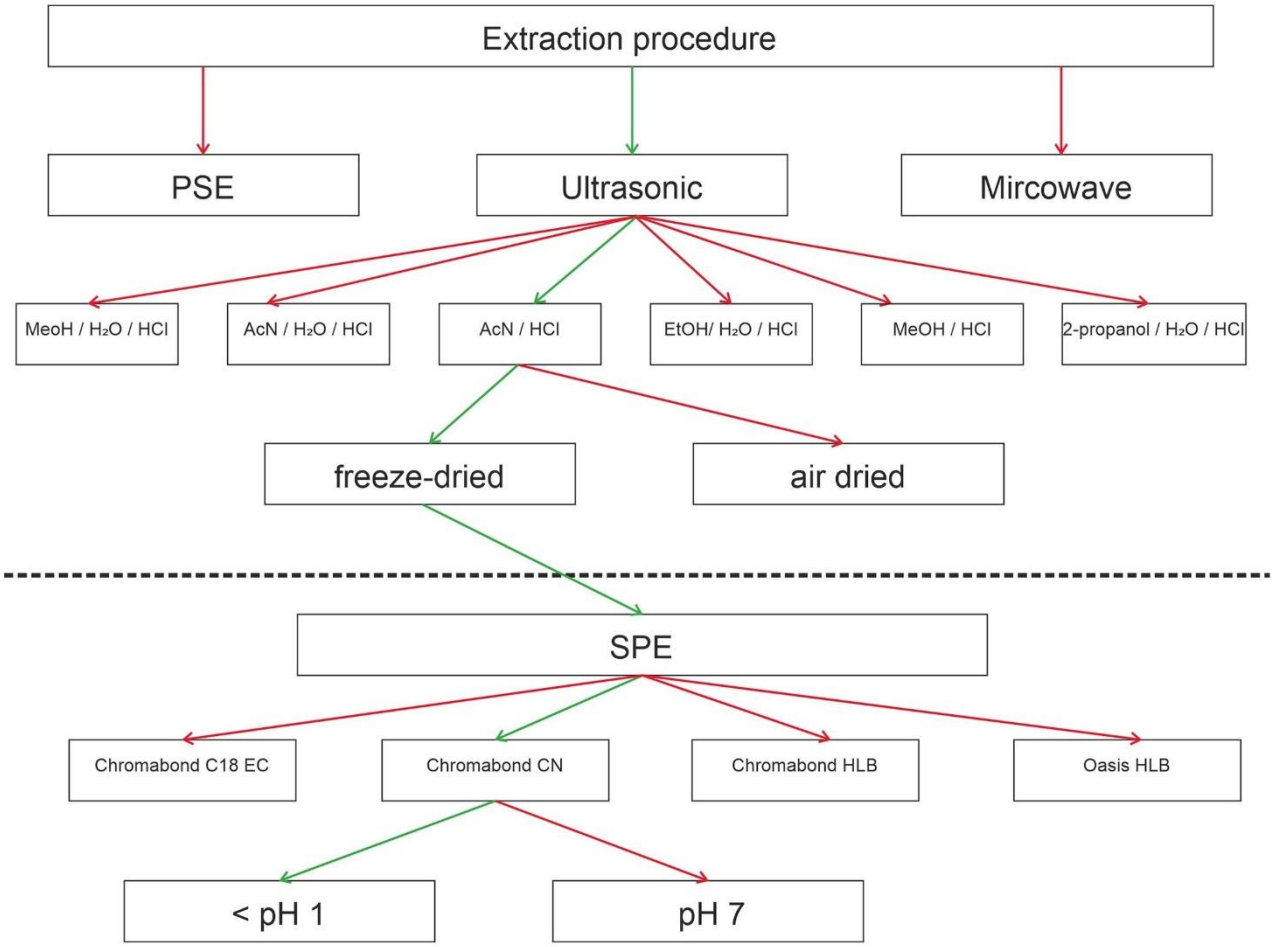

Fig 1. Schematic overview of the development of the extraction method. Green arrows indicate the chosen optimum procedure, red arrows represent dead ends with suboptimal results that were not further followed. The initial method development was performed for soil samples, and was subsequently also applied to sewage sludge samples.

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Table 2a. Settings and Parameter for the HPLC as used to analyze QAACs.

Time	90% H_2O + buffer 10% AcN (A)	10% H_2O + buffer 90% AcN (B)	Flow
	[%]	[%]	[mL min^{-1}]
0	100	0	0.25
3	25	75	0.25
14.5	0	100	0.25
21	0	100	0.25
21.01	100	0	0.25
26	100	0	0.25

Waters XSelect Phenyl Hexyl Column; 38˚C column temperature; 20 μL injection; buffer = 50 mM formic acid + 10 mM ammonium formate.

https://doi.org/10.1371/journal.pone.0237020.t002

Table 2b. Settings and Parameter for the MS/MS as used to analyze QAACs. Qualifier ion is given in brackets behind the quantifier ion.

	RT [min]	Precursor ion [m z^{-1}]	Product ion [m z^{-1}]	Collision [V]
ATMAC-C8	6.3*	172*	60* (57*)	25*
ATMAC-C10	7.0*	200†	60† (57†)	25*
ATMAC-C12	7.6*	228†	60† (57†)	25*
ATMAC-C14	8.4*	256†	60† (57†)	27*
ATMAC-C16	9.3*	284†	60† (57†)	27*
BAC-C8	7.2*	248†	91† (58†)	23*
BAC-C10	7.8*	276†	91† (58†)	23*
BAC-C12	8.6*	304†	91† (58†)	25*
BAC-C14	9.6*	332†	91† (58†)	25*
BAC-C16	10.8*	361†	91† (58†)	27*
BAC-C18	12.4*	389†	91† (58†)	29*
DADMAC-C8	8.4*	270†	158† (57†)	25*
DADMAC-C10	10.3*	326†	186† (57†)	29*
DADMAC-C12	13.1*	383†	214† (57†)	32*
DADMAC-C14	16.5*	439†	242† (57†)	35*
DADMAC-C16	20.5*	495†	270† (57†)	39*
Chlormequat	1.6*	122†	58† (63*)	33* (35*)
Benzethonium	8.9*	413†	91† (320*)	50* (30*)
BAC-C12-D7	8.6*	311†	98*	37*
DADMAC-C10-D6	10.3*	332†	192*	41*

Cone voltage [V] = 43; Capillary voltage [kV] = 3.5; Source [˚C] = 130; Desolvation ˚C [450]; Desolvation Gas N^2 [L hr-1] = 600.

* determined in our lab

† Ruan et al. [10].

https://doi.org/10.1371/journal.pone.0237020.t003

Calibration and quantification. For quantification, an external eight-point calibration (2.5, 5.0, 10, 20, 40, 80, 160 and 300 μg L^{-1}) was prepared in AcN before each analysis, with the working solution warmed to room temperature before use. As injection standard, a deuterated BAC-12 (BAC-12-D7) was added to every single extract and calibration sample immediately before sample injection. The concentration of the injection standard was consistently 100 μg L^{-1}. Peak analysis was performed with Waters MassLynx 4.0, data evaluation and illustration with Systat Sigma Plot 12.0. In order to correct the analysis for instrumental drift, the quotient of the signal of the analyte and the injection standard was used for quantification.

The limit of Detection (LOD) x_{LOD} and limit of quantification (LOQ) x_{LOQ} (μg kg^{-1}) were calculated as shown below,

$$x_{LOD} = 4 \times \sigma \quad (1)$$

$$x_{LOQ} = 10 \times \sigma \quad (2)$$

with σ (as μg kg^{-1}) denoting the standard deviation of the blank values (n = 12).

Results and discussion

Procedure optimization

In the following, different steps in our method development procedure are presented and discussed. The flow chart in Fig 1 offers a schematic guide through our optimization procedure.

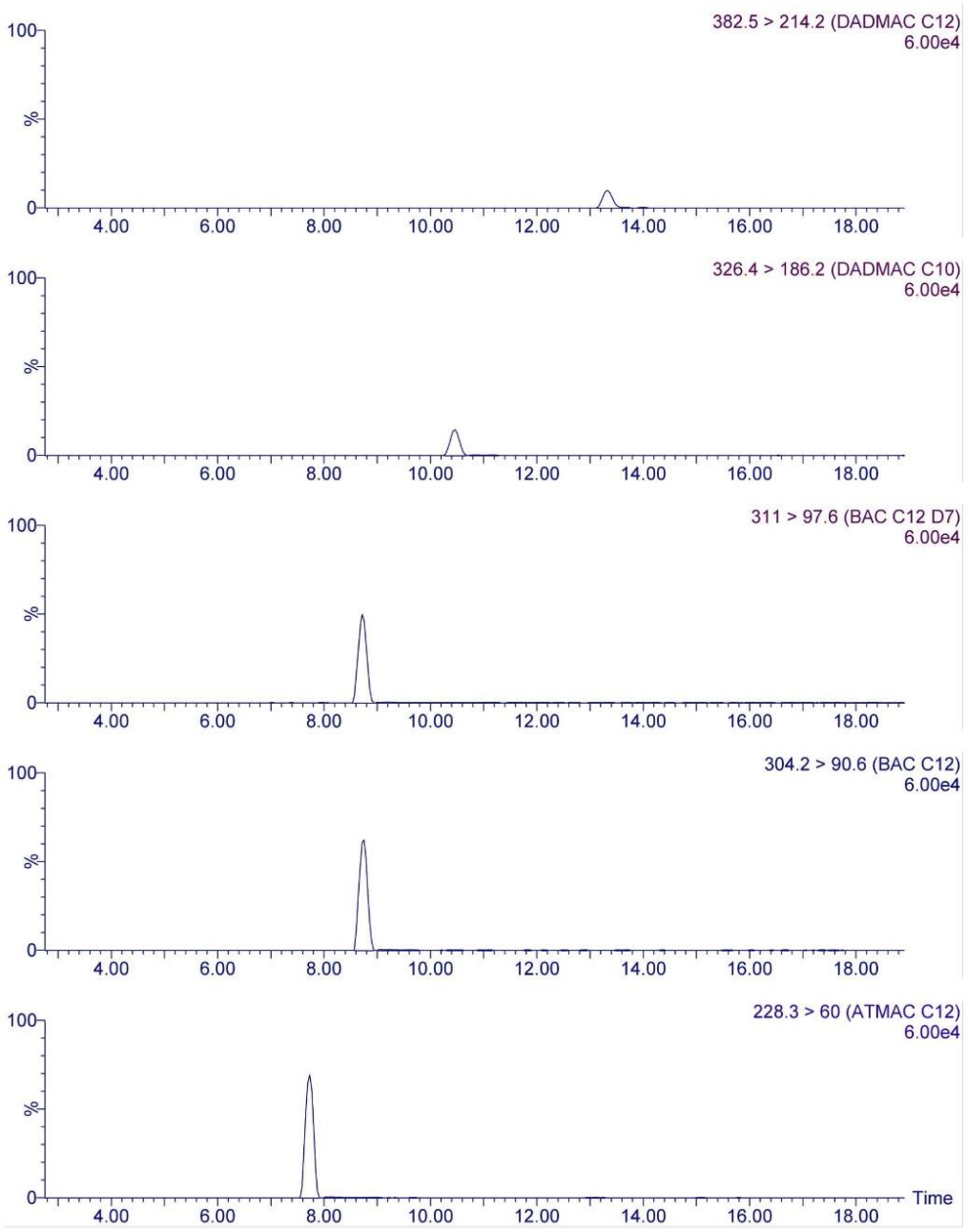

Fig 2. Chromatogram of DADMAC-C10, BAC-C12-D7, BAC-C12 and ATMAC-C12 in concentrations of 200 μg L^{-1} in AcN.

https://doi.org/10.1371/journal.pone.0237020.g002

The initial method development was performed for soil samples, and was subsequently also applied to sewage sludge samples that required SPE cleanup.

Sample extraction. Acidified MeOH [15, 16, 19, 21] and AcN [22, 23] are the most commonly used solvents for the QAAC extraction of sludges, water and sediments. In our

Table 3. Extraction efficiency of spiked soil samples expressed as recovery, as well as precision, Limit of Detection (LOD), Limit of Quantification (LOQ) and blank value of ultrasonic extraction with AcN (99.9%) and HCl (0.1%) using 0.5 g of soil (n = 3).

	PSE A	PSE B	MWE	Ultrasonic extraction				
	Recovery			Recovery	Precision (SD)	LOD	LOQ	Blank values
	[%]			[%]	[%]	[μg kg^{-1}]		
ATMAC-C12	< LOD	24	4	47	2.5	2.2	2.5	< LOD
ATMAC-C16	< LOD	< LOD	< LOD	51	1.3	3.9	6.0	< LOD
DADMA-C12	18	< LOD	< LOD	55	3.0	3.5	4.3	< LOD
DADMAC-C10 D6	< LOD	< LOD	< LOD	33	1.7	4.5	6.8	< LOD
BAC-C12 D7	< LOD	3	< LOD	43	1.8	2.9	3.3	< LOD
BAC-C16	< LOD	14	51	57	2.4	3.2	4.6	< LOD

https://doi.org/10.1371/journal.pone.0237020.t004

extraction trials we found that AcN with HCl worked best for soils (Table 3). Recovery rates of spiked QAACs extracted with USE and other extracting agents (EtOH, MQ-water, 2-Propanol, see Table 3) were all $\leq$ 10%. Mixtures of water and organic solvents had poor recoveries in our corresponding extraction tests, which is contradictory to Ferrer and Furlong [13], whose work showed that the extraction of BAC-C14 from sediment samples worked better with a mixture of organic solvent and water compared to pure organic solvents. PSE and Microwave extraction were tested as well, but QAAC recoveries were insufficient (Table 3).

The final optimal extraction method using USE and acidic (0.1% HCl) AcN provided recovery rates of the unlabeled QAACs between 47% (ATMAC-C12) and 57% (BAC-C16) with standard deviations $\leq$ 3% for replicate extractions (Table 4). Surprisingly, the extraction efficiencies for deuterated standards added were roughly 10% lower, possibly due to substitution of the H^2 with H^1 in the labeled BAC and DADMAC. The work by Davison et al. supports this hypothesis as they also observed deprotonation and substitution of deuterium for 5-hydroxyindole-4,6,7-d3-3-acetic-2,2-d2 acid within their labeled standard [24].

Importantly, for our extraction procedure we found it to be essential that freeze-dried samples were used. Recovery rates for air-dried samples were only half as high as for freeze-dried samples. Potential reasons for higher recoveries from freeze-dried samples than from air-dried samples are a better accessibility of sorbed QAACs in the fine powder of freeze-dried samples and the prevention of biodegradation that might occur during the air-drying procedure.

The extracts of sewage sludge consisted of two visible liquid phases, which also contained large concentrations of dissolved organic matter and suspended particles. Therefore, SPE was necessary for the sewage sludge extracts for sample cleanup. As it is clear from Fig 1, out of the four SPE cartridges tested by following its application guides and Östman et al. [25], only CN

Table 4. Extraction methods that were tested for the highest recovery of QAAC from soil samples.

Extraction method	Extracting agent [%]
Microwave extraction (MWE)	AcN [60] / H_2O [40]
Pressurized solvent extraction (PSE A)	AcN [60] / H_2O [40]
Pressurized solvent extraction (PSE B)	MeOH [50] / H_2O [50]
Ultrasonic extraction (1)	MeOH [99.9] / HCl [0.1]
Ultrasonic extraction (2)	MeOH [59.45] / H_2O [39.45] / HCl [0.1]
Ultrasonic extraction (3)	AcN[59.45] / H_2O [39.45] / HCl [0.1]
Ultrasonic extraction (4)	2-Propanol [59.45] / H_2O [39.45] / HCl [0.1]
Ultrasonic extraction (5)	EtOH [59.45] / H_2O [39.45] / HCl [0.1]
Ultrasonic extraction (6)	AcN [99.9] / HCl [0.1]

https://doi.org/10.1371/journal.pone.0237020.t005

columns eluted with 1.5 mol L^{-1} HCl acidified MeOH showed satisfying recovery rates around 50% (further detail in S1 Fig). DADMACs with an alkyl chain longer than 12 C atoms had very low recovery rates around 20% (S1 Fig), compared to reference samples that were not subject to SPE, highlighting their stronger tendency to interact with the cartridge surface due to increasing hydrophobicity with increasing chain length [3]. Also, chlormequat is almost fully eliminated in this step, possibly because of the strong polarity of its chlorogroup. Another reason could be the specific interaction of the free electron pair of the CN group with the electro negative chlorgroup, since the CN cartridge mechanism is, according to providers catalogue, based on weakly hydrophobic interactions and specific interaction.

Chromatography. First chromatographic experiments were performed with three eluents (MeOH, water, acetic acid and ammonium acetate in iso-propanol) and a Zorbax RX-C8 analytical column (2.1 mm i.d. × 150 mm length, 5 μm, Agilent) as described by Ruan et al. [10]. We reduced the number of eluents to two and also used the aqueous phase rather than the organic phase to buffer the pH in order to prevent precipitation of salts. The organic and the aqueous phase were premixed (90% / 10%, v / v) with each other for the same reasons and also to reduce the risk of bubbles in the system.

We then tested three different HPLC columns, XSelect, Zorbax RX-C8 and Nucleodur π^2. The best results were obtained with the XSelect column, with optimal separation and sharp peaks (Fig 2; S2 Fig). The final gradient program, which ends with a five minute re-equilibration step, is shown in Table 2a.

The pH values of the SPE extracts were very acid (below 1), hence NaOH was used to adjust the values to around pH 7. However, recovery rates were lower with adjusted pH values. We assume that the formation of salt crystals resulted from the reaction of dissolved Na^+ and Cl^- (from the QAAC counterion). Coprecipitation phenomena of the QAACs such as the adsorption to or the occlusion in the NaCl crystals could have led to the reduced QAAC recovery.

We concluded, that in the light of the acid tolerance of the analytical column of choice, the lengthy procedure of pH adjustment was not necessary. Regarding to retention times, the peak analysis confirmed that the adjusted and non-adjusted samples were identical.

The negative effect of SPE on recoveries of DADMACs with longer alkylchains (> 12 Cs) did also occur in chromatography. Retention times increased due to the strong hydrophobic interaction of the alkylchains with the analytical column and peak broadening occurred. Therefore, we excluded DADMAC-C18 from the method. In contrast to the effect of the SPE, the small molecule chlormequat showed only little interaction with the XSelect column and was first to elute after 1.6 minutes (Table 2B).

Method validation

Limit of detection and quantification. Table 5 lists the LODs and LOQs for all analytes. Additionally, LOQs determined over an extended period of time during routine operation are shown in Fig 3. From Fig 3 it is apparent, that the robustness of the method depends on the analytes. The best results were obtained for ATMACs in general and, in particular, for ATMAC-C10. In contrast, DADMACs were analytically challenging, especially those with alkyl chains longer than C12. LODs for the most common QAACs BAC-C12 and DADMAC-C10 were 0.4 μg kg^{-1} and 1.3 μg kg^{-1}, respectively. In general, our achieved LODs were lower compared to methods for sewage sludge and sediment rivers as described by Merino et al., Bergé et al., Ruan et al. and Martinez-Carballo et al. [10, 11, 15, 26]. An overview of the studies and their LOQs compared to our work is given in Table 6.

Matrix effect. The internal standard that was added immediately before the HPLC-MS/MS analysis should compensate possible matrix-effects on ionization and quantification of

Table 5. Limit of Detection (LOD) and Limit of Quantification (LOQ) and coefficient of determination (R^2) of calibration from 2.5 µg L^{-1} to 300 µg L^{-1}.

	LOD [µg kg^{-1}]	LOQ [µg kg^{-1}]	R^2
ATMAC-C8	0.2	0.3	0.996
ATMAC-C10	0.1	0.2	0.997
ATMAC-C12	0.3	0.5	0.998
ATMAC-C14	0.1	0.3	0.999
ATMAC-C16	0.5	1.1	0.999
BAC-C8	0.2	0.3	0.996
BAC-C10	0.3	0.5	0.998
BAC-C12	0.4	0.8	0.999
BAC-C14	0.2	0.4	0.999
BAC-C16	0.4	0.9	0.999
BAC-C18	0.3	0.5	0.997
DADMAC-C8	0.3	0.5	0.998
DADMAC-C10	1.3	2.1	0.997
DADMAC-C12	0.7	1.4	0.999
DADMAC-C14	0.9	1.3	0.996
DADMAC-C16	0.8	1.7	0.997
Chlormequat	0.1	0.1	0.992
Benzethonium	0.2	0.4	0.999

https://doi.org/10.1371/journal.pone.0237020.t006

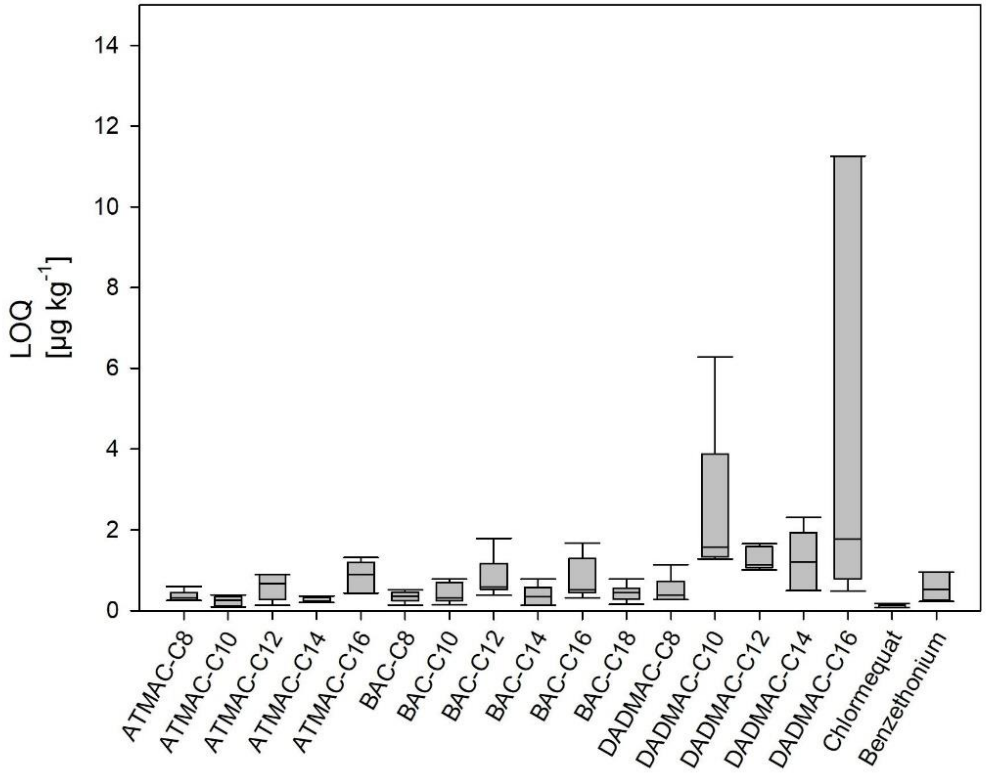

Fig 3. Limits of quantification of the HPLC-MS/MS method during two years of routine analyzes.

https://doi.org/10.1371/journal.pone.0237020.g003

Table 6. Limit of Quantification (LOQ) of our work compared to works with similar Matrices and their number of the analyzed homologues. Extraction methods were Acid-Induced Cloud-Point (ACPE), Solid Phase Extraction (SPE) an Ultrasonic Extracition (USE).

	Matrix	Extraction	Detection	LOQ [μg kg^{-1}]	# homologues
Merino et al. [11]	Raw sewage	ACPE	LC—MS	40–70 *	3 ATMACS; 4 BACs; 3 DADMACs
Bergé et al. [26]	Biological sludge	SPE / QuEChERS	LC—MS/MS	20–40	2 BACs
Ruan et al. [10]	Sewage sludge	USE	LC—MS/MS	1.3–4.2	7 ATMAC; 6 BACs; 7 DADMACs
Martinez-Carballo et al. [15]	Sediment and Sludge	Soxhlet	LC—MS/MS	0.6–3	3 ATMAC; 4 BACs; 5 DADMACs
This work	Soil and sewage Sludge	USE	LC—MS/MS	0.1–2.1	5 ATMAC; 6 BACs; 5 DADMACs

* LOQ were not shown by this work, so their LOD are displayed here.

https://doi.org/10.1371/journal.pone.0237020.t007

target analytes. In order to determine the magnitude of matrix-effects on the ionization and quantification of QAACs, we recorded the signal of a concentration of 100 μg L^{-1} of BAC-C12 in pure solvents that were used for the extractions (MeOH and AcN), in MQ-water and in spiked soil extracts. Fig 4 shows similar signal intensities in different matrices and slightly elevated signals in AcN soil extracts. Fig 4 also shows that the instrumental drift is higher than the matrix effect, which has to be corrected by the internal standard.

Precision and reproducibility. Retention times were well reproducible and only showed a maximum overall inter-day shift of 0.2 min, regardless of analyte. This shift did not affect the results, since the MS/MS confirmed analyte identification. However, the linearity of the calibration is a key factor in detection precision. In the concentration range of 2.5 μg L^{-1} to 300 μg L^{-1} for each QAAC analyte, the regression coefficient R^2 was never below 0.996, with a median value of 0.998, see Table 5. The precision of the extraction process expressed as standard deviation of the QAAC concentrations recovered after three repeated extractions of spiked soil

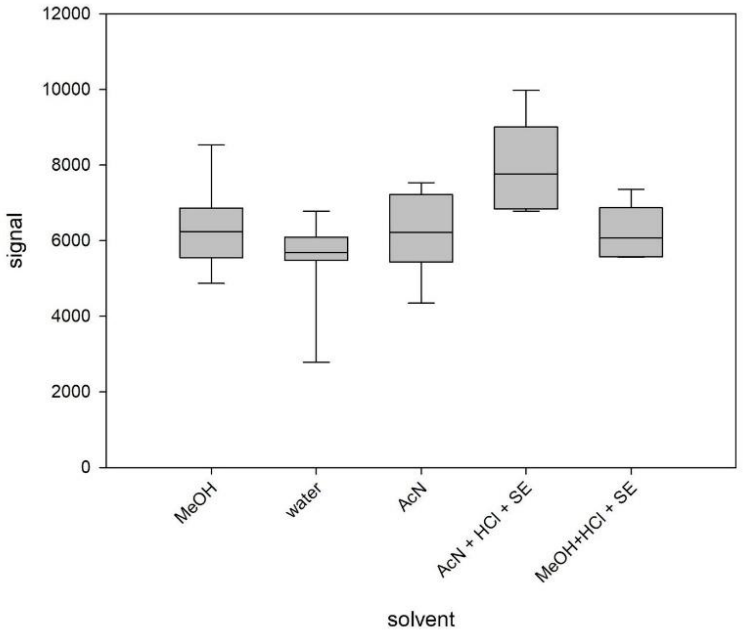

Fig 4. Signal intensity of BAC-C12 (100 μg/L) depending on solvent matrix (methanol; deionized water; acetonitrile [n = 8]; acetonitrile with HCl and Soil Extract (SE); methanol with HCl and SE [n = 6]).

https://doi.org/10.1371/journal.pone.0237020.g004

Table 7. Soil characteristics of German Luvisol and Mexican Vertisol.

	Mexican Vertisol	German Luvisol
C_{total} [%]	2.2	1.4
N_{total} [%]	0.2	0.2
pH	7.1	6.8
Clay content [%]	44.6	28.9
Carbonate [%]	0.3	0.5

https://doi.org/10.1371/journal.pone.0237020.t008

samples lies between 1.3% for ATMAC-C12 and 3.0% for DADMAC-C12 (Table 3). Stable retention times, R^2 values for the linearity $\geq$ 0.996 and the standard deviation of analyte recoveries from spiked replicate samples are confirming a good reproducibility and precision of the method. Nevertheless, we recommend using the qualifier ions (Table 2B) to confirm the identity of the analytes for less well characterized samples.

Environmental samples. After establishing the analytical method, we analyzed the QAAC concentration in different samples: Sewage sludge samples from Germany and soil samples from a Mexican Vertisol (Tlahuelilpan, Mexico) and a German luvisol (Hungen. Germany), a short overview of the soil characteristics is shown in Table 7. Sewage sludge A and B were from the same wastewater treatment plant in Giessen (Hesse) but sludge A was freeze-dried, whereas sludge B was air-dried. Sewage sludge C was from Bad Nauheim (Hesse) and freeze-dried as well. All wastewater treatment plants use a mechanical primary treatment and a biological secondary treatment. The sewage sludges were all dewatered sludges. As shown in Table 7 we found QAACs in all of those samples. Each of the analyzed QAAC was found in sewage sludge B and C. Soils and sewage sludge samples contained mainly BACs and DADMACs and less ATMACs, with DADMAC-C10 and BAC-C12 being detected in greatest concentrations of 24000 μg kg^{-1} and 38600 among all QAACs. This corresponded to the large

Table 8. QAAC concentrations of agricultural soil from Mexico and Germany and sewage sludges from Germany analyzed with the developed method.

	Sewage sludge A	Sewage sludge B	Sewage sludge C	Mexican Vertisol irrigated with untreated wastewater	German Luvisol ten days after application of sewage sludge (0–30 cm)
	[μg/kg]	[μg/kg]	[μg/kg]	[μg/kg]	[μg/kg]
ATMAC-C8	17.0	3.0	10.4	< LOD	1.1
ATMAC-C10	322	27.0	194	< LOD	1.3
ATMAC-C12	388	34.0	795	3.5	0.8
ATMAC-C14	58.0	7.0	167	< LOD	1.4
ATMAC-C16	439	26.0	2470	7.0	1.4
BAC-C8	14.0	2.0	11.2	3.8	1.8
BAC-C10	123	18.0	328	3.0	1.6
BAC-C12	4780	562	38600	80.6	6.9
BAC-C14	933	55.0	19940	22.4	2.5
BAC-C16	68.0	10.0	3203	3.2	1.7
BAC-C18	38.0	9.0	919	2.1	1.8
DADMAC-C8	2260	247	3710	5.2	< LOD
DADMAC-C10	2870	222	24000	3.7	4.8
DADMAC-C12	44.0	20.0	16.6	< LOD	1.6
DADMAC-C14	< LOD	< LOD	22.1	< LOD	2.4
DADMAC-C16	< LOD	2.0	162	< LOD	< LOQ; > LOD
Chlormequat	< LOD	< LOD	< LOD	2.3	< LOD
Benzethonium	18.0	9.0	62.7	3.0	1.1

https://doi.org/10.1371/journal.pone.0237020.t009

amounts of these compounds used in numerous applications. BAC-C12 is one of the main components of industrial BAC mixtures and DADMAC-C10 is the main component of DADMAC mixtures ($\geq$ 90%). Both mixtures are used for the cleaning of machines and rooms in all production sectors [27].

Conclusion

QAACs are challenging analytes due to their surfactant nature requiring a specific analytical method for reliable extraction and quantification. In this work we present a fast and robust method for the simultaneous extraction of ATMACs, BACs, DADMACs, benzethonium and chlormequat from soils and sewage sludge and the subsequent quantification of their concentrations using HPLC-MS/MS. As far as we know, there is no method for the analysis of both environmental matrices. The reduction to only two eluents in HPLC makes the proposed method easier to handle and less error-prone compared to previously reported method(s). The achieved LOQ and LOD are equal to or lower than those reported in the literature for QAACs in comparable matrices. The good recovery rates and excellent precision of the proposed method enable and justify its use for enlarging our data base on the distribution and concentration levels of QAACs in soils and sewage sludge. First results of QAAC concentrations in soil and sewage sludge samples emphasize the importance of these analyzes.

Supporting information

S1 Fig. SPE Recovery of four different Cartridges, Chromabond HLB, Oasis HLB, Chromabond C18 EC and Chromabin CN. The challenging homologues of DADMAC (C14, C16) had the lowest recovery, Chlormequat is fully eliminated.
(TIF)

S2 Fig. Chromatogram of 20 analytes in concentrations of 200 μg L^{-1} in Acn.
(TIF)

S3 Fig. TIC-chromatogram of 20 analytes in concentrations of 200 μg L^{-1} in Acn.
(TIF)

S4 Fig. Extracts during the SPE cleanup step.
(TIF)

S1 File.
(XLSX)

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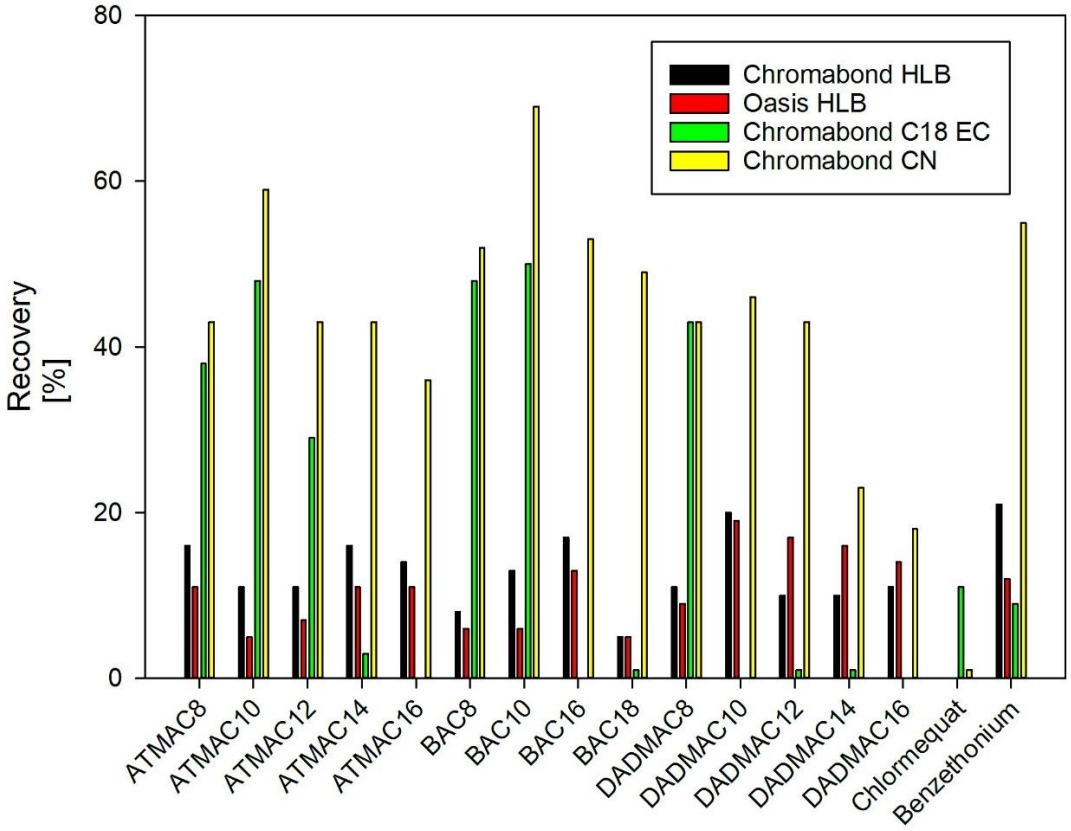
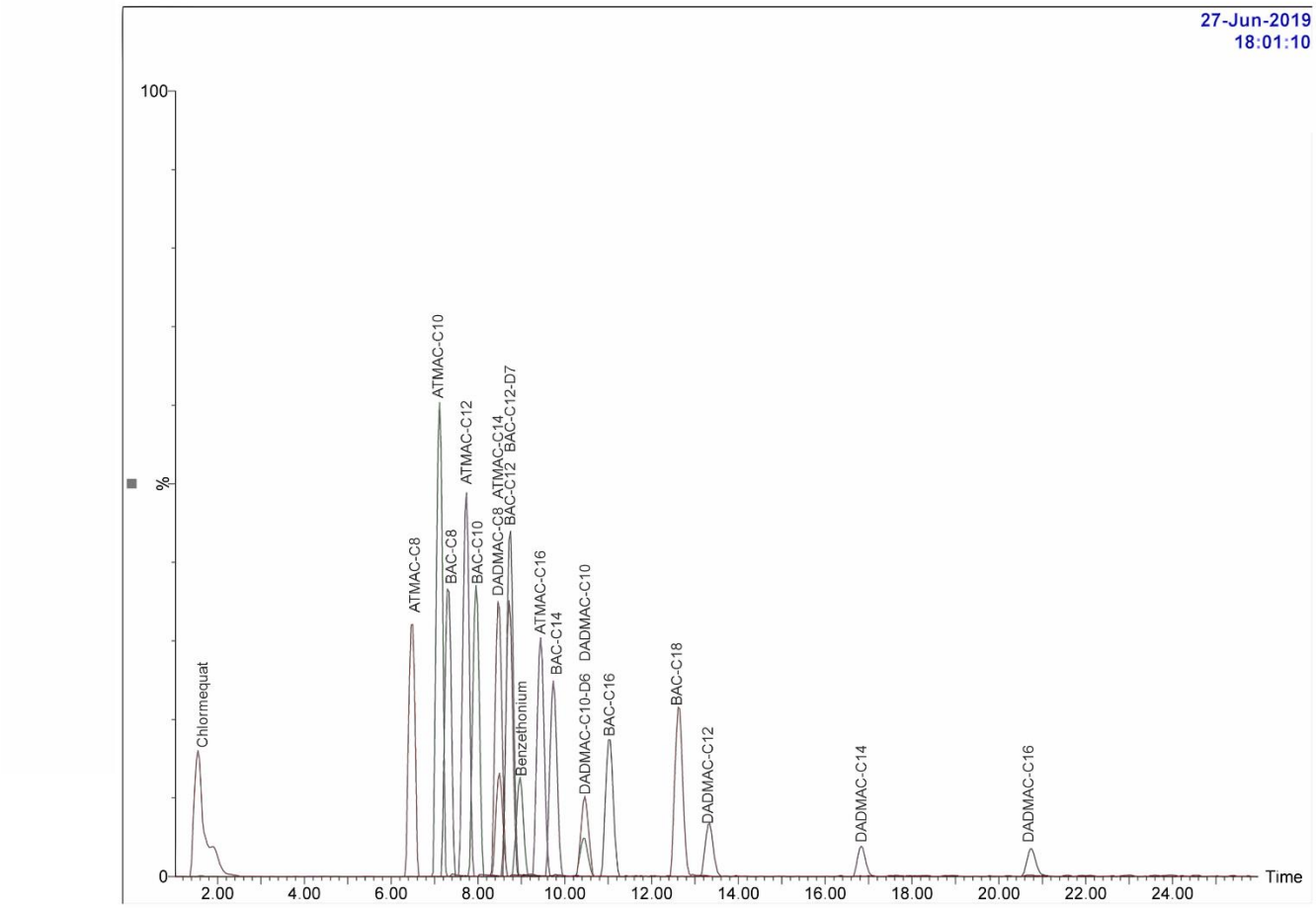
Supporting Information

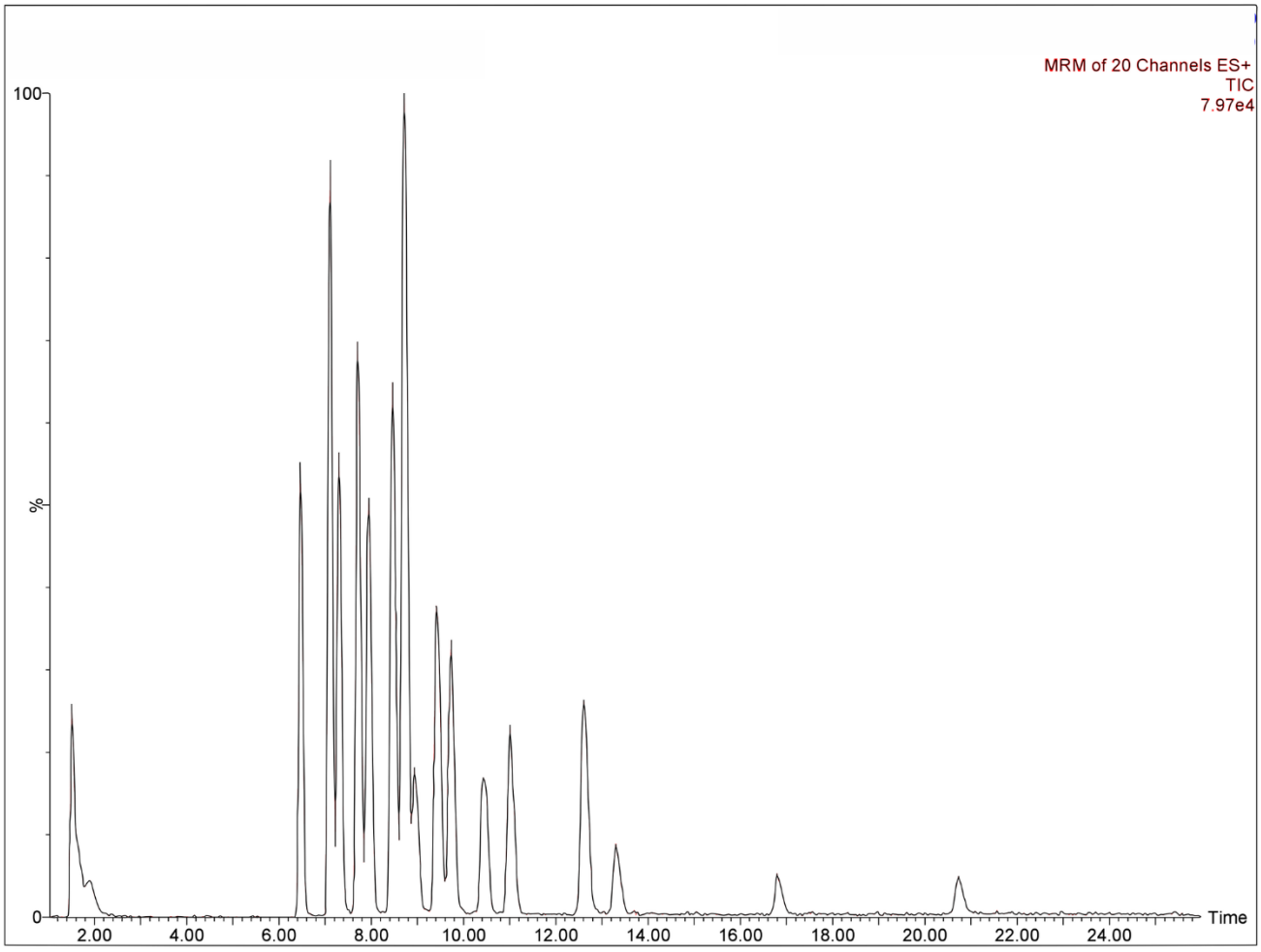


S1 Fig. SPE Recovery of four different Cartridges, Chromabond HLB, Oasis HLB, Chromabond C18 EC and Chromabond CN.

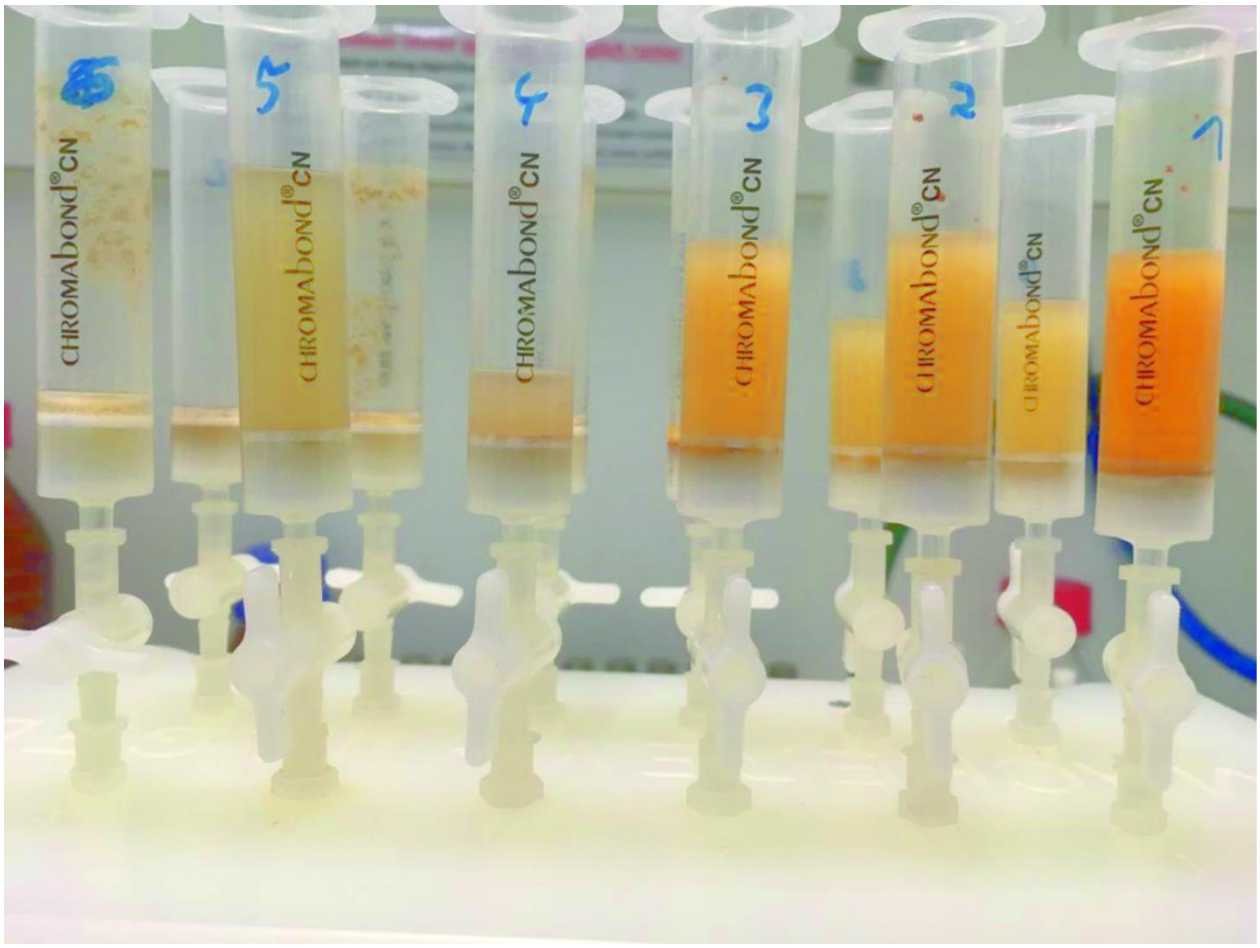
The challenging homologues of DADMAC (C14, C16) had the lowest recovery, Chlormequat is fully eliminated.



S2 Fig. Chromatogram of 20 analytes in concentrations of 200 μg L^{-1} in AcN.



S3 Fig. TIC-chromatogram of 20 analytes in concentrations of 200 µg L^{-1} in AcN.



S4 Fig. Extracts during the SPE cleanup step.

Fig 3 Data

S1 File: This (XLSX) file is published as:
https://doi.org/10.1371/journal.pone.0237020.s005

It consists of four table sheets which are shown below.

Fig 3 Data

	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6
ATMAC-C8	0.3980	0.5995	0.2568	0.2568	0.3398	0.2865
ATMAC-C10	0.0861	0.3887	0.2558	0.2558	0.1271	0.3357
ATMAC-C12	0.1281	0.3203	0.8907	0.8907	0.7139	0.6339
ATMAC-C14	0.2249	0.3647	0.2027	0.2027	0.2463	0.3146
ATMAC-C16	1.0368	0.7531	0.4330	0.4330	1.1576	1.3141
BAC-C8	0.1271	0.5147	0.3615	0.3615	0.4402	0.2833
BAC-C10	0.1419	0.3616	0.2738	0.2738	0.6689	0.7768
BAC-C12	0.3840	0.5711	0.5814	0.5814	0.9620	1.7932
BAC-C14	0.5018	0.7828	0.1322	0.1322	0.3500	0.3297
BAC-C16	0.3223	0.5311	0.4860	0.4860	1.6760	1.1736
BAC-C18	0.3261	0.4882	0.4480	0.4480	0.1551	0.7804
DADMAC-C8	0.3620	0.4148	0.2738	0.2738	1.1359	0.5933
DADMAC-C10	6.2769	3.0826	1.3461	1.3461	1.7992	1.2823
DADMAC-C12	1.5588	1.0090	1.0858	1.0858	1.6512	1.1817
DADMAC-C14	2.3082	1.1038	0.4974	0.4974	1.8003	1.3209
DADMAC-C16	1.2605	0.8813	11.2597	11.2597	2.2949	0.4859
Chlormequat	0.1234	0.0819	0.1782	0.1782	0.0738	0.1445
Benzethonium	0.2655	2.0000	0.9517	0.9517	0.2286	0.6516

Fig 4 Data

MeOH	Water	AcN	AcN + HCl + SE	MeOH + HCl + SE
6868	2780	6153	6867	7358
5548	5957	6787	6789	5564
6091	6785	6184	7445	5583
8536	6146	7368	8084	6709
6402	5481	6260	9980	6132
6868	5791	7534	8688	6016
5587	5579	5204		
4873	5497	4349		

Table 3 Data

	WFR 1	WFR 2	WFR 3	SD
ATMAC12	45%	50%	45%	2.5%
ATMAC16	49%	52%	50%	1.3%
DADMAC12	51%	58%	55%	3.0%
DADMAC10_D6	34%	35%	31%	1.7%
BAC12_D7	42%	45%	41%	1.8%
BAC16	54%	60%	57%	2.4%

Fig SI 1 Data

	Chromabond_HLB	Oasis_HLB	Chromabond_C18_EC	Chromabond_CN
ATMAC-C8	16%	11%	38%	43%
ATMAC-C10	11%	5%	48%	59%
ATMAC-C12	11%	7%	29%	43%
ATMAC-C14	16%	11%	3%	43%
ATMAC-C16	14%	11%	0%	36%
BAC-C8	8%	6%	48%	52%
BAC-C10	13%	6%	50%	69%
BAC-C16	17%	13%	0%	53%
BAC-C18	5%	5%	1%	49%
DADMAC-C8	11%	9%	43%	43%
DADMAC-C10	20%	19%	0%	46%
DADMAC-C12	10%	17%	1%	43%
DADMAC-C14	10%	16%	1%	23%
DADMAC-C16	11%	14%	0%	18%
Chlormequat	-1%	0%	11%	1%
Benzethonium	21%	12%	9%	55%

3 Quaternary alkylammonium disinfectant concentrations in soils rise exponentially after long-term wastewater irrigation

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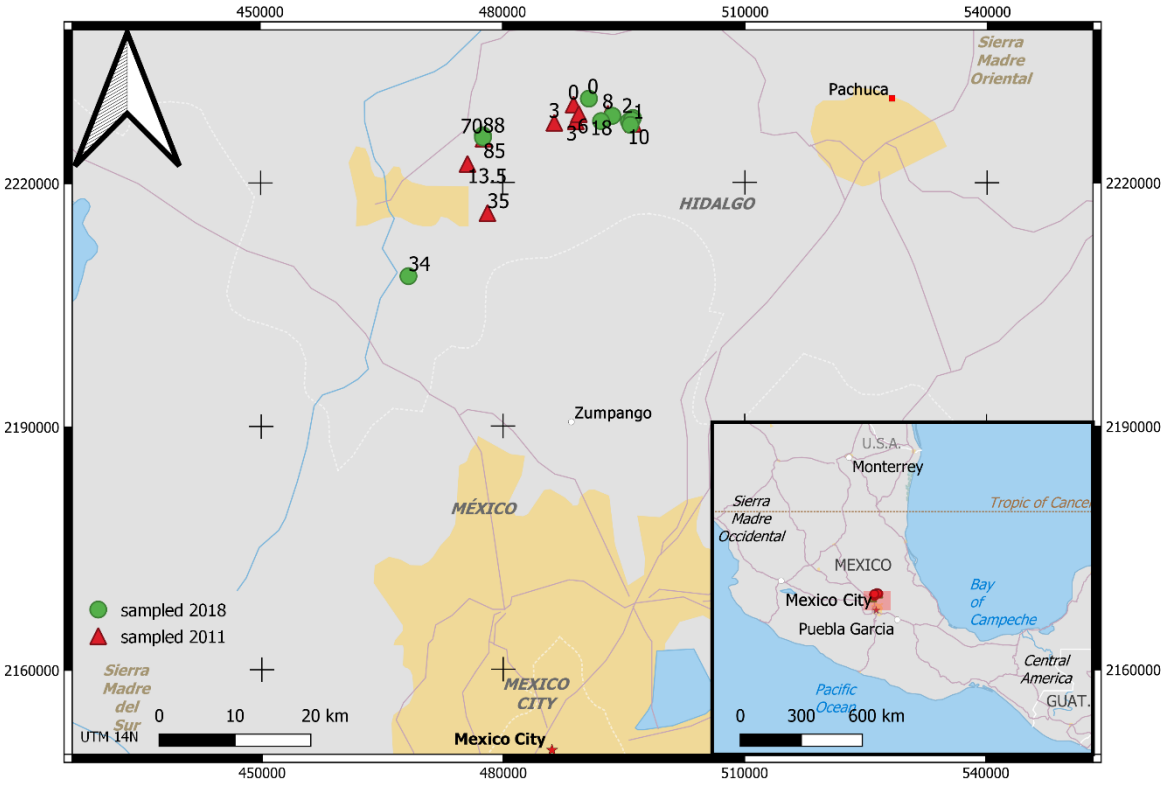

Figure 3.1 Sample points of 2011 (red) and 2018 (green) in Mezquital Valley in the north of Mexico City. Numbers indicate how many years the soils have been irrigated with wastewater

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ENVIRONMENTAL RESEARCH
LETTERS

LETTER

Quaternary alkylammonium disinfectant concentrations in soils rise exponentially after long-term wastewater irrigation

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Abstract

Quaternary alkylammonium compounds (QAACs) are used as disinfectants and surfactants worldwide, with their usage currently increasing as a result of the COVID-19 pandemic. QAACs are released into the environment with manure, sewage sludge and wastewater. The fate of QAACs in soils is poorly understood, although QAACs are inflicted in the selection of antibiotic-resistant bacteria. We studied the temporal accumulation of QAACs in soils of the Mezquital Valley that have been irrigated with Mexico City wastewater from 0 to 88 years. Concentrations of 16 QAACs, including alkyltrimethylammonium compounds (ATMACs), dialkyldimethylammonium compounds (DADMACs) and benzylalkyldimethylethylammonium compounds (BACs), were determined using HPLC-MS/MS after ultrasonic extraction. The most abundant QAAC-homologues in the soils were BACs > ATMACs > DADMACs. The concentrations of QAACs increased linearly and slowly during the first years of irrigation ($\sum$QAAC: 2–23 $\mu g\,kg^{-1}$), but after 40 years of wastewater irrigation we observed an exponential increase in QAAC concentrations (up to 155 $\mu g\,kg^{-1}$). QAACs accumulate in soils of the Mezquital Valley during long-term wastewater irrigation. In contrast to pharmaceuticals, no apparent 'steady state' concentration is reached after decades of wastewater irrigation.

1. Introduction

Quaternary alkylammonium compounds (QAACs), such as alkyltrimethylammonium compounds (ATMACs), dialkyldimethylammonium compounds (DADMACs) and benzylalkyldimethylethylammonium compounds (BACs), are used worldwide as surfactants in household chemicals, the chemical and food industry and the agricultural sector (Mulder *et al* 2018). They consist of a permanently positively charged nitrogen atom, which is the hydrophilic part of the molecule, and four covalently bound organic rests, such as methyl groups, benzyl group or alkyl-chains of variable length that form the hydrophobic part. Thus, they are amphiphilic compounds. The critical micelle concentration best describes their partitioning in a soil–aqueous system (table 1). Ismail *et al* (2010) described that CMC represents the hydrophobic as well as the ionic properties of QAAC.

The large scale production and use of QAACs started in the first half of the 20th century. QAACs were first described in the literature in 1928 as '*saure seifen*' (acidic soaps) by Hartmann and Kägi (1928). Domagk (1935) described the use of 'Zephirol' in 1935, which is BAC-C12, as a new class of disinfectants. In the following years BAC-C12 was widely used in the medical sector (Frankl 1941). Botwright (1946) advocated the use of these tasteless, for humans non-toxic compounds as cost-effective germicides for the food industry. By the end of the 1940, QAACs were used in the whole food industry (quick-freezing, canning, baking, butcher-shops), at food-markets, in eating and drinking establishments, medicine, veterinary medicine and more (However, as early as 1961, first signs of resistance development of microorganism were observed (Lee and Fialkow 1961)).

Nevertheless, even today QAACs belong to the class of so-called high production volume chemicals

Table 1. Compound properties of QAACs.

	CAS #	mp (°C)[a]	CMC (mM)	Mol. mass
ATMAC-C8	2083-68-3	198-200	190 ± 10[c]	252
ATMAC-C10	10108-87-9	N/a	60 ± 1[c]	236
ATMAC-C12	112-00-5	37	15 ± 1[c]	264
ATMAC-C14	4574-04-3	N/a	4.1 ± 0.1[c]	292
ATMAC-C16	112-02-7	77	1.6 ± 0.1[c]	320
BAC-C8	959-55-7	N/a	188 ± 16[b]	284
BAC-C10	965-32-2	N/a	34 ± 1.8[b]	312
BAC-C12	1390-07-1	42	8.3 ± 0.2[b]	340
BAC-C14	139-08-2	63	1.8 ± 0.05[b]	368
BAC-C16	122-18-9	59	0.4 ± 0.015[b]	396
BAC-C18	122-19-0	57	0.1 ± 0.01[b]	424
DADMAC-C8	3026-69-5	60–68	N/a	350
DADMAC-C10	7173-51-5	88	N/a	362
DADMAC-C12	3401-74-9	175	3.5×10^{-2} [d]	418
DADMAC-C14	68105-02-2	N/a	1.6×10^{-3} [d]	519
DADMAC-C16	70755-47-4	159–162	7.8×10^{-5} [d]	575

[a] Mulder *et al* (2018).
[b] Mulder *et al* (2020).
[c] Cepeda *et al* (2013).
[d] Diress *et al* (2007).

(HPVCs) (OECD 2004), because of their antimicrobial and surfactant properties. During the COVID-19 pandemic hygiene is one of the key measures for infection control and prevention. Consequently, the use of disinfectants has risen substantially, even though exact data on disinfectant consumption are difficult to obtain. Hora *et al* (2020) found that in the U.S the consumption of disinfection wipes of one manufacturer in May 2020 was 146% that of the year before. Almost half of all disinfectants (600 of 1285) listed by the German Association of Applied Hygiene (Association for Applied Hygene 2020) contain QAACs as active ingredients. It should be expected that the amount of QAACs released into the environment is increasing as a consequence of the COVID-19 pandemic.

After their use, QAAC are introduced into the environment with wastewater, sewage sludge and manure (Mulder *et al* 2018). Data on the environmental concentrations and fate of QAACs are scarce and predominantly available for wastewater and sewage sludge (Merino *et al* 2003, Clara *et al* 2007, Ruan *et al* 2014), followed by data on river water and river sediments (Ferrer and Furlong 2002, Lara-Martín *et al* 2010). Information about their concentrations in soils is rarely available (Gerike *et al* 1994).

Studies have shown that microorganism can degrade QAACs under aerobic conditions with half-lives in the range of hours (Games *et al* 1982, Zhang *et al* 2015) in aerated laboratory systems. Regarding their effects in the environment, the main concern is that their presence promotes the selection and spread of antibiotic-resistant bacteria and resistance genes (Gaze *et al* 2005, Tandukar *et al* 2013, Mulder *et al* 2018). The ecotoxicologial effects of QAACs in soils and sediments are strongly reduced by their sorption. Previous work has shown, that QAACS can sorb in interlayer spaces of smectite soil clay minerals (Zanini *et al* 2013), which reduces their toxicological effects (and hence their bio-accessibility) at least under controlled laboratory conditions (Heyde *et al* 2020b). By reducing the bio-accessible concentration of molecules, sorption is also known to slow down the dissipation and degradation of organic compounds, thus increasing their persistence in strongly bound-forms (Rosendahl *et al* 2012). Sorption occurs via cation exchange (due to the positively charged N atom) and via hydrophobic interactions of the alkyl chains with organic matter (Ismail *et al* 2010, Ren *et al* 2011, Mulder *et al* 2018).

In semiarid and arid areas of the world, municipal wastewater is reused for irrigation of agricultural land (Asano 1998). The reuse of wastewater for irrigation is regarded as key for relieving regional water shortages, increasing food production and improving income and livelihoods of people (e.g. Bahri *et al* 2009, Drechsel *et al* 2015). Wastewater irrigation also has positive effects on the soil concentration of nutrients, the content of soil organic matter and the biomass as well as activity of soil microorganisms (Siebe 1998, Friedel *et al* 2000, Dalkmann *et al* 2012). Disadvantages of wastewater irrigation are the input of pollutants and the spread of resistance genes due to the input of pathogenic bacteria (Igbinosa *et al* 2011, Munir and Xagoraraki 2011, Dalkmann *et al* 2012). One of the largest agriculturally used areas under wastewater irrigation in the world (Siebe and Cifuentes 1995), the Mezquital Valley, covers an area of 900 km^2 and is located 80 km north of Mexico City (figure 1). Wastewater irrigation in this area started in 1912 and the irrigated

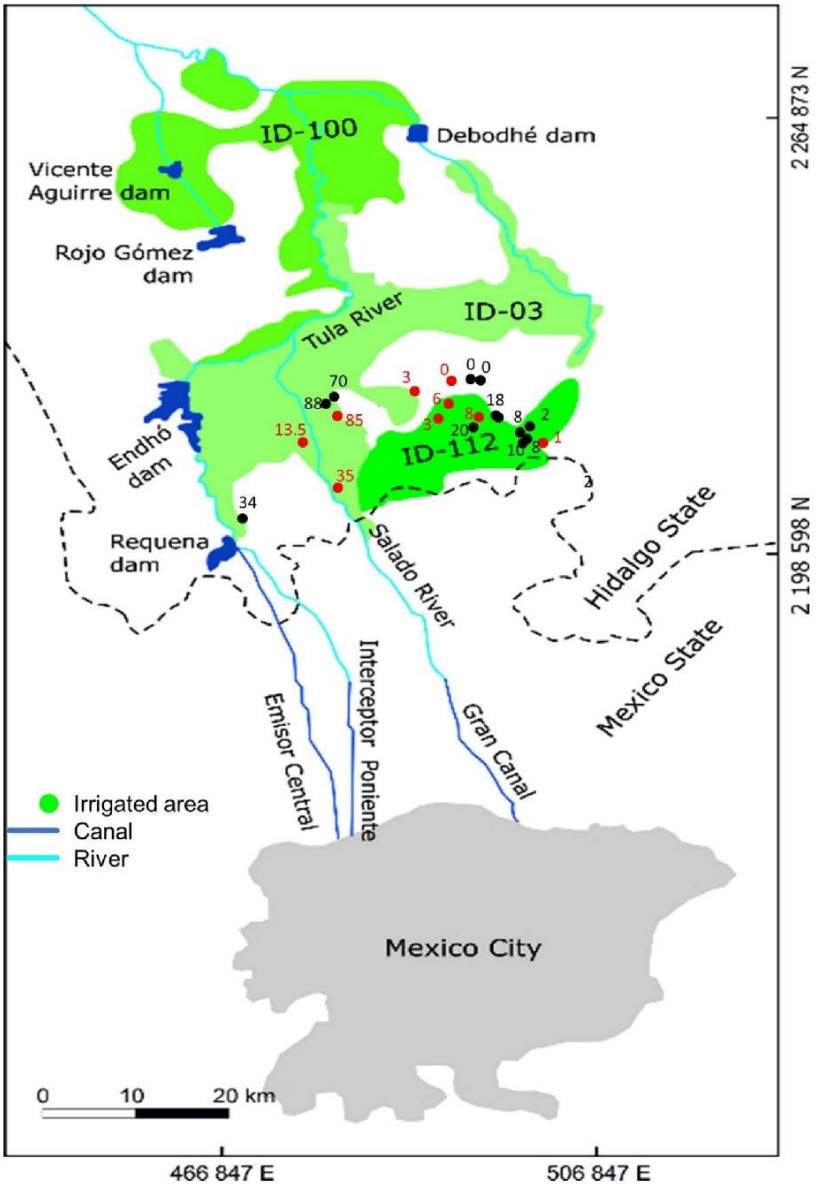

Figure 1. Sample points of 2011 (red) and 2018 (black) in Mezquital Valley in the north of Mexico City. Numbers indicate how many years the soils have been irrigated with wastewater and ID describes the irrigation areas (adapted from Siebe *et al* 2016). Reproduced with permission from Siebe *et al* (2016). Adapted from Safe Use of Wastewater in Agriculture: Good Practice Examples (Case 7, figure 1), available at: https://collections.unu.edu/view/UNU:5764 © United Nations University Institute for Integrated Management of Material Fluxes and of Resources (UNU-FLORES). Reproduced with the permission of UNU-FLORES.

area has been growing over the past 100 years due to the expansion of Mexico City and is still expanding (Broszat *et al* 2014). Until 2017 most of the irrigated 80 000 ha land received untreated wastewater for irrigation; since 2018 approximately 60% of the wastewater is treated prior to irrigation (pers. Comm. Ing. Cruz Atienza, director of the wastewater treatment plant).The wastewater is pumped and diverted frequently until it reaches the fields (Dalkmann *et al* 2012). While in the pipe system, the water is anaerobic until it reaches the fields where it is aerated. Main characteristics and metal concentrations of the water are provided in table SI1 (available online at stacks.iop.org/ERL/16/064002/mmedia). The concentrations of trace metals in the wastewater canals are high but below the Mexican wastewater

guidelines. The concentrations of Al, As, Pb and methylmercury in the Tula River, the river into which the wastewater is partially discharged, exceeded the surface water guidelines (Guédron *et al* 2014). Few trace metal concentrations (0.024 mg l^{-1} Cd, 0.510 mg l^{-1} Pb) in the irrigation canal were exceeding the surface water guideline concentrations (Ramirez-Fuentes *et al* 2002).

Together with the wastewater, all kinds of chemicals that are used in households are introduced into the fields and soils. Siebe Grabach (1994) and Siebe and Cifuentes (1995) documented the accumulation of heavy metals in soils of the Mezquital Valley with increasing time under irrigation by sampling a chronosequence of soils for which irrigation started in different years in the course of the expansion of the irrigated area. The concentrations of heavy metals in the soils increased linearly with increasing time of irrigation, because these metals are continuously introduced and sorbed to clay, iron oxides and organic matter. Later studies showed that the soils are also enriched with pharmaceuticals and endocrine disruptors over time of irrigation (Durán-Alvarez *et al* 2009, Gibson *et al* 2010). Dalkmann *et al* (2012) revealed that concentrations of pharmaceutical residues reach an apparent steady state over time, which can be theoretically explained as an equilibrium reached between constant input and first order degradation (dissipation) of the organic compounds. QAAC concentrations in wastewater are known to be quite high, often an order of magnitude higher than commonly observed pharmaceuticals. For example, treatment plant influents in the range of 2–6000 μg l^{-1} were reported for Austria (Clara *et al* 2007, Martínez-Carballo *et al* 2007). Effluent concentrations between 14 and 78 μg l^{-1} are reported for Northern Europe by Kaj *et al* (2014).

Therefore, as was observed in the Mezquital Valley soils for pharmaceuticals, a similar pattern enrichment and attainment of apparent steady-state could also be expected for QAACs, which are also biodegradable under aerobic conditions (Tandukar *et al* 2013). We therefore hypothesized that (a) soil concentrations of QAACs increase with the duration of untreated wastewater irrigation and (b) the soil concentrations of QAAC will reach a steady state with time, analogously to the documented enrichment of pharmaceuticals in the Mezquital Valley. For testing these hypothesis we sampled an irrigation chronosequence of soils in 2018 and quantified concentrations of BACs, ATMACs and DADMACS. Results were confirmed and compared by extracting samples that were already analyzed for concentrations of pharmaceuticals by Dalkmann *et al* (2012). Finally, the accumulation of QAACs was compared to the accumulation of heavy metals with increasing time of irrigation.

2. Materials and methods

2.1. Study area description

The Mezquital Valley is mostly located in the northeastern part of the Transmexican volcanic belt and extends into the Sierra Madre Oriental sedimentary system in its northern part. The valley bottom consists of extended piedmonts of relatively compacted and partly $CaCO_3$-cemented volcanoclastic sediments of Tertiary age which grade into fluvial and lacustrine Quaternary deposits in its lower parts. Climate is temperate (mean annual temperatures between 16 °C and 18 °C) semi-arid in the South with annual mean precipitations ranging from up to 700 mm in the southern parts to less than 435 mm in the North concentrated in the summer months. Dominant soils are rendzic Leptosols, haplic, calcaric and vertic Phaeozems and pellic Vertisols. Irrigated areas are mostly cultivated with lucerne—maize rotations (3 years of lucerne followed by 2 years of maize combined with rye grass or different kinds of vegetables). Non-irrigated areas are nowadays scarce and used to cultivate dominantly rain-fed maize or agave species (Siebe Grabach 1994).

2.2. Soil samples

The first set of samples was taken in 2011 for the Dalkmann *et al* (2012) study from 0 to 30 cm depth from nine different fields in the Mezquital Valley representing eight periods of irrigation with untreated wastewater (0, 1, 3, 6, 8, 13.5, 35, 85 years). These samples were taken as composite samples with a soil auger (~1 kg), collected in polyethylene bags and stored at −21 °C. The second set of samples was collected in 2018 as composite sample from 0 to 20 cm depth from ten fields representing nine irrigation durations (0, 2, 8, 10, 18, 20, 34, 70, 88 years). Sampling in 2018 was performed with a shovel (~300–500 g) and the soil samples were wrapped in alumina foil and stored at −21 °C in polyethylene bags.

For all analyzes, except for pH measurements, the samples of 2018 and 2011 were freeze-dried (Beta 1 8 LSCplus, CHRIST, Osterode am Harz, Germany) in the lab for 5 d, sieved < 2 mm (Retsch®, Haan, Germany) and stored in high density polyethylene (HDPE) containers.

2.3. Characterization of soil samples

Soil pH-values were determined with a pH electrode (SenTix® 940–3 WTW, Weinheim, Germany) and a pH meter (handylab 2, SCHOTT Insturments, Mainz, Germany) in 0.01 M $CaCl_2$. Five grams of air-dried (40 °C until constant weight) soil was mixed with 12.5 ml $CaCl_2$ solution and shaken at 80 rpm in a horizontal shaker (KS 10, Edmund Buehler GmbH, Bodelshausen, Germany) for 30 min. After shaking,

suspensions were allowed to settle for 30 min and pH was determined in the supernatant.

Carbonates were determined volumetrically as CO_2 released from $CaCO_3$ with a Scheibler apparatus (Blume *et al* 1996, DIN EN ISO 1069 2014). Total carbon and total nitrogen content of the samples were determined from 5 mg soil using a CN analyzer with a thermal conductivity detector (Unicube®, Elementar, Langenselbold, Germany).

The organic carbon (C_{org}) content was calculated by subtracting the content of carbonate-C from the total organic carbon content (Nelson and Sommers 2001).

For soil texture analysis all particles $\geqslant 20$ μm were separated with a wet sieving process and quantified gravimetrically, and particles smaller than 20 μm were separated by sedimentation according to Köhn (1928) and quantified also gravimetrically. Prior to soil texture analysis, carbonates were destroyed with HCl (10%) and soil organic matter was destroyed with H_2O_2 (30%). Tetrasodium pyrophosphate (0.4 mol l^{-1}) was added as dispersion agent to prevent flocculation of soil particles (Jensen *et al* 2017).

Soil trace element contents were determined by inductively coupled plasma coupled to an optical emission spectrometer (Agilent 720ES) after microwave assisted aqua regia extraction of the pseudo-total metal content based on U.S. EPA. Method 3051A (2007) modified by Öztan and Düring (2012).

2.4. QAAC analyzes

The soils were analyzed for ATMACs (chain length C-8 to C-16), BACs (C-8 to C-18) and DADMACs (C-8 to C-16). Firstly, the soils were repeatedly extracted with acidified acetonitrile (99.9% AcN/0.1% HCl v/v) and ultrasonic irradiation according to Heyde *et al* (2020a). The extracts were cleaned with solid phase extraction (Chromabond CN, Machery Nagel, Dueren, Germany). The analytes were separated and quantified with high performance liquid chromatography (Waters™ alliance 2690, Eschborn, Germany) and a Waters XSelect CSH Phenyl-Hexyl-Column (130 Å, 150 mm length, 2.1 mm ID, 3.5 μm particle size) with a column guard of the same material coupled to a mass spectrometer (Micromass Quattro Micro, Waters, Eschborn, Germany) following the method described elsewhere by Heyde *et al* (2020a).

A list of QAACs analyzed along with available compound parameters is provide in table 1.

2.5. Data evaluation

Peak integration of the QAAC analyzes was performed with Waters MassLynx 4.0, data evaluation and illustration with Systat Sigma Plot 12.0. RStudio 4.0.2 was used to perform correlation analysis i.e. the correlation matrix (tables SI2/SI3) and the correlograms (figures 2 and SI1). In order to analyze the linear correlation between irrigation time, soil properties and QAAC concentration, the Pearson correlation coefficient (r) was used. We tested the correlation of soil properties and QAAC irrigation time additionally with Spearman rank correlation to confirm the results (tables SI4, SI5).

Systat Sigma Plot 12.0 was also used to calculate regressions between (log) QAAC-concentrations (y) and duration of irrigation (x). The same was performed for the concentrations of the trace metals, pH, $CaCO_3$, C_{org}, Clay, and N_{total} (y) and the duration of irrigation (x). We judged the increase of concentrations (values) with increasing duration of irrigation as significant, if the confidence intervals of the slope parameter of the regression did not overlap with zero. In this case the H_0-hypotheses was: slope parameter b is 0, which means the slope did not differ significantly from 0. The level of significance was set to $\alpha = 0.05$. Hence, the increase of concentrations with duration of irrigation was significant if $p < 0.05$.

Multiple Regression was performed to test which variable (pH, irrigation time, clay content) explains most of the observed increase in the sum of QAAC concentrations ($p < 0.05$).

3. Results and discussion

3.1. Accumulation of QAACs in wastewater-irrigated soils

The smallest total concentrations of QAACs were found in the samples of 2011 after 0 and 3 years of wastewater irrigation. Across both sample sets the most abundant QAACs belonged to the group of BACs (2018; 2.3–131.2 mg kg^{-1}), followed by ATMACs (2018; 0.1–12.7 mg kg^{-1}) and DADMACs (2018; 0–11.3 mg kg^{-1}) (table 2). The prevalence of BACs was expected since they are widely used in households and four of six BAC homologues are HPVCs, whereas only two of the five analyzed ATMACs and DADMACs are HPVCs (Mulder *et al* 2018). Our results were in the same concentration range as data of Li *et al* (2018), who also found DADMACs in lowest concetrations among the three group of QAACs in sewage-impacted estuarine sediments.

In every single sample BAC-C12 was detected, which is one of the most commonly used QAACs in disinfectant agents (Wagner 2017). We also found BAC-C14 in all samples, with the exception of one sample of the 2011 sampling year. Both homologues were found at highest concentrations among all QAACs. The greatest concentration of each sum of the three homologue groups (ATMAC, BAC, DADMAC) was in the soils that were irrigated for 88 years. BAC-C12 was found in highest concentrations among all homologues in soil that was irrigated 88 years (83.6 μg kg^{-1}). The greatest increase of QAACs from 0 to 88 years of irrigation was observed for BAC-C12 (+4100%), BAC-C14 (+5500%) and ATMAC-C16

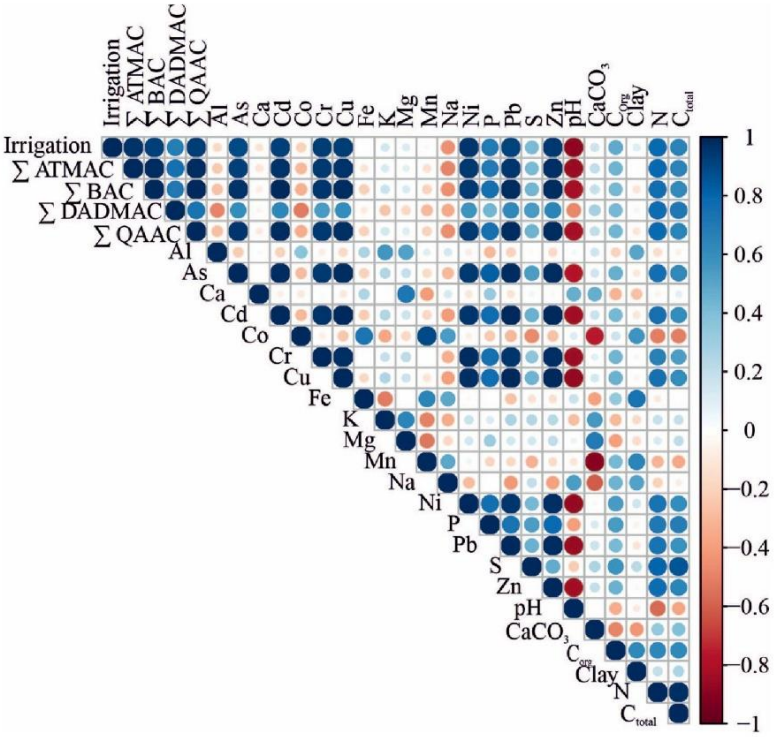

Figure 2. Pearsons correlations of ∑ATMACs, ∑BACs, ∑DADMACs and the sum of QAACs (all $\mu g\,kg^{-1}$), irrigation time (a), soil trace metals ($mg\,kg^{-1}$), pH, carbonates (%), C_{org} (%), Clay (%), N (%) and C (%) in Mezquital Valley. Sampled in 2018.

(+3500%). Concentrations of ATMAC-C8 and C10 (+0%) remained at a similarly low level.

In general, the samples taken in 2018 contained higher QAAC concentrations compared to those taken in 2011. This could be caused by aging effects of the soils and a loss due to microbial activity during storage, since QAACs are biodegradable by microorganisms (Nishihara *et al* 2000), especially by *Pseudomonas spec.* which can use QAACs as carbon source (Dean-Raymond and Alexander 1977, Geftic *et al* 1979, Kaech and Egli 2001, Takenaka *et al* 2007, Liffourrena *et al* 2008). Samples taken in 2011 were stored for 7 years longer before analysis than samples from 2018. Since we cannot rule out the suspected aging effects, we focus the discussion mainly on the samples of 2018.

The duration of irrigation was strongly and positively correlated to ∑ATMACs ($r = 0.89$), ∑BACs ($r = 0.89$) and ∑QAACs ($r = 0.88$) (figure 2 and tables SI2, SI3), while for ∑DADMACs the correlation was weaker, with $r = 0.60$. This was supported by the data of the 2011 samples (figure SI1 and tables SI2, SI3). One possible reason is, as mentioned before, the lower production volumes of DADMACs (OECD 2004) resulting in lower discharges through the wastewater into soils. Further, DADMACs, especially those with alkyl chains longer than 12 C atoms, have lower extraction recovery rates (Heyde *et al* 2020a) and possibly stronger interaction with soil particles since they have two hydrophobic alkyl chains. The time of irrigation showed the smallest p-values ($p = 0.044$ in 2018 and $p = 0.013$ in 2011), compared to pH and clay content ($p = 0.355$; $p = 0.272$ in 2018 and $p = 0.0.707$; $p = 0.156$ in 2018) and hence explained most of the observed increase of the sum of QAAC concentrations in multiple linear regressions considering pH and clay content in addition to irrigation duration. The equation of the multiple linear regression of 2018 ($R^2 = 0.76$) was:

$$\sum\text{QAAC} = 455.828 + (1.103 \times \text{irrigation}) - (55.331 \times \text{pH}) - (21.123 \times \text{Clay})$$

And in 2011 ($R^2 = 0.92$):

$$\sum\text{QAAC} = -28.662 + (0.577 \times \text{irrigation}) + (6.135 \times \text{pH}) - (0.430 \times \text{Clay})$$

Plotting the logarithmic concentrations of ∑ATMAC and ∑BAC versus the irrigation time resulted in highly significant linear increases (figures 3(a) and (c)), which illustrates an exponential increase of these concentrations. This pattern was also observed in soils sampled in 2011 for all three classes of QAACs, including DADMACs (figures SI 3(a), (c), (e)).

Table 2. Concentrations of all analyzed QAAC homologues ($\mu g\ kg^{-1}$) from samples taken in 2011 and 2018.

	Irrigation time (years)	ATMAC-C8	ATMAC-C10	ATMAC-C12	ATMAC-C14	ATMAC-C16	BAC-C8	BAC-C10	BAC-C12	BAC-C14	BAC-C16	BAC-C18	DADMAC-C8	DADMAC-C10	DADMAC-C12	DADMAC-C14	DADMAC-C16	∑ QAAC
LOQ		0.075	0.03	0.25	0.06	0.11	0.03	0.05	0.16	0.05	0.01	0.29	0.15	0.47	0.5	0.13	0.6	
		($\mu g\ kg^{-1}$)																
Sampled in 2018	0	< LOQ	< LOQ	< LOQ	< LOQ	0.2	< LOQ	< LOQ	1.5	0.8	< LOQ	< LOQ	1.8	< LOQ	< LOQ	< LOQ	< LOQ	4.3
	0	0.2	0.1	< LOQ	< LOQ	< LOQ	0.1	< LOQ	6.5	0.6	< LOQ	< LOQ	0.5	2.5	< LOQ	< LOQ	< LOQ	10.5
	2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	5.3	0.5	0.2	< LOQ	< LOQ	0.8	< LOQ	0.2	< LOQ	6.9
	8	0.3	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	4.7	0.7	0.2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	5.9
	10	0.1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.1	4.2	0.7	0.5	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	5.6
	10	0.1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	3.2	0.5	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	3.7
	18	< LOQ	< LOQ	0.7	< LOQ	0.6	0.0	0.0	3.0	1.5	1.1	1.0	1.8	1.2	< LOQ	0.2	< LOQ	11.1
	20	< LOQ	< LOQ	0.5	< LOQ	0.3	< LOQ	0.1	5.2	0.9	0.3	< LOQ	0.5	< LOQ	< LOQ	< LOQ	< LOQ	7.7
	34	0.1	< LOQ	0.7	< LOQ	1.1	0.1	0.2	8.3	5.0	1.9	1.4	1.8	2.0	< LOQ	< LOQ	< LOQ	22.7
	70	< LOQ	< LOQ	0.6	0.1	2.0	0.4	0.3	15.5	7.2	1.9	2.7	0.6	< LOQ	< LOQ	0.1	< LOQ	31.5
	88	0.2	0.1	3.8	0.3	8.4	4.1	3.4	83.6	28.6	6.6	4.9	6.1	4.9	< LOQ	0.3	< LOQ	155.2
Sampled in 2011	0	<LOQ	<LOQ	<LOQ	<LOQ	0.1	0.1	<LOQ	0.5	0.4	<LOQ	<LOQ	1.3	<LOQ	<LOQ	<LOQ	<LOQ	2.3
	1	0.1	<LOQ	<LOQ	<LOQ	0.1	0.1	<LOQ	1.1	0.6	<LOQ	<LOQ	1.0	<LOQ	<LOQ	<LOQ	<LOQ	2.9
	3	<LOQ	<LOQ	<LOQ	<LOQ	0.2	<LOQ	0.1	1.8	0.8	0.3	<LOQ	1.1	<LOQ	<LOQ	0.1	<LOQ	2.3
	3	0.2	<LOQ	<LOQ	<LOQ	0.1	<LOQ	<LOQ	0.9	0.6	0.3	0.7	1.1	<LOQ	<LOQ	<LOQ	<LOQ	3.7
	6	0.1	<LOQ	<LOQ	<LOQ	0.2	0.0	<LOQ	1.2	1.1	0.3	0.9	1.5	<LOQ	<LOQ	<LOQ	<LOQ	5.3
	8	<LOQ	0.1	0.8	<LOQ	0.2	0.0	0.1	1.4	1.2	1.2	0.9	1.6	<LOQ	<LOQ	<LOQ	<LOQ	5.4
	13.5	<LOQ	<LOQ	1.2	<LOQ	<LOQ	0.1	0.2	5.1	1.4	<LOQ	<LOQ	2.0	<LOQ	<LOQ	<LOQ	1.2	10.9
	35	<LOQ	<LOQ	0.9	<LOQ	0.1	<LOQ	<LOQ	2.3	<LOQ	<LOQ	<LOQ	1.7	<LOQ	<LOQ	<LOQ	<LOQ	5.0
	85	<LOQ	<LOQ	2.0	0.4	2.7	1.3	1.1	25.2	9.4	3.1	3.6	2.5	1.6	<LOQ	<LOQ	<LOQ	49.6

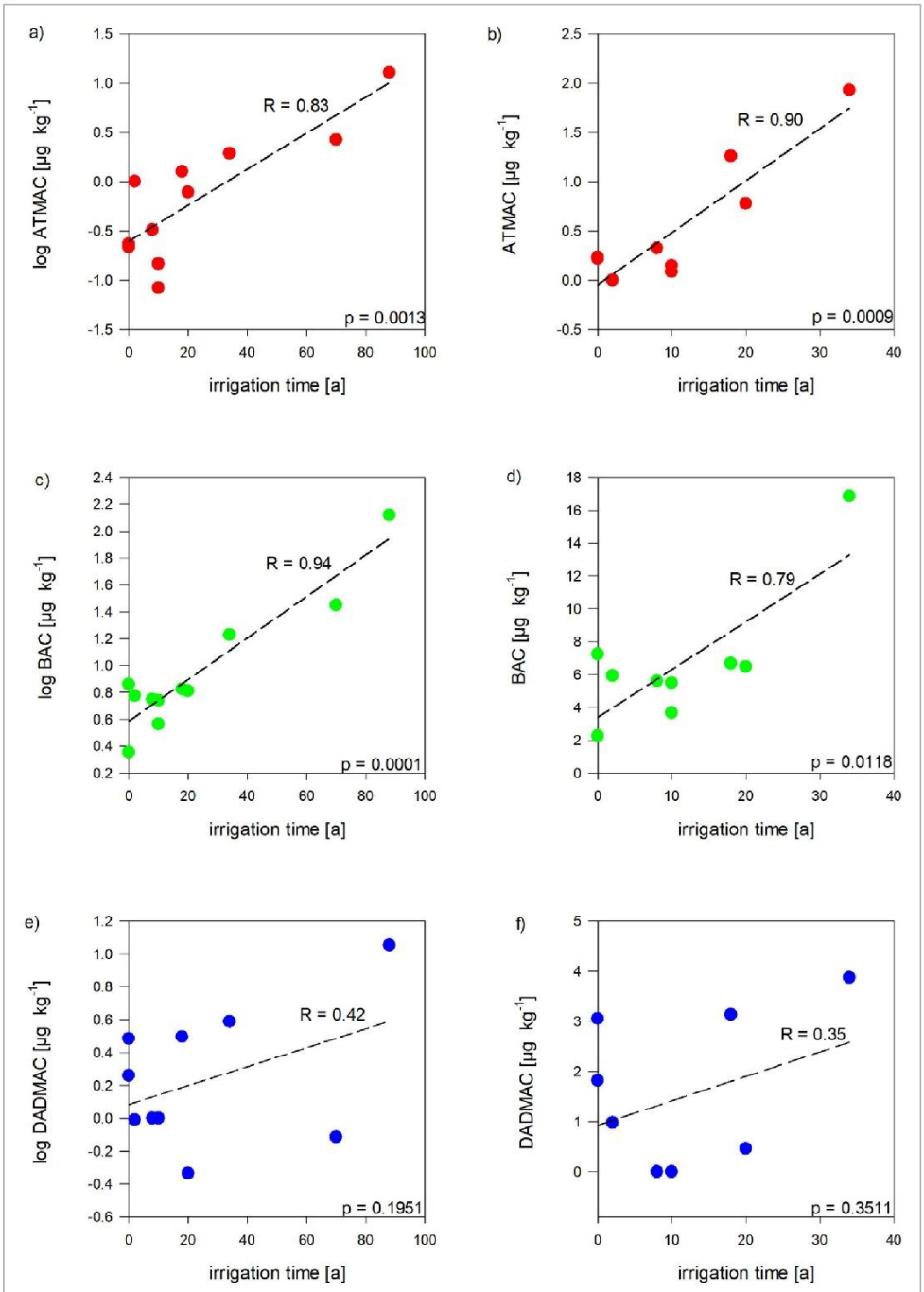

Figure 3. Logarithmic concentrations of ∑ATMACs (a), ∑BACs (c) and ∑DADMACs (e) in soils of Mezquital Valley irrigated with untreated wastewater and (de-log) concentrations of ∑ATMACs (b), ∑BACs (d) and ∑DADMACs (f) of the same soils (*x*-axes cut at 40 years). Significance ($\alpha = 5\%$) of the slope if $p < 0.05$. Sampled in 2018.

When narrowed down to the first 40 years of irrigation, a closer look at the temporal trend of ∑ATMAC and ∑BAC concentrations during the first 40 years of irrigation revealed a linear increase with the duration of irrigation (figures 3(b), (d) and SI 3(b), (d)). This suggested a biphasic accumulation in soil with time: an initial phase with linear accumulations, as typically observed for metals (Siebe Grabach 1994, Siebe and Cifuentes 1995), followed by an exponential rise of concentrations for time

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Table 3. Soil properties of samples taken in 2011 and 2018. pH value was determined in 0.01 $CaCl_2$. C_{total} and N_{total} were measured in triplicates with a standard deviation of 0.0.

	Irrigation time (years)	pH[ab]	$CaCO_3$ (wt. %)	C_{org} (wt. %)	C_{total} (wt. %)[a]	Clay (wt. %)	N_{total} (wt. %)
Sampled in 2018	0	7.5	0.3	1.5	1.8	50	0.2
	0	7.5	0.2	1.3	1.5	71	0.1
	2	7.9	0.6	0.3	0.9	29	0.1
	8	7.7	0.7	0.4	1.1	38	0.1
	10	7.9	0.5	0.8	1.3	36	0.1
	10	7.8	0.2	2.1	2.3	55	0.2
	18	7.6	0.3	1.6	1.9	53	0.2
	20	7.7	0.1	1.9	2.0	57	0.2
	34	7.3	2.8	0.2	3.0	42	0.2
	70	7.3	0.1	2.1	2.2	50	0.2
	88	7.1	0.3	2.0	2.2	45	0.2
Sampled in 2011	0	7.8	0.0	0.9	0.9	43	0.1
	1	7.8	1.3	0.1	1.4	33	0.1
	3	7.9	0.2	1.3	1.4	40	0.1
	3	7.9	0.5	1.6	2.1	49	0.2
	6	7.8	0.4	1.3	1.7	53	0.1
	8	7.5	0.1	1.1	1.2	41	0.1
	13.5	7.9	0.9	1.0	1.9	36	0.2
	35	7.5	0.5	1.3	1.8	55	0.2
	85	7.1	0.7	1.7	2.4	41	0.2

[a] Statistically significant slope ($p < 0.05$) of the regression (y = irrigation; x = soil property) in 2018.
[b] Statistically significant slope ($p < 0.05$) of the regression (y = irrigation; x = soil property) in 2011.

periods >40 years. This observation is in contrast to the behavior of pharmaceuticals in those soils, for which an apparent 'steady state' is reached over time (Dalkmann *et al* 2012). We suppose the biphasic accumulation of BACs and ATMACs in soil during long-term irrigation with wastewater was caused either by (a) massive QAAC-inputs during the first half of the 20th century that ceased towards the end of the 20th century, or by (b) an increasing strength of QAAC sorption to the soils, slowing down their biodegradation in soils with a very long history of wastewater irrigation.

Indeed, QAACs were intensively used following their invention as disinfectants in 1935 (Domagk 1935) as described in the introduction. Speculatively, the upcoming concerns about resistance could then have led to a drop of consumption, with subsequently lower QAAC concentrations in the wastewater and eventually in the soils, which were first irrigated during the 2nd half of the 20th century or later. However, since BACs and ATMACs are biodegradable, their accumulation requires an efficient sequestration of these compound caused by an increasingly strong sorption.

Interestingly, Zanini *et al* (2013) described a shift in sorption processes with increasing concentration of BACs. They found that the sorption of BAC occurred at low concentrations to soil minerals and at higher concentration to humic acids. At low concentrations QAACs act as polar as molecules and behave like other (metal) cations, but at high concentrations they form micelles and act like a nonpolar compound, which changes their behavior in solutions (Mulder *et al* 2020). Also the sorption to soil minerals occurred in two steps (Zanini *et al* 2013). At low concentrations, BACs were sorbed on the surface and in the interlayer of clay minerals and with rising concentrations the sorption occurred in bi-layers due to hydrophobic-hydrophobic interaction at the clay surface. However, it is unclear whether sorption to organic matter and in bi-layers would stabilize BACs against biodegradation. Sorption in the interlayer space of clay minerals is known to protect organic molecules against biodegradation (Amelung *et al* 2018). As we and others have shown in previous work, QAACs widen the interlayer spaces of clay minerals (Paiva *et al* 2008, Heyde *et al* 2020b). In those interlayer spaces they can form bilayers and paraffin-like structures (Zhu *et al* 2003), which are poorly accessibly for enzymes and microorganisms. Possibly, such widening of interlayer spaces and the efficient diffusion of QAACs into these spaces requires the build-up of certain minimum QAAC concentrations and time, which are only given after long-term irrigation.

Since there is no ecotoxicological information available regarding no-effects concentrations of QAACs in soils, we cannot judge potential risks associated with the accumulation of the compounds in soils.

3.2. Change of soil properties with increasing duration of irrigation

The pH-values decreased the longer soils were irrigated (up to 85 years and 88 years, table 3). The duration of irrigation and pH-value were correlated

Table 4. Total elemental analysis of the soils (mg kg^{-1}) in the Mezquital Valley. Sampled in 2018 and 2011.

	Irrigation time	Al	As[b]	Ca	Cd[ab]	Co	Cr[ab]	Cu[ab]	Fe	K[a]	Mg	Mn	Na	Ni[ab]	P[ab]	Pb[ab]	S[a]	Zn[ab]
	(years)	(mg kg^{-1})																
Sampled in 2018	0	63 700	1.1	12 300	0.0	17.5	51.6	11.9	34 400	8000	9976	702	2700	22.3	243	3.8	150	66.8
	0	66 300	0.0	11 000	0.1	17.3	51.7	15.3	36 400	10 100	11 798	638	1200	25.9	448	3.3	248	80.0
	2	57 700	1.0	16 600	0.1	17.8	47.9	11.6	30 400	8700	9335	808	3500	17.8	434	3.7	179	64.5
	8	58 900	1.5	13 300	0.1	16.2	48.5	12.0	30 600	8500	9599	713	3200	17.6	454	4.9	217	66.3
	10	59 600	1.7	12 800	0.1	15.8	50.2	12.7	30 900	8400	9894	662	3400	17.9	482	3.2	245	68.3
	10	63 200	1.5	8800	0.1	16.1	48.7	16.4	32 300	9400	9161	693	2600	19.4	820	6.3	394	78.5
	18	58 000	0.4	8700	0.1	17.2	47.0	15.0	31 600	7200	7437	819	3000	19.9	542	6.8	290	72.3
	20	62 000	1.2	8000	0.1	15.3	46.4	15.0	32 900	7300	6601	672	3100	20.5	596	5.2	299	76.6
	34	49 300	7.8	27 500	0.3	14.2	53.1	22.8	29 400	7100	8523	617	2800	28.4	1025	6.4	476	97.8
	70	58 500	2.7	7700	1.4	16.1	80.3	44.0	32 900	7300	8012	755	2600	35.4	879	35.1	430	185
	88	55 600	2.3	9900	1.2	15.7	74.6	50.6	33 600	7000	7671	702	3000	33.5	1303	37.5	532	215
Sampled in 2011	0	56 600	1.4	15 200	0.0	19.5	52.8	12.0	36 500	5000	7711	880	4500	20.3	518	0.8	130	62.5
	1	59 800	1.3	16 000	0.1	15.3	44.8	12.6	29 800	9000	10 241	609	2500	17.6	514	3.2	234	67.0
	3	54 200	1.5	15 000	0.1	17.6	46.4	13.1	34 000	6200	8014	803	4400	19.8	920	0.8	202	65.9
	3	57 700	1.8	13 900	0.1	16.2	43.7	13.4	33 300	7000	8075	735	5200	19.9	746	3.5	727	71.7
	6	60 800	1.0	12 100	0.1	18.2	50.3	13.3	36 000	6500	8076	810	4000	21.3	546	1.7	241	70.2
	8	54 800	1.2	6700	0.1	16.7	42.6	13.8	29 900	6400	5626	828	3200	19.1	333	5.4	207	59.5
	13.5	51 400	1.3	15 300	0.1	14.9	42.6	11.7	33 400	4600	7213	676	3100	18.1	643	1.5	334	70.3
	35	59 200	1.4	13 200	0.1	18.2	54.2	15.6	38 500	6300	8171	867	3600	23.6	593	1.2	311	81.3
	85	55 000	5.1	12 900	2.5	15.9	102.7	58.0	32 100	7300	8520	735	2700	38.7	1191	57.3	548	242.5

[a] Statistically significant slope ($p < 0.05$) of the regression (y = irrigation; x = trace metal concentration) in 2018.
[b] Statistically significant slope ($p < 0.05$) of the regression (y = irrigation; x = trace metal concentration) in 2011.

negatively in the samples of 2018 (figure 2), which was confirmed by the data of samples collected in 2011 (figure SI1). The range of pH was only from 7.1 to 7.9 in both sample sets, but the negative correlation corresponds to findings of Xu *et al* (2010), who showed that the pH-value of wastewater irrigated soils decreased in the upper 20 cm over time of irrigation. Siebe *et al* (2016) also observed this decrease in the range of neutral to slightly alkaline values due to wastewater irrigation in the Mezquital Valley. Hernandez-Martínez *et al* (2018) proposed that proton production by nitrification is accountable for the slightly changing pH values over time.

Apart from the soil that was irrigated for 34 years, all soils that were sampled in 2018 had a carbonate content below 1%. Among the samples from 2011, only one soil irrigated during 1 year had a carbonate content of 1.3%. Neither for the samples collected in 2011 nor for the ones collected in 2018, could we observe a significant correlation between the irrigation time and the $CaCO_3$ content in the soils (figures 2 and SI1). These results are similar to those of Adrover *et al* (2012), who found no significant change in soil carbonate content after wastewater irrigation for 30 years.

C_{total} and N_{total} were strongly correlated with each other with correlation coefficients of 0.93 (2018) and 0.97 (2011), but only weakly correlated with irrigation time in both sample sets. N_{total} ranged from 0.1% to 0.2% in 2018 and 2011 and C_{total} ranged from 0.9% to 3.0% in 2018 and up to 2.4% in 2011 (table 3 and figure SI2). Considering that C_{org} ranged from 0.3% to 2.1% in 2011 samples, the results are in good agreement with the results from Dalkmann *et al* (2014), who obtained the same concentrations of N_{total} and slightly lower C_{org} concentrations. The highest amounts of organic carbon with 3% and 2.4% were detected for a soil that was irrigated more than 34 years (2018) and one irrigated for 85 years (2011). An increasing C_{org} content with irrigation time was expected, since it is known that wastewater irrigation can drive the build-up of soil organic matter content (Friedel *et al* 2000). Sánchez-González *et al* (2017) observed that organic carbon stocks increase 1.5 fold after 40 years of irrigation and reach thereafter a new steady state in the Mezquital Valley.

The determined clay contents ranged from 36% to 57% in samples form 2018 and from 33% to 55% in 2011. An exception was the field that was irrigated for 0 years (table 3), where the soil had a clay content of 71%. Dalkmann *et al* (2014) described a clay content between 39% and 54% for soils in that area, which is close to our findings. Apparently, the clay content was independent of the irrigation duration, since the amounts of fine material introduced into the soils with wastewater were too small to increase the bulk mass of clay-sized material significantly. This is also in agreement with a study by Mamedov *et al* (2001), who found no significant difference in clay content between wastewater and freshwater-irrigated soils.

3.3. Accumulation of trace metals with increasing duration of irrigation

The regression between soil concentrations and irrigation duration showed that concentrations of Cd, Cr, Cu, Ni, P, Pb and Zn increased significantly with time in both sets of samples (table 4). The concentrations of Cu, Mn, Zn, Cd, Cr, Co and Pb (table 4) were similar to those that were found by Cajuste *et al* (1991) in wastewater-irrigated soils in Mezquital Valley, but our Ni-concentrations were about four times higher (17.6–35.4 mg kg^{-1}). Pb contents in the soils that were irrigated for 70 or more years ($\geqslant$35.1 mg kg^{-1}) were elevated. This coincided with the fact that the Pb-concentration in wastewater were increased. The lowest Cr-content that we found was 42.6 mg kg^{-1} (table 4) and background concentration of rainfed soils were around 50 mg kg^{-1}. These comparably large background concentrations of Cr were most likely inherited from the volcanic rocks in the Mezquital Valley area, which form the parent material for soil formation.

Strong positive correlations between duration of irrigation and the contents of Cd, Cr, Cu, Ni, P, Pb, S and Zn occurred for the samples of 2018 (figure 2). These correlations were confirmed by the data of the samples from 2011. This leads us to conclude, that metals were accumulated in the soils due to the irrigation with wastewater. Similar accumulation of Cu, Cr, Pb, Zn and Cd in wastewater irrigated soils of the Mezquital Valley was reported previously by Siebe and Cifuentes (1995). Hence, the temporal change of metal contents with increasing irrigation time underlines the suitability of our set of chronosequence samples for testing the potential accumulation of QAACs in wastewater-irrigated soils.

In summary, wastewater irrigation over a time period of 88 years leads to an accumulation of QAACs in soils. Contradictory to our second hypotheses, QAACs behaved differently to what we expected from pharmaceuticals, as their concentrations did not reach a steady state after 88 years of irrigation.

4. Conclusion

This study provides first evidence that wastewater irrigation leads to an increase of QAACs concentrations in the soil of the Mezquital Valley, Mexico. In fact, our study is to our knowledge is the first to report QAAC concentrations determined for an extended set of (unspiked) soil samples. We show that the concentrations of QAACs exceed the concentrations of other antibiotics and pharmaceuticals often by an order of magnitude, highlighting the need to keeping this group of substances on the radar when considering mechanisms of antibiotic resistance gene

evolution. The temporal trend of QAAC accumulation is opposite to the trend observed for pharmaceuticals. No 'steady state' concentration caused by a balance between inputs and dissipation is reached after 88 years of irrigation. Instead, QAAC concentrations in long-term irrigated soils rise exponentially, potentially due to massive inputs of QAACs into the soils in the first half of the 20th century or due to a strong sorption and effective sequestration (e.g. in clay mineral interlayer spaces).

The intensive use of disinfectants during the COVID-19 pandemic will likely leave a footprint of QAACs in wastewater-irrigated soils.

The fate of QAACs in soils is different to the fate of metal cations and pharmaceuticals. Future work should address the processes and kinetics of QAAC sorption and sequestration in soils with a focus on sorption and sequestration in clay mineral interlayers.

Data Availability

All data that support the findings of this study are included within the article (and any supplementary files).

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Quaternary alkylammonium disinfectant concentrations in soils rise exponentially after long-term wastewater irrigation

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S1

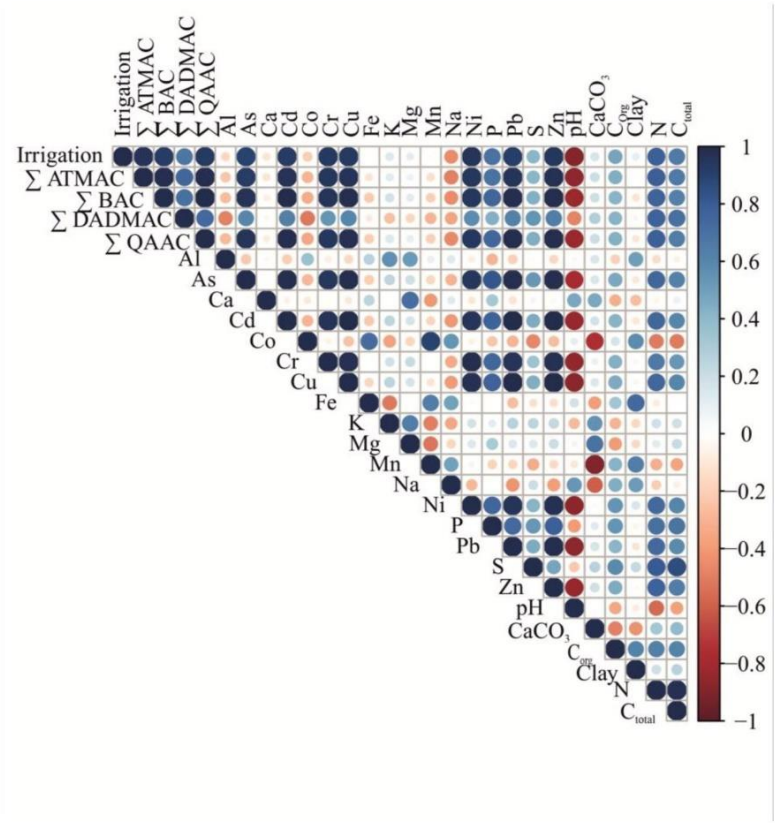

Figure SI1. Pearsons Correlations of ∑ATMACs, ∑BACs, ∑DADMACs and the sum of QAACs [all µg kg^{-1}], irrigation time [a], soil trace metals [mg kg^{-1}], pH, carbonates [%], C_{org} [%], Clay [%], N [%] and C [%] in Mezquital Valley. Sampled in 2011.

S2

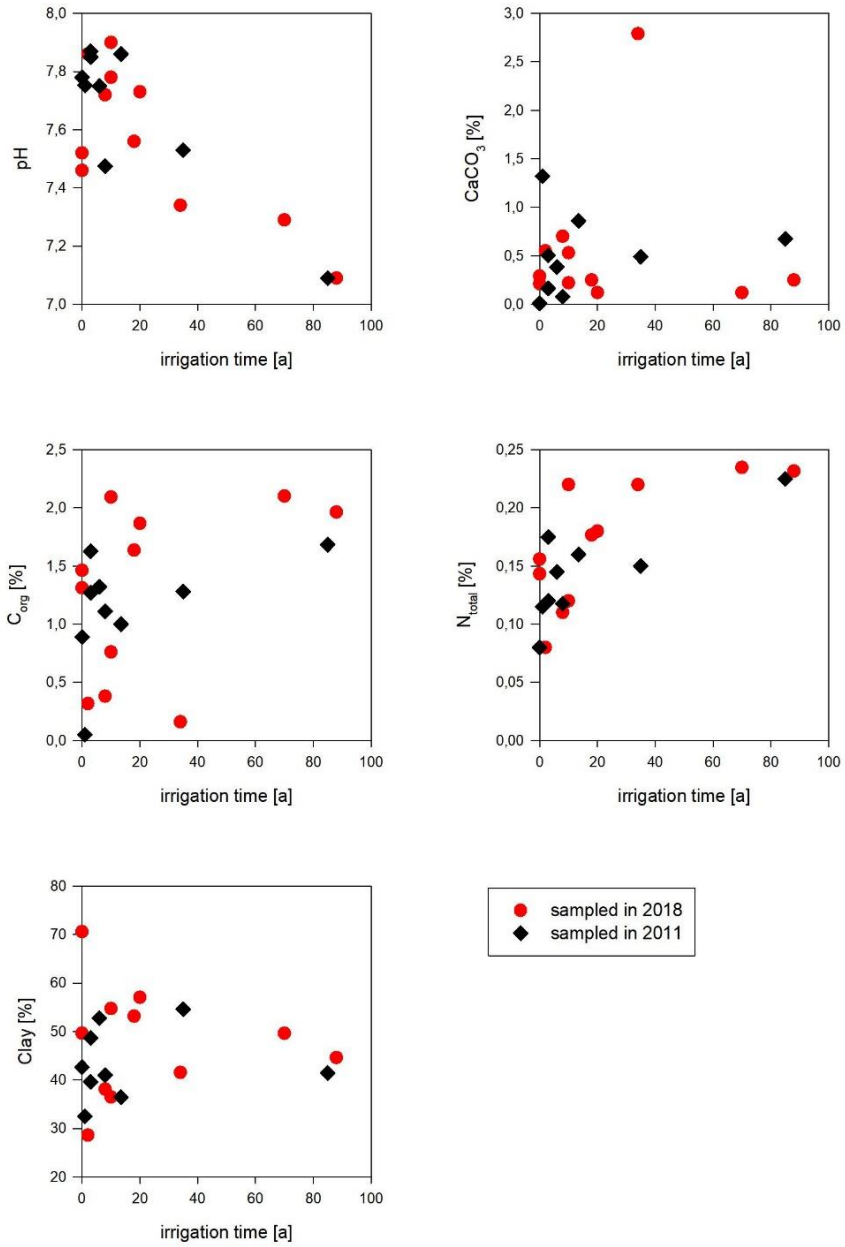

Figure SI2. Soil properties depending on irrigation time from samples taken in 2018 (red) and 2011 (black).

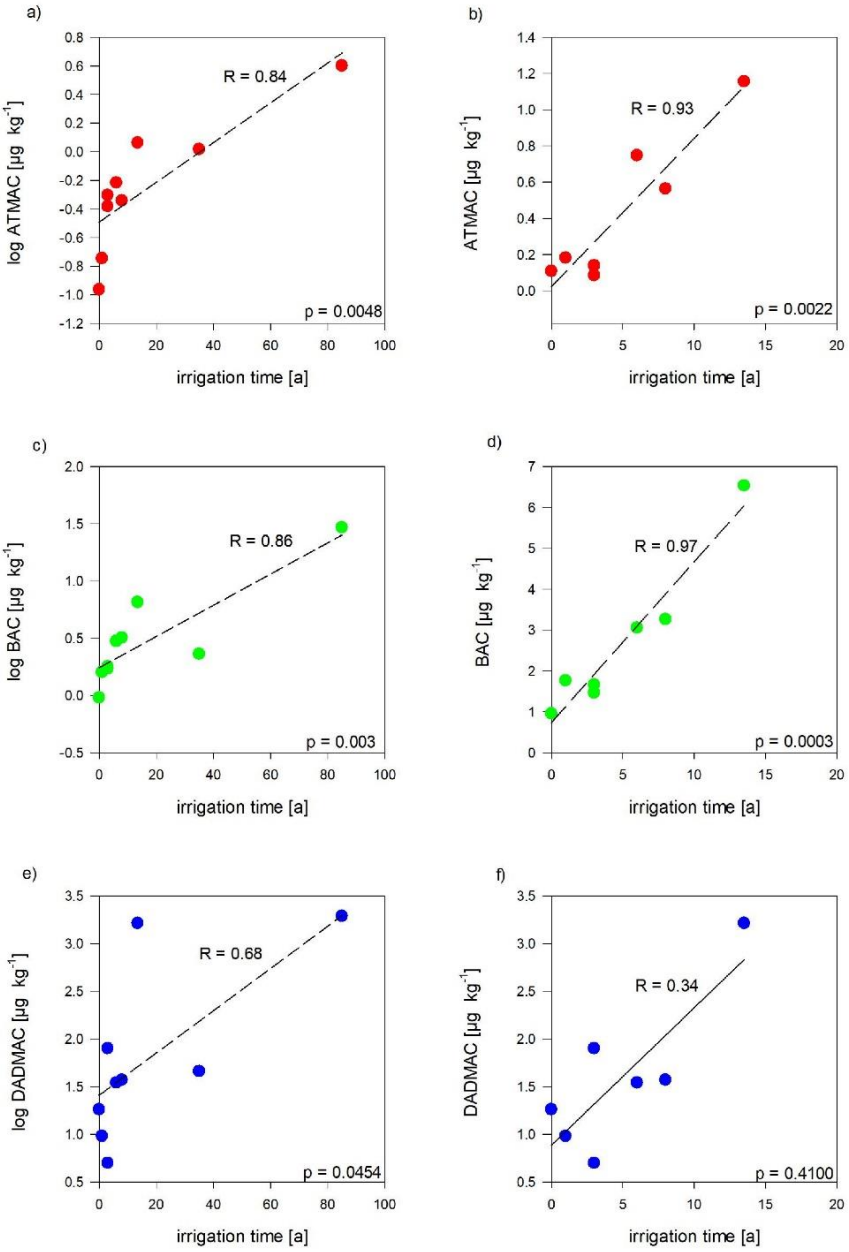

Figure SI3. Logarithmic concentrations of ∑ATMACs (a), ∑BACs (b) and ∑DADMACs (c) in soils of Mezquital Valley irrigated with untreated wastewater frequently and concentrations of ∑ATMACs (d), ∑BACs (e) and ∑DADMACs (f) of the same soils (X-axes cut at 20 years). Significance (α = 5%) of the slope if p < 0.05. Sampled in 2011.

S4

Table SI1. Characteristics of the untreated wastewater in the Mezquital Valley

	Untreated wastewater[a]	Tula river	Irrigtation canal
pH	7.26-8.07 [a]		7.9 [d]
el. cond. [µS cm^{-1}]	1013-1559 [a]		
BOD [mg L^{-1}]	78-131[b]		
COD [mg L^{-1}]	294-315[b]		
Total N [mg L^{-1}]	23-23[b]		
NH-N [mg L^{-1}]	3.6-4.0 [a]		
NO-N [mg L^{-1}]	0 [a]		
Total P [mg L^{-1}]	1.9-2.1 [b]		0.5 [d]
Ca [mg L^{-1}]	33-47 [a]		15.65 [d]
Mg [mg L^{-1}]	15-30 [a]		0.454 [d]
K [mg L^{-1}]	21-37 [a]		5.72 [d]
Na [mg L^{-1}]	101-203 [a]		12.3 [d]
Al [mg L^{-1}]		0.64 [c]	0.110 [d]
As [mg L^{-1}]		0.026 [c]	
Cd [mg L^{-1}]		< LOD [c]	0.024 [d]
Cr [mg L^{-1}]		0.002 [c]	0.004 [d]
Cu [mg L^{-1}]		0.006 [c]	0.017 [d]
Mn [mg L^{-1}]		0.13 [c]	0.053 [d]
Ni [mg L^{-1}]		0.003 [c]	0.015 [d]
Pb [mg L^{-1}]		0.103 [c]	0.510 [d]
Se [mg L^{-1}]		0.006 [c]	
Zn [mg L^{-1}]		0.029 [c]	0.167 [d]
THg [ng L^{-1}]		100.4 [c]	
Cl [mg L^{-1}]	81-162 [a]		
HCO_3^- [mg L^{-1}]	400-720 [a]		
SO_4^{2-} [mg L^{-1}]	31-103 [a]		

[a] (Dalkmann *et al* 2012, Dalkmann *et al* 2014)
[b] (Comisión Nacional del Agua *et al* 1998)
[c] (Guédron *et al* 2014)
[d] (Ramirez-Fuentes *et al* 2002)

Table SI2. Pearson correlation coefficients of ∑ATMACs, ∑BACs, ∑DADMACs and the sum of QAACs [all µg kg^{-1}], irrigation time [a], soil trace metals [mg kg^{-1}], pH, carbonates [%], Corg [%], Clay [%], N [%] and C [%] in Mezquital Valley. Sampled in 2018.

	Irrigation	∑ ATMAC	∑ BAC	∑ DADMAC	∑ QAAC	Al	As	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	Pb	S	Zn	pH	$CaCO_3$	C_{org}	Clay	N	C_{total}
Irrigation	1.00	0.89	0.89	0.60	0.88	-0.45	0.41	-0.13	0.98	-0.46	0.94	0.95	0.03	-0.61	-0.51	0.04	0.05	0.87	0.78	0.94	0.79	0.94	-0.73	0.02	0.41	-0.05	0.71	0.53
∑ ATMAC	0.89	1.00	0.98	0.86	0.99	-0.31	0.29	-0.19	0.87	-0.24	0.85	0.77	0.06	-0.52	-0.42	0.22	-0.06	0.79	0.50	0.81	0.56	0.75	-0.62	-0.05	0.40	0.05	0.61	0.43
∑ BAC	0.89	0.98	1.00	0.83	1.00	-0.25	0.26	-0.19	0.87	-0.21	0.89	0.78	0.09	-0.40	-0.30	0.17	-0.09	0.80	0.48	0.81	0.53	0.77	-0.57	-0.08	0.37	0.02	0.54	0.35
∑ DADMAC	0.60	0.86	0.83	1.00	0.85	-0.21	0.31	0.03	0.57	-0.04	0.61	0.48	0.15	-0.30	-0.11	0.14	-0.35	0.71	0.23	0.48	0.33	0.46	-0.57	0.12	0.21	0.23	0.45	0.41
∑ QAAC	0.88	0.99	1.00	0.85	1.00	-0.25	0.27	-0.18	0.86	-0.20	0.87	0.76	0.10	-0.41	-0.30	0.18	-0.11	0.80	0.47	0.80	0.52	0.75	-0.58	-0.06	0.36	0.04	0.55	0.37
Al	-0.45	-0.31	-0.25	-0.21	-0.25	1.00	-0.82	-0.68	-0.38	0.57	-0.26	-0.38	0.72	0.66	0.45	-0.04	-0.49	-0.32	-0.60	-0.30	-0.55	-0.34	0.35	-0.75	0.44	0.60	-0.27	-0.41
As	0.41	0.29	0.26	0.31	0.27	-0.82	1.00	0.78	0.27	-0.77	0.23	0.31	-0.54	-0.47	-0.24	-0.41	0.16	0.41	0.58	0.17	0.60	0.26	-0.38	0.90	-0.38	-0.33	0.46	0.67
Ca	-0.13	-0.19	-0.19	0.03	-0.18	-0.68	0.78	1.00	-0.21	-0.36	-0.21	-0.17	-0.57	-0.09	0.18	-0.35	0.14	0.00	0.12	-0.30	0.09	-0.20	-0.05	0.95	-0.81	-0.47	-0.12	0.20
Cd	0.98	0.87	0.87	0.57	0.86	-0.38	0.27	-0.21	1.00	-0.30	0.97	0.98	0.12	-0.54	-0.44	0.15	0.02	0.88	0.75	0.99	0.73	0.97	-0.76	-0.10	0.45	-0.05	0.66	0.43
Co	-0.46	-0.24	-0.21	-0.04	-0.20	0.57	-0.77	-0.36	-0.30	1.00	-0.21	-0.36	0.39	0.46	0.44	0.60	-0.18	-0.33	-0.66	-0.24	-0.70	-0.32	0.26	-0.58	0.05	0.09	-0.56	-0.67
Cr	0.94	0.85	0.89	0.61	0.87	-0.26	0.23	-0.21	0.97	-0.21	1.00	0.95	0.25	-0.41	-0.25	0.06	-0.11	0.90	0.66	0.96	0.65	0.95	-0.76	-0.13	0.44	0.00	0.60	0.37
Cu	0.95	0.77	0.78	0.48	0.76	-0.38	0.31	-0.17	0.98	-0.36	0.95	1.00	0.18	-0.51	-0.41	0.02	-0.06	0.90	0.83	0.98	0.81	1.00	-0.82	-0.05	0.47	0.02	0.71	0.50
Fe	0.03	0.06	0.09	0.15	0.10	0.72	-0.54	-0.57	0.12	0.39	0.25	0.18	1.00	0.32	0.31	-0.19	-0.76	0.33	-0.12	0.20	-0.08	0.22	-0.36	-0.60	0.62	0.79	0.17	0.00
K	-0.61	-0.52	-0.40	-0.30	-0.41	0.66	-0.47	-0.09	-0.54	0.46	-0.41	-0.51	0.32	1.00	0.84	-0.20	-0.51	-0.42	-0.48	-0.50	-0.48	-0.49	0.47	-0.24	-0.15	0.26	-0.48	-0.48
Mg	-0.51	-0.42	-0.30	-0.11	-0.30	0.45	-0.24	0.18	-0.44	0.44	-0.25	-0.41	0.31	0.84	1.00	-0.31	-0.53	-0.22	-0.49	-0.42	-0.50	-0.38	0.22	0.01	-0.38	0.11	-0.51	-0.45
Mn	0.04	0.22	0.17	0.14	0.18	-0.04	-0.41	-0.35	0.15	0.60	0.06	0.02	-0.19	-0.20	-0.31	1.00	0.42	-0.16	-0.19	0.17	-0.24	0.03	0.14	-0.42	0.13	-0.30	-0.21	-0.37
Na	0.05	-0.06	-0.09	-0.35	-0.11	-0.49	0.16	0.14	0.02	-0.18	-0.11	-0.06	-0.76	-0.51	-0.53	0.42	1.00	-0.36	0.03	0.01	-0.06	-0.06	0.37	0.12	-0.28	-0.83	-0.22	-0.20
Ni	0.87	0.79	0.80	0.71	0.80	-0.32	0.41	0.00	0.88	-0.33	0.90	0.90	0.33	-0.42	-0.22	-0.16	-0.36	1.00	0.71	0.85	0.74	0.89	-0.92	0.11	0.38	0.23	0.73	0.61
P	0.78	0.50	0.48	0.23	0.47	-0.60	0.58	0.12	0.75	-0.66	0.66	0.83	-0.12	-0.48	-0.49	-0.19	0.03	0.71	1.00	0.74	0.98	0.81	-0.67	0.29	0.29	-0.04	0.79	0.72
Pb	0.94	0.81	0.81	0.48	0.80	-0.30	0.17	-0.30	0.99	-0.24	0.96	0.98	0.20	-0.50	-0.42	0.17	0.01	0.85	0.74	1.00	0.71	0.98	-0.75	-0.20	0.52	-0.01	0.64	0.38
S	0.79	0.56	0.53	0.33	0.52	-0.55	0.60	0.09	0.73	-0.70	0.65	0.81	-0.08	-0.48	-0.50	-0.24	-0.06	0.74	0.98	0.71	1.00	0.78	-0.68	0.30	0.35	0.09	0.87	0.81
Zn	0.94	0.75	0.77	0.46	0.75	-0.34	0.26	-0.20	0.97	-0.32	0.95	1.00	0.22	-0.49	-0.38	0.03	-0.06	0.89	0.81	0.98	0.78	1.00	-0.81	-0.10	0.47	0.02	0.68	0.46

pH	-0.73	-0.62	-0.57	-0.57	-0.58	0.35	-0.38	-0.05	-0.76	0.26	-0.76	-0.82	-0.36	0.47	0.22	0.14	0.37	-0.92	-0.67	-0.75	-0.68	-0.81	1.00	-0.16	-0.33	-0.27	-0.70	-0.61
$CaCO_3$	0.02	-0.05	-0.08	0.12	-0.06	-0.75	0.90	0.95	-0.10	-0.58	-0.13	-0.05	-0.60	-0.24	0.01	-0.42	0.12	0.11	0.29	-0.20	0.30	-0.10	-0.16	1.00	-0.68	-0.35	0.12	0.43
C_{Org}	0.41	0.40	0.37	0.21	0.36	0.44	-0.38	-0.81	0.45	0.05	0.44	0.47	0.62	-0.15	-0.38	0.13	-0.28	0.38	0.29	0.52	0.35	0.47	-0.33	-0.68	1.00	0.62	0.63	0.37
Clay	-0.05	0.05	0.02	0.23	0.04	0.60	-0.33	-0.47	-0.05	0.09	0.00	0.02	0.79	0.26	0.11	-0.30	-0.83	0.23	-0.04	-0.01	0.09	0.02	-0.27	-0.35	0.62	1.00	0.38	0.32
N	0.71	0.61	0.54	0.45	0.55	-0.27	0.46	-0.12	0.66	-0.56	0.60	0.71	0.17	-0.48	-0.51	-0.21	-0.22	0.73	0.79	0.64	0.87	0.68	-0.70	0.12	0.63	0.38	1.00	0.93
C_{total}	0.53	0.43	0.35	0.41	0.37	-0.41	0.67	0.20	0.43	-0.67	0.37	0.50	0.00	-0.48	-0.45	-0.37	-0.20	0.61	0.72	0.38	0.81	0.46	-0.61	0.43	0.37	0.32	0.93	1.00

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Table SI3. Pearsons Correlations of ∑ATMACs, ∑BACs, ∑DADMACs and the sum of QAACs [all µg kg^{-1}], irrigation time [a], soil trace metals [mg kg^{-1}], pH, carbonates [%], C_{org} [%], Clay [%], N [%] and C [%] in Mezquital Valley. Sampled in 2011.

	Irrigation	∑ ATMAC	∑ BAC	∑ DADMAC	∑ QAAC	Al	As	Ca	Cd	Co	Cr	Cu	Fe	K	Mg	Mn	Na	Ni	P	Pb	S	Zn	pH	$CaCO_3$	C_{org}	Clay	N	C_{total}
Irrigation	1.00	0.97	0.93	0.68	0.93	-0.16	0.90	-0.12	0.92	-0.21	0.93	0.94	0.01	0.14	0.11	-0.03	-0.45	0.96	0.69	0.91	0.42	0.95	-0.89	0.17	0.48	0.11	0.78	0.66
∑ ATMAC	0.97	1.00	0.98	0.75	0.99	-0.23	0.93	-0.12	0.96	-0.30	0.94	0.97	-0.11	0.12	0.09	-0.14	-0.49	0.95	0.70	0.96	0.41	0.97	-0.86	0.20	0.45	-0.01	0.80	0.65
∑ BAC	0.93	0.98	1.00	0.70	1.00	-0.26	0.97	-0.08	0.99	-0.33	0.95	0.99	-0.22	0.18	0.12	-0.19	-0.45	0.95	0.75	0.99	0.44	0.99	-0.83	0.20	0.42	-0.12	0.77	0.62
∑ DADMAC	0.68	0.75	0.70	1.00	0.74	-0.48	0.60	-0.05	0.63	-0.52	0.55	0.61	-0.08	-0.25	-0.17	-0.29	-0.36	0.57	0.43	0.62	0.54	0.64	-0.47	0.29	0.42	-0.04	0.78	0.71
∑ QAAC	0.93	0.99	1.00	0.74	1.00	-0.28	0.96	-0.08	0.99	-0.34	0.95	0.98	-0.20	0.15	0.10	-0.20	-0.46	0.94	0.74	0.98	0.45	0.98	-0.82	0.20	0.44	-0.11	0.79	0.64
Al	-0.16	-0.23	-0.26	-0.48	-0.28	1.00	-0.23	0.03	-0.21	0.36	-0.08	-0.17	0.26	0.56	0.53	0.10	0.13	-0.07	-0.29	-0.20	-0.03	-0.16	0.04	0.15	-0.19	0.51	-0.14	-0.06
As	0.90	0.93	0.97	0.60	0.96	-0.23	1.00	-0.01	0.99	-0.29	0.95	0.98	-0.21	0.24	0.19	-0.17	-0.30	0.95	0.82	0.98	0.53	0.98	-0.79	0.15	0.47	-0.10	0.75	0.63
Ca	-0.12	-0.12	-0.08	-0.05	-0.08	0.03	-0.01	1.00	-0.07	-0.09	-0.02	-0.10	0.26	0.00	0.72	-0.41	0.13	-0.11	0.33	-0.12	0.05	-0.03	0.47	0.47	-0.32	-0.27	-0.05	0.08
Cd	0.92	0.96	0.99	0.63	0.99	-0.21	0.99	-0.07	1.00	-0.29	0.97	1.00	-0.22	0.24	0.17	-0.17	-0.40	0.96	0.77	1.00	0.45	0.99	-0.83	0.17	0.43	-0.10	0.75	0.60
Co	-0.21	-0.30	-0.33	-0.52	-0.34	0.36	-0.29	-0.09	-0.29	1.00	-0.07	-0.24	0.72	-0.37	-0.18	0.89	0.55	-0.07	-0.23	-0.30	-0.47	-0.26	0.11	-0.75	0.16	0.58	-0.51	-0.50
Cr	0.93	0.94	0.95	0.55	0.95	-0.08	0.95	-0.02	0.97	-0.07	1.00	0.98	0.00	0.18	0.22	0.00	-0.33	0.99	0.74	0.96	0.35	0.98	-0.84	0.08	0.44	0.05	0.67	0.53
Cu	0.94	0.97	0.99	0.61	0.98	-0.17	0.98	-0.10	1.00	-0.24	0.98	1.00	-0.18	0.25	0.17	-0.12	-0.39	0.98	0.76	0.99	0.45	1.00	-0.86	0.14	0.45	-0.04	0.75	0.60
Fe	0.01	-0.11	-0.22	-0.08	-0.20	0.26	-0.21	0.26	-0.22	0.72	0.00	-0.18	1.00	-0.52	0.00	0.65	0.50	0.03	0.01	-0.26	-0.12	-0.14	0.18	-0.38	0.33	0.73	-0.08	0.01
K	0.14	0.12	0.18	-0.25	0.15	0.56	0.24	0.00	0.24	-0.37	0.18	0.25	-0.52	1.00	0.65	-0.50	-0.35	0.17	0.11	0.27	0.25	0.24	-0.27	0.55	-0.29	-0.16	0.19	0.19
Mg	0.11	0.09	0.12	-0.17	0.10	0.53	0.19	0.72	0.17	-0.18	0.22	0.17	0.00	0.65	1.00	-0.53	-0.18	0.15	0.31	0.15	0.14	0.21	0.06	0.69	-0.39	-0.16	0.12	0.21
Mn	-0.03	-0.14	-0.19	-0.29	-0.20	0.10	-0.17	-0.41	-0.17	0.89	0.00	-0.12	0.65	-0.50	-0.53	1.00	0.49	0.05	-0.18	-0.17	-0.33	-0.15	-0.11	-0.90	0.43	0.64	-0.32	-0.36
Na	-0.45	-0.49	-0.45	-0.36	-0.46	0.13	-0.30	0.13	-0.40	0.55	-0.33	-0.39	0.50	-0.35	-0.18	0.49	1.00	-0.28	0.01	-0.42	0.20	-0.39	0.53	-0.60	0.44	0.51	-0.20	-0.08
Ni	0.96	0.95	0.95	0.57	0.94	-0.07	0.95	-0.11	0.96	-0.07	0.99	0.98	0.03	0.17	0.15	0.05	-0.28	1.00	0.75	0.95	0.43	0.98	-0.86	0.04	0.55	0.15	0.74	0.61
P	0.69	0.70	0.75	0.43	0.74	-0.29	0.82	0.33	0.77	-0.23	0.74	0.76	0.01	0.11	0.31	-0.18	0.01	0.75	1.00	0.74	0.54	0.79	-0.39	0.12	0.55	-0.05	0.71	0.68
Pb	0.91	0.96	0.99	0.62	0.98	-0.20	0.98	-0.12	1.00	-0.30	0.96	0.99	-0.26	0.27	0.15	-0.17	-0.42	0.95	0.74	1.00	0.45	0.99	-0.85	0.17	0.42	-0.11	0.74	0.59
S	0.42	0.41	0.44	0.54	0.45	-0.03	0.53	0.05	0.45	-0.47	0.35	0.45	-0.12	0.25	0.14	-0.33	0.20	0.43	0.54	0.45	1.00	0.47	-0.23	0.26	0.58	0.23	0.81	0.85
Zn	0.95	0.97	0.99	0.64	0.98	-0.16	0.98	-0.03	0.99	-0.26	0.98	1.00	-0.14	0.24	0.21	-0.15	-0.39	0.98	0.79	0.99	0.47	1.00	-0.83	0.19	0.45	-0.03	0.78	0.65

pH	-0.89	-0.86	-0.83	-0.47	-0.82	0.04	-0.79	0.47	-0.83	0.11	-0.84	-0.86	0.18	-0.27	0.06	-0.11	0.53	-0.86	-0.39	-0.85	-0.23	-0.83	1.00	-0.01	-0.35	-0.09	-0.56	-0.38
$CaCO_3$	0.17	0.20	0.20	0.29	0.20	0.15	0.15	0.47	0.17	-0.75	0.08	0.14	-0.38	0.55	0.69	-0.90	-0.60	0.04	0.12	0.17	0.26	0.19	-0.01	1.00	-0.48	-0.42	0.34	0.39
C_{Org}	0.48	0.45	0.42	0.42	0.44	-0.19	0.47	-0.32	0.43	0.16	0.44	0.45	0.33	-0.29	-0.39	0.43	0.44	0.55	0.55	0.42	0.58	0.45	-0.35	-0.48	1.00	0.61	0.64	0.62
Clay	0.11	-0.01	-0.12	-0.04	-0.11	0.51	-0.10	-0.27	-0.10	0.58	0.05	-0.04	0.73	-0.16	-0.16	0.64	0.51	0.15	-0.05	-0.11	0.23	-0.03	-0.09	-0.42	0.61	1.00	0.20	0.26
N	0.78	0.80	0.77	0.78	0.79	-0.14	0.75	-0.05	0.75	-0.51	0.67	0.75	-0.08	0.19	0.12	-0.32	-0.20	0.74	0.71	0.74	0.81	0.78	-0.56	0.34	0.64	0.20	1.00	0.97
C_{total}	0.66	0.65	0.62	0.71	0.64	-0.06	0.63	0.08	0.60	-0.50	0.53	0.60	0.01	0.19	0.21	-0.36	-0.08	0.61	0.68	0.59	0.85	0.65	-0.38	0.39	0.62	0.26	0.97	1.00

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Table SI4. Spearman's rank correlation coefficients (ρ) of correlations between the sum of QAACs and soil properties of the 2018 samples. When $p \leq 0.05$, parameters are correlated significantly.

	pH	$CaCO_3$	Corg	Ctotal	Clay	Ntotal	∑ QAAC	
yrs_irrigation	-0.505	0.315	0.545	0.643	0.193	0.652	0.853	ρ
	0.153	0.381	0.111	0.0501	0.58	0.0501	**0.000392**	P
pH		0.0479	-0.176	-0.0087	-0.274	-0.045	-0.444	ρ
		0.878	0.612	0.948	0.462	0.878	0.204	P
$CaCO_3$			0	0.639	-0.412	0.522	0.378	ρ
			0.983	0.0583	0.243	0.138	0.285	P
Corg				0.706	0.485	0.528	0.357	ρ
				0.0301	0.169	0.124	0.331	P
Ctotal					0.143	0.87	0.592	ρ
					0.676	**0.0000002**	0.0769	P
Clay						0.217	0.0084	ρ
						0.55	0.948	P
Ntotal							0.522	ρ
							0.138	P

Table SI5. Spearman's rank correlation coefficients (ρ) of correlations between the sum of QAACs and soil properties of the 2011 samples. When p ≤ 0.05, parameters are correlated significantly.

	pH	$CaCO_3$	Corg	Ctotal	Clay	Ntotal	∑ QAAC	
yrs_irrig	-0.491	-0.162	0.405	0.691	-0.0206	0.6	0.699	ρ
	0.116	0.614	0.199	**0.0165**	0.946	**0.0467**	**0.0145**	P
pH		0.151	-0.246	-0.577	-0.257	-0.543	-0.743	ρ
		0.633	0.45	0.0602	0.433	0.0762	**0.0068**	P
$CaCO_3$			-0.841	-0.303	-0.788	-0.394	-0.0922	ρ
			0.0000002	0.353	**0.00211**	0.221	0.776	P
Corg				0.491	0.591	0.599	0.114	ρ
				0.116	0.051	**0.0467**	0.714	P
Ctotal					0.395	0.839	0.378	ρ
					0.221	**0.0000002**	0.233	P
Clay						0.419	0.0547	ρ
						0.188	0.86	P
Ntotal							0.299	ρ
							0.353	P

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4 Smectite clay minerals reduce the acute toxicity of quaternary alkylammonium compounds towards potentially pathogenic bacterial taxa present in manure and soil

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Figure 4.1 Photography of *E. Coli* cultures grown on agar.

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Smectite clay minerals reduce the acute toxicity of quaternary alkylammonium compounds towards potentially pathogenic bacterial taxa present in manure and soil

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Quaternary alkylammonium compounds (QAACs) are a group of cationic surfactants which are disinfectants with numerous industrial and agricultural applications and frequently released into the environment. One recent hypothesis is that bacteria present in soil will be protected from acute toxic effects of QAACs in the presence of expandable layer silicates due to interlayer sorption. We therefore studied bacterial growth kinetics with high temporal resolution and determined minimal inhibitory concentrations (MICs) of two QAACs, benzyldimethyldodecylammonium chloride (BAC-C12) and didecyldimethylammonium chlorid (DADMAC-C10), for eight strains of different bacterial taxa (*Escherichia coli*, *Acinetobacter*, *Enterococcus faecium*, *Enterococcus faecalis*, and *Pseudomonas fluorescens*) in relation to QAAC sorption to smectite and kaolinite. The MICs of BAC-C12 and DADMAC-C10 were in the absence of smectite and kaolinite in the order of 10 to 30 µg mL^{-1} and 1.0 to 3.5 µg mL^{-1} for all strains except the more sensitive *Acinetobacter* strain. For all tested strains and both tested QAACs, the presence of smectite increased apparent MIC values while kaolinite had no effect on MICs. Sorption curves without bacteria showed that smectite sorbed larger amounts of QAACs than kaolinite. Correcting nominal QAAC concentrations employed in toxicity tests for QAAC sorption using the sorption curves explained well the observed shifts in apparent MICs. Transmission electron microscopy (TEM) demonstrated that the interlayer space of smectite expanded from 13.7 ± 1 Å to 19.9 ± 1.5 Å after addition of BAC-C12. This study provides first evidence that low charge 2:1 expandable layer silicates can play an important role for buffering QAAC toxicity in soils.

The 2009 OECD list of High Production Volume Chemicals (HPVC) catalogues those chemical substances with annual production exceeding 1000 Mg in at least one OECD member country "in order to identify those which are potentially hazardous to the environment and/or to the health of the general public or worker"[1]. Surfactants are an important class of HPVCs and several neutral and anionic surfactants have extensively been investigated in various environmental compartments. However, the number of studies concerned with the transport, fate, and possible adverse effects of cationic surfactants in the environment is small, despite the fact that electrostatic and hydrophobic interactions with negatively charged surfaces in soil and sediments could promote their persistence[2].

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Within the group of cationic surfactants, quaternary alkylammonium compounds (QAACs) are a heterogeneous group of organic compounds comprising a quaternary nitrogen atom. This nitrogen atom carries one permanent positive charge and at least one alkyl chain rest[3], which together cause their amphiphilic properties used for disinfection and sanitation purposes. The antimicrobial activity of QAACs is mainly based on the interaction of QAAC molecules with cell membranes due to the positively charged nitrogen atom, causing disturbance of membrane integrity and subsequent leakage of cellular contents[4].

Production and consumption data for these QAACs are scattered, but volumes that are released into the environment are tentatively orders of magnitude higher than is the case for example for pharmaceuticals[2]. For agricultural soils, direct application of QAACs with manure, sewage sludge, during irrigation with wastewater or as biocides or adjuvants in agricultural pesticide formulations are the main entry pathways. The use of QAACs as adjuvants for pesticide application enhances solubility of poorly soluble pesticide compounds, rain fastness and penetration of pesticides. Tezel[5] provided consumption data for the biocidal adjuvants in the State of California for BAC-C12-16, DADMAC-C10, DADMAC-C8-10, and DADMAC-C8, totaling about 5000 kg in one year. Typical QAAC concentrations in agrochemical tank-mixed sprays range from 0.05 to 0.5% v/v[6]. According to Mulder et al.[2] predicted environmental concentrations (PEC) for QAACs that are applied to soils with manure are in the order of 3.5 mg kg^{-1} and the median PEC of QAACs in sewage sludge amended soils was reported to be 25 µg kg^{-1}. In a screening study on Swedish sewage sludge and wastewater, Östman et al.[7] demonstrated that QAACs, with the exception of metals, are the biocides encountered at highest concentrations. The concentrations determined in sewage sludge were at least one order of magnitude higher than other antimicrobial active substances with average concentrations often exceeding 100 µg g^{-1} d.w.

The release of large quantities of QAACs into the environment poses a risk for environmental and human health especially with regard to the evolution and spreading of disinfectant and antibiotic resistant bacteria. For example, wastewater irrigated agroecosystems that are exposed to high QAAC loads were spotlighted as "hot spots" of selection for antibiotic resistant bacteria[8,9]. Decisive for the evolution and propagation of antibiotic resistant bacteria within these systems is possibly not only the direct selection by antibiotic agents, but also co- and cross-selection by other compounds such as QAACs, which are used in much higher quantities than antibiotic substances[8,10–14]. Gaze et al.[15] and Tandukar et al.[16] reported the occurrence of antibiotic and QAAC resistance genes in soil and the alteration of microbial communities as a result of long-term exposure to QAACs. Plasmid and genome sequence based studies showed that several QAAC resistance genes are located on the same genetic units as antibiotic resistance genes, promoting the co-selection of antibiotic resistance genes in the presence of QAACs[15–18].

The eco-toxicological effects of organic pollutants in the environment depend largely on their bio-accessibility and persistence, both of which are heavily affected by the pollutant's sorption to constituents of soils and sediments[19]. Due to the molecular nature of QAACs, based on results of sorption studies, and as known from the preparation of organoclays for industrial applications, it appears likely that these compounds are adsorbed and retained by soil clay minerals and soil organic matter. More specifically, in soils, potential adsorbents include soil organic matter, clay minerals, oxides, proteins, and microbial cell walls[2,20]. Sorption of the QAACs can potentially retard their biodegradation and reduce their toxicity, with interlayers of expandable layer silicate minerals appearing particularly suitable to sequester QAACs.

We hypothesized that bacteria exhibit strain specific growth response to QAAC exposure leading to QAAC specific MICs, which are shifted to higher apparent concentrations due to a reduction to acute toxicity as a consequence of QAAC sorption to clay minerals. We further hypothesized that a stronger binding of QAACs to interlayers of expandable smectite clay minerals causes a stronger reduction of toxicity compared to the sorption to non-expandable 1:1 layer kaolinite. In order to test these hypotheses, we determined (1) growth behavior and minimal MICs of BAC-C12 and DADMAC-C10 for a selection of eight bacterial strains including Gram-negative and Gram-positive bacteria representing potential pathogens that can originate in soils for example from the application of manures and (2) investigated the MIC values and sorption curves of BAC-C12 and DADMAC-C10 in the presence of smectite and kaolinite.

Results

Strain specific growth response to QAAC exposure and QAAC specific MICs. MIC values for BAC-C12 and DADMAC-C10 were in a similar range of 10 to 30 µg mL^{-1} and 1.0 to 3.5 µg mL^{-1}, respectively, for all tested strains according to their compound specific susceptibilities (Table 1). Only the *Acinetobacter* strain showed a higher sensitivity to BAC-C12 with a MIC below 5 µg mL^{-1}.

Growth responses to the exposure of QAACs were different among the tested strains. Most often, lower QAAC concentrations had no effect on the growth kinetics of the strains, while higher concentrations totally inhibited the bacterial growth. For individual strains, inhibitory, but sublethal QAAC concentrations led either to an elongated lag phase without changes of the growth kinetic after the extended lag phase or to an elongated lag phase combined with a changed growth kinetic, often characterized by a reduced doubling time. Growth responses differed among the two different studied QAACs. Strain-specific growth effects are visualized by growth curves in Fig. 1. *E. coli* strain ESBL37B15_13_1E grew in the presence of 5 and 10 µg mL^{-1} of BAC-C12 with the same kinetic as under control conditions without QAAC addition. In the presence of 10 and 15 µg mL^{-1} the same growth kinetics were observed, however, the lag phases were extended by 4 and 12 h, respectively (Fig. 1a). This effect was not observed for DADMAC-C10. The strain showed the same growth kinetic as under control conditions up to the addition of 3.0 µg mL^{-1} of DADMAC-C10 but did not grow at higher concentrations. *E. coli* strain ESBL37B15_13_2E showed a different response kinetic in the presence of BAC-C12. In the presence of 10 and 15 µg mL^{-1} the growth was again as without QAACs. *E. coli* ESBL370B15_13_2A in contrast showed up to concentrations of 10 µg mL^{-1} of BAC-C12 and 2 µg mL^{-1} of DADMAC-C10 the same growth kinetic as under

Strains	MIC BAC-C12 [µg mL^{-1}]	MIC DADMAC-C10 [µg mL^{-1}]
E. coli ESBL37B15_13_1E	15 (1x)/25 (1x)	3 (1x)/ 3.5 (1x)
E. coli ESBL232B15_13_2E	15 (2x)/20 (2x)	2.5 (2x)
E. coli ESBL370B15_13_2A	15 (2x)	2.5 (2x)
E. coli ConF4	10 (2x)	2/2.5 (3x)
Acinetobacter sp. KPC-SM-21	< 5 (2x)	2.5 (2x)
P. fluorescens DSM 50090^{T}	25 (4x)/30 (1)	3 (3x)/ 3.5 (1)
E. faecium DSM 20477^{T}	15 (2x)	2 (2x)
E. faecalis DSM 20478^{T}	15 (2x)	1 (1x)/1.5 (1x)

Table 1. Overview of minimal inhibitory concentrations (MICs) of BAC-C12 and DADMAC-C10 on the growth of tested reference strains. Values in brackets indicated the number of replicates which shared a respective value.

control conditions and did not grow at higher QAAC concentrations. *E. coli* ConF4 showed a slight shift in the lag phase (approximately two hours) in the presence of 5 µg mL^{-1} of BAC-C12 and 1.0 µg mL^{-1} of DADMAC-C10, but a reduced growth rate and a lower final OD at a BAC-C12 concentration of 10 µg mL^{-1} (Fig. 1d).

The studied *Acinetobacter* did not grow in the presence of BAC-C12 and showed the same growth kinetic up to a concentration of 2 µg mL^{-1} DADMAC-C10 as under control conditions and did not grow in the presence of higher DADMAC-C10 concentrations (Fig. 1e).

Plenty of different growth kinetics including reduced growth rates and a reduced final OD (cultured biomass) and extended lag phases were obtained for *P. fluorescens* DSM 50090^{T} after QAAC exposure. This was especially observed in the presence of different BAC-C12 and partially in the presence of different DADMAC-C10 concentrations (Fig. 1f). The *P. fluorescens* strain thereby showed growth in several single wells above the MIC values. This may be due to the growth of a resistant subpopulation present in the inoculum or spontaneously developed within the wells.

E. faecium DSM 20477^{T} showed extended lag phases (approx. 4 h) in the presence of 5 and 10 µg mL^{-1} BAC-C12 and 1.5 µg mL^{-1} DADMAC-C10. At lower DADMAC-C10 concentrations, the growth kinetic was identical to that of the control culture (Fig. 1g). *E. faecalis* DSM 20478^{T} showed different growth kinetics in the presence of different BAC-C12 concentrations. In the presence of 10 µg mL^{-1} BAC-C12 the lag phase was shorter but the growth rate higher; in contrast, in the presence of 5 µg mL^{-1} the exponential growth phase started in parallel to the control culture but with a lowered growth rate.

Additionally, Fig. 3 gives a strain-specific and detailed overview of the growth kinetic results for all four strains: *E. coli* ConF4, *E. coli* ESBL37B15_13_1E, *E. faecalis* DSM 20478^{T}, and *P. fluorescens* DSM 50090^{T}.

Clay specific effects on the susceptibility of bacteria to QAACs. The effect of smectite and kaolinite on growth inhibition by QAACs was analyzed with 0.03 mg mL^{-1} of smectite and 0.09 mg mL^{-1} of kaolinite for two *E. coli* strains and the type strains of *P. fluorescens* and *E. faecalis*. For all strains, the addition of smectite apparently increased the MIC values (reduced the QAAC bioavailability) of BAC-C12 by one, partially two concentration steps; for DADMAC-C10 by two to even three concentration steps. In contrast, kaolinite had no effect on the determined MIC values (Table 2). The growth kinetic showed that at strain-specific inhibiting QAAC concentrations, the presence of smectite enabled the bacteria to grow as in the absence of the respective QAACs, as exemplified for strain *E. coli* ESBL37B15_13_1E in Fig. 2. The presence of kaolinite had no effect on the bacterial growth inhibition by either QAAC concentration (Fig. 2a–c). The addition of the two clay minerals itself had no effect on the growth dynamics as was confirmed by the results in control runs without BAC-C12 or DADMAC-C10 addition.

Only a slight increase of the starting OD values was obtained in some of the measurements, presumably due to an increased turbidity with the clay suspension added. The reduction of acute toxicity in the presence of clay minerals at QAAC concentrations close to MICs is summarized for all tested bacterial taxa and QAACs in Fig. 3.

Dissolved QAAC concentration versus total concentration. Sorption curves allowed the calculation of dissolved (bio-accessible) QAAC concentrations in the presence of clay minerals from the nominal or total QAAC concentration. This calculation showed that the freely available or dissolved concentration of BAC-C12 hardly changed when kaolinite was added (Fig. 4a). When smectite was added instead of kaolinite, the dissolved concentration of BAC-C12 decreased over the whole tested concentration range compared to the control resulting in a reduction of more than 10 µg mL^{-1} at the nominal concentrations corresponding to the MIC (Fig. 4b).

For DADMAC-C10 concentrations after kaolinite addition, we observed a slight reduction of the dissolved concentration (Fig. 4c), which was, however, smaller than the reduction observed after smectite addition (Fig. 4d). The dissolved concentration of DADMAC-C10 was reduced by about half of the total concentration at concentrations < 5 µg mL^{-1}. In contrast, at larger concentrations, the reductions of dissolved concentrations compared to total concentrations were smaller. Addition of smectite was able to reduce the dissolved concentration from 7.5 µg mL^{-1} to 2.5 µg mL^{-1} at total concentration of 10 µg mL^{-1}, which is the MIC-value of *E. coli*

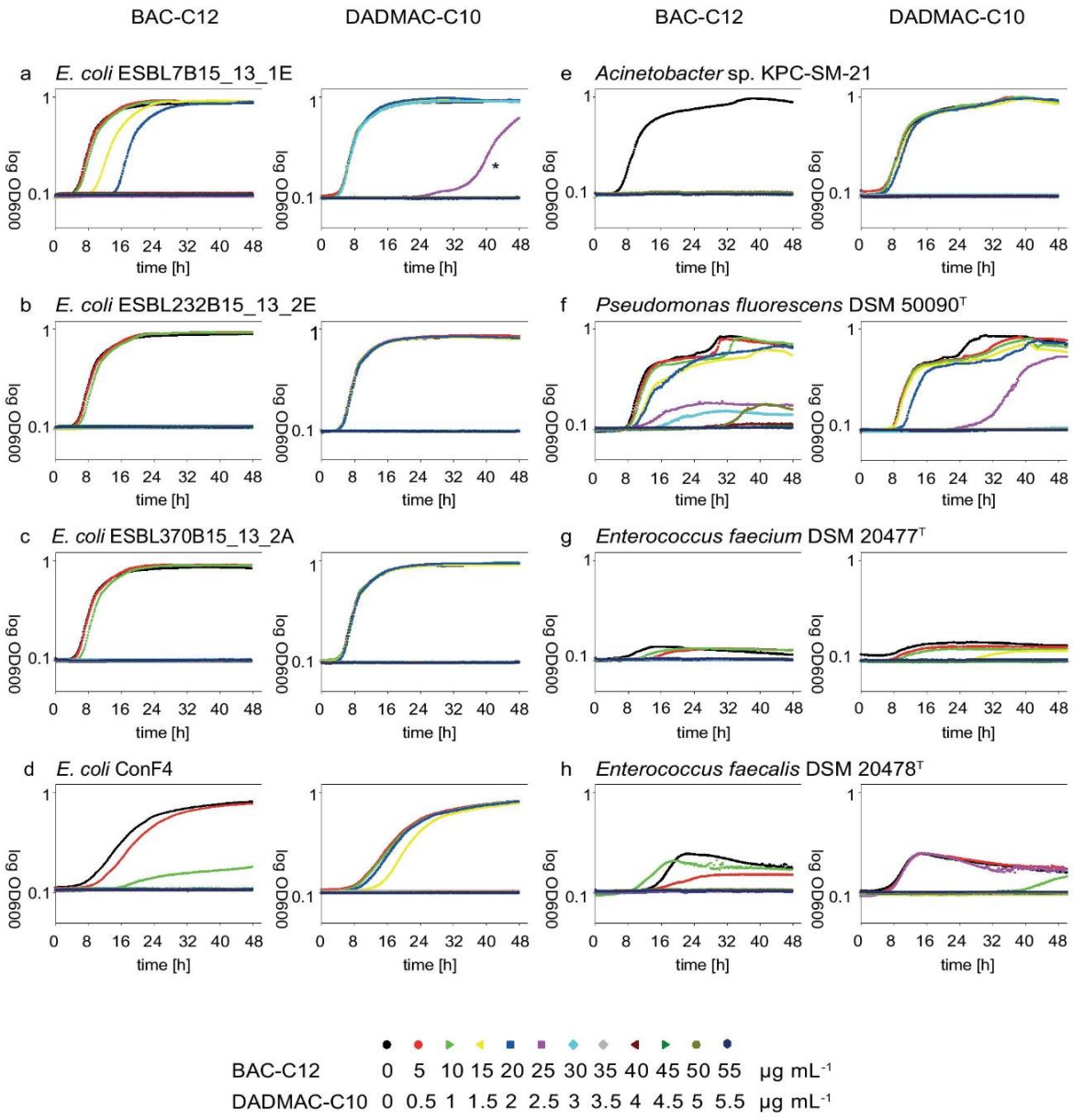


Figure 1. Growth curves for all eight tested bacterial strains in the presence of several concentrations of BAC-C12 and DACMAC-C10. *Bacterial growth in one replicate at 2.5 µg L^{-1}.

ESBL37B15_13_1E und *P. fluorescens* DSM 50090^T (Fig. 4d). Remarkably, for the curve of DADMAC-C10 with smectite added, the slope of the curve was tentatively s-shaped. The second increase of the slope was observed after the concentration $C_{dissolved}$ corresponding to a saturation exceeding 100% of the CEC of the smectite. This showed that $C_{dissolved}$ stayed below the MIC of 2.5 µg mL^{-1} for total concentrations $C_{total} < 10$ µg mL^{-1}. The data thus demonstrated that sorption occurred beyond the CEC, both in smectite and kaolinite experiments with BAC-C12 and DADMAC-C10. Our data also clearly demonstrated that smectite was able to sequester both, BAC-C12 and DADMAC-C10, so that at nominal MIC the QAACs antimicrobial activity could be buffered by reducing the dissolved concentration of the agents.

Beyond the sorption curves that we obtained, we observed that the addition of QAACs to clay minerals led to flocculation and particle aggregation, as might have been expected from the addition of a salt. This phenomenon was observed especially in smectite samples and became more pronounced at higher QAAC and thus increasing salt concentration.

Test strain	Clay mineral	MIC BAC-C12 [µg mL^{-1}]	MIC DADMAC-C10 [µg mL^{-1}]
E. coli ESBL37B15_13_1E	None	12.5	2.5
	Kaolinite	< 6.25/12.5[a]	2.5
	Smectite	25	20
E. coli ConF4	None	< 6.25	1.25
	Kaolinite	< 6.25	1.25
	Smectite	12.5/25[a]	10
P. fluorescens DSM 50090^{T}	None	12.5	2.5
	Kaolinite	12.5	2.5
	Smectite	25	10/20[a]
E. faecalis DSM 20478^{T}	None	> 6.25	1.25
	Kaolinite	> 6.25	1.25
	Smectite	12.5/25[a]	10

Table 2. Shift in apparent MIC [µg mL^{-1}] of four tested strains with BAC-C12 and DADMAC-C10 while adding smectite or kaolinite compared to the control with no addition of clay. [a]Two values due to differences in duplicates.

TEM analysis and molecular considerations of sorption. Both QAAC-treated and untreated clay mineral samples showed frequent folding or cloud like appearance as is typical for Wyoming bentonite[21]. Diffraction patterns showed diffuse rings or no signs of crystallinities and EDX spectra produced Si/Al-ratios of 1:2 as is typical for smectites. For control and BAC-C12-treated samples, folded edge sites, where lattice fringes of the mineral interlayers were visible, were analyzed by measuring stacks of layers (n = 9) using Image J (W. Rasband, Maryland, USA). Average layer thicknesses were 1.37 ± 0.10 nm for the untreated smectite and 1.99 ± 0.15 nm for the BAC-C12-treated smectite (Fig. 5).

The calculated molar diameters were 0.99 nm and 1.06 nm for BAC-C12 and DADMAC-C10, respectively (Eq. (1), see Table 3). Accordingly, the area occupied by one molecule adsorbed to mineral surfaces equaled roughly 0.77 nm^2 for BAC-C12 and 0.88 nm^2 for DADMAC-C10. We used the molar diameter and the BET surface area to estimate the potential number of QAAC molecules adsorbed to the BET surface per ng of smectite and kaolinite. Our estimation did not consider the orientation of the QAACs on clay surfaces. Values for smectite are on the order of 5.5×10^{10} molecules DADMAC-C10 per ng and 4.8×10^{10} molecules BAC-C12 per ng. Theoretically, one ng of kaolinite provides surface area sufficient for 2.9×10^{10} molecules DADMAC-C10 or 2.5×10^{10} molecules of BAC-C12. Combining the mass of the QAAC molecule and the potential sorption space of the clays leads to a ratio (ng/ng) of 2.8×10^{-2} BAC-C12/smectite (2.6×10^{-2} DADMAC-C10/smectite) and a ratio of 1.44×10^{-2} for BAC-C12/kaolinite (1.35×10^{-2} DADMAC-C10/kaolinite). Considering the experimental conditions, with an addition of 30 µg mL^{-1} smectite and 90 µg mL^{-1} kaolinite, we conclude that theoretically, based on our calculations, the surface was completely covered by QAAC at $C_{total} = 1.2$ µg mL^{-1} for smectite and at $C_{total} = 0.75$ µg mL^{-1} of QAAC for the kaolinite experiments.

Discussion

The performed high throughput growth kinetic measurements of selective Gram-negative and Gram-positive bacterial strains in the presence of different concentrations of two different QAACs combined with two different clay minerals provided information on bacterial strain specific responses to QAACs and the effects of different soil minerals on the QAAC toxicity.

The two different QAACs showed substance specific differences in toxicity. MIC values of DADMAC-C10 were approximately ten-fold lower, indicating a higher toxicity for bacterial growth than BAC-C12[22,23].

The detailed study of growth kinetics further illustrated the different response of bacterial taxa and even different bacterial strains within one genus to the exposure of QAACs which are often overseen in endpoint MIC measurements (see[24]). The kinetic data showed a broad range of different reactions to QAAC exposure while endpoint MIC determinations differentiated only between endpoint growth as in controls, reduced growth (reduced endpoint OD) or total growth inhibition by specific QAAC concentrations. In contrast, kinetic data also showed that QAAC concentrations, which had no effect on growth measured at the end of the incubation experiment, partially affected bacterial growth by an extension of lag phases or changing growth rates. The growth experiments performed showed for example that different *E. coli* strains exhibit one or both of the aforementioned growth characteristics depending on the QAAC (Fig. 1). Especially the studied *P. fluorescens* strain showed a broad range of different reactions to QAAC exposure, which differed between the two QAACs, different QAAC concentrations, but also among replicate cultures. The growth response of *P. fluorescens* DSM 50090^{T} indicated a high potential for a spontaneous adaptation to QAAC exposure. This was also reported in a previous experiment in which the growth response of the *P. fluorescens* type strain to QAACs was investigated[25]. Changes in the fatty acid composition of the cell membranes or expression of resistance genes as QAAC-efflux were reported[26,27].

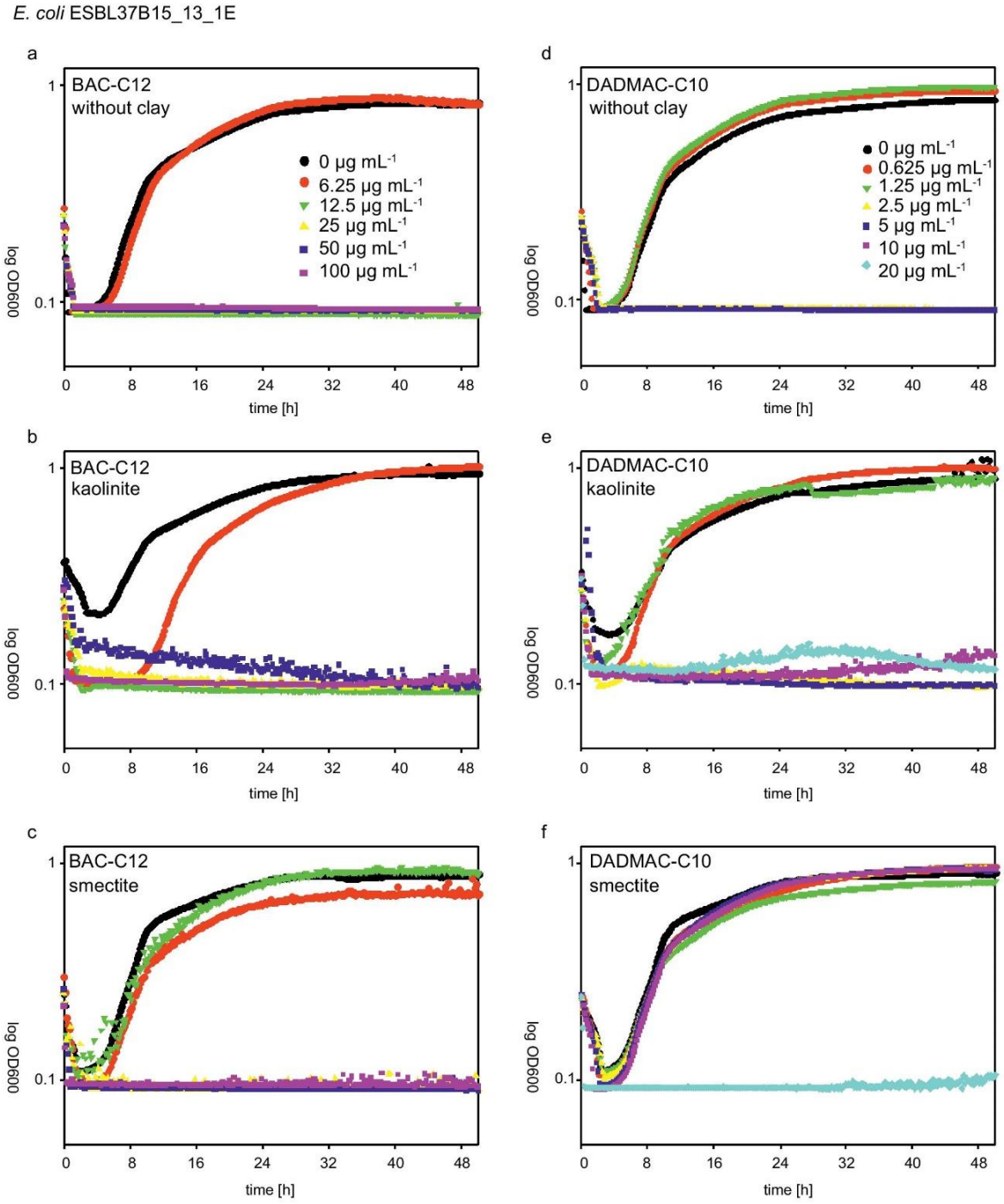


Figure 2. Growth curves of *E. coli* ESBL37B15_13_1E in the presence of different BAC-C12 (**a**–**c**) and DADMAC-C10 (**d**–**f**) concentrations without clay mineral addition (**a**, **d**) and in the presence of smectite (**b**, **e**) and kaolinite (**c**, **f**). Visible are growth curves at QAAC concentrations that were inhibitory in the absence of clay minerals and the presence of kaolinite while growth was not inhibited in the presence of smectite.

Reported concentrations of QAACs in wastewater range from 9 to 42 µg mL^{-1}[28]. Since the MICs determined in our experiments were in a comparable concentration range, an inhibition of the growth of susceptible bacteria

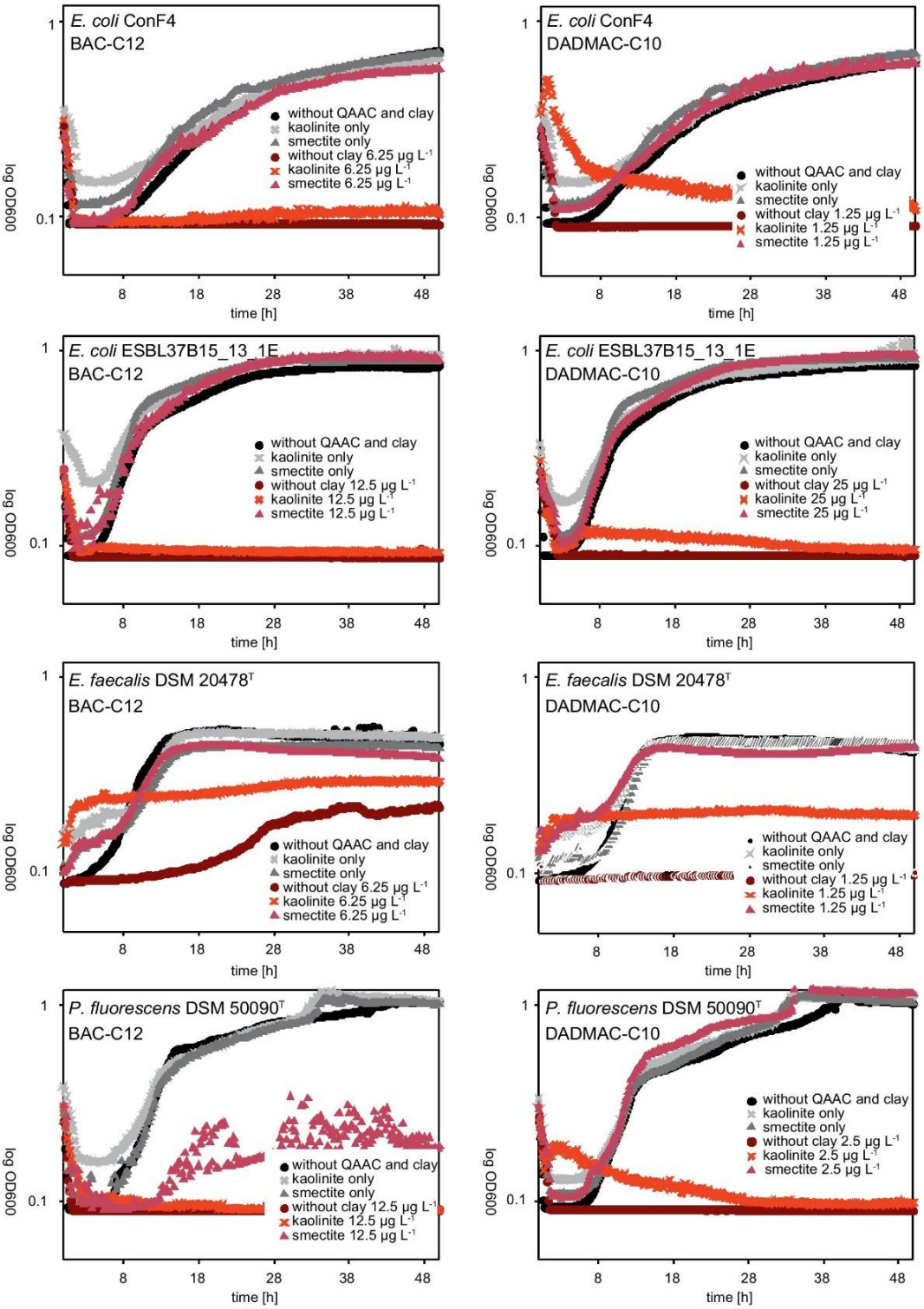


Figure 3. Growth curves of *E. coli* ConF4, *E. coli* ESBL37B15_13_1E, *E. faecalis* DSM 20478^T, and *P. fluorescens* DSM 50090^T in the presence of MICs of BAC-C12 and DADMAC-C10 without clay mineral addition. Same concentrations of QAAC are plotted for kaolinite and smectite experiments. Greyscale plots are without the addition of QAAC while reddish plots are with QAAC.

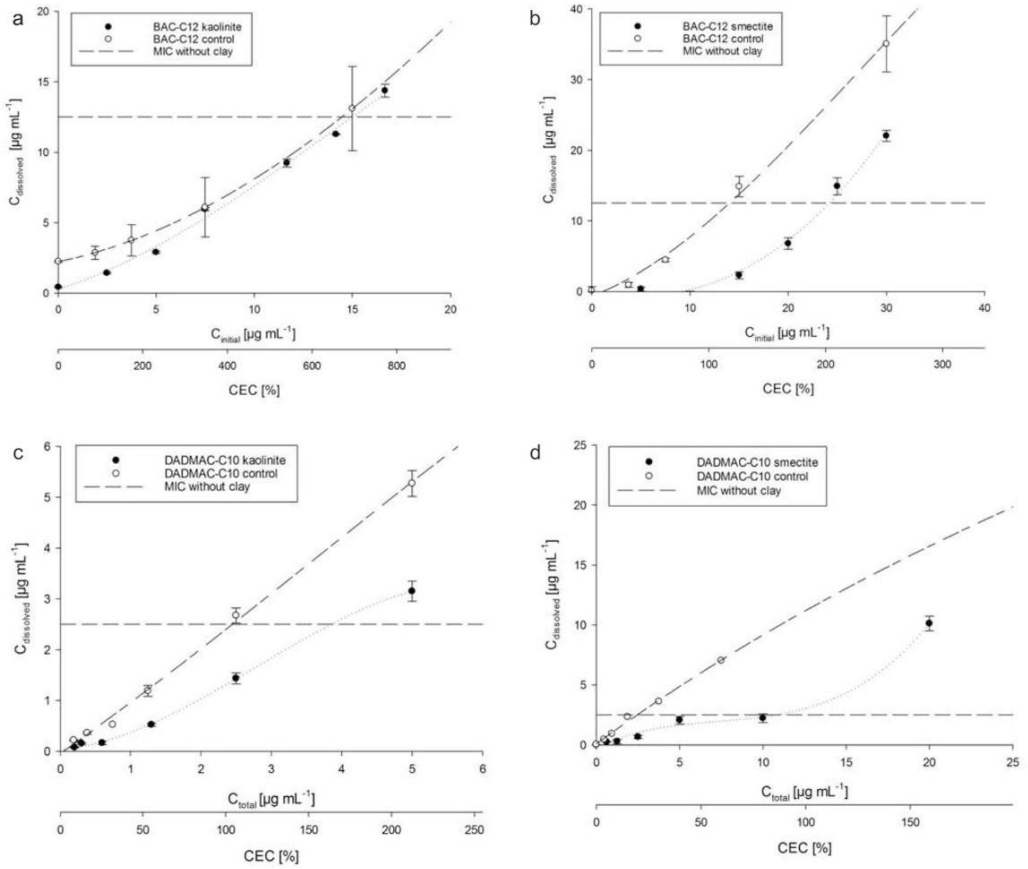


Figure 4. Dissolved concentration [$C_{dissolved}$] versus initial concentration [C_{total}] of BAC-C12 and kaolinite (**a**), BAC-C12 and smectite (**b**), DADMAC-C10 and kaolinite (**c**), DADMAC-C10 and smectite (**d**) and MICs shown as long dash lines for *E. coli* ESBL37B15_13_1E and *P. fluorescens* DSM 50090^T. The according controls without clay minerals are shown as empty circle.

seems principally possible. It is important to note in this context that for the selection of resistant strains of bacteria, minimum selective concentrations (MSCs) are more relevant than MICs and MSCs are often several orders of magnitude smaller than MICs[29], especially when bacteria are exposed to mixtures of chemicals[30]. Hence, a selection of bacteria resistant to QAACs and a potential co-selection of antibiotic-resistant bacteria in wastewater or manure containing QAACs as well as in soils receiving them as fertilizer appears even more probable. However, wastewater, manure, and soils contain particles including clay particles, which can bind QAACs to their surfaces, inactivating them with regard to their effects on bacteria.

For all tested strains, the addition of smectite showed a clear buffering effect on the toxicities of the employed QAACs confirming our hypothesis that 2:1 layer silicates with their interlayer regions, high specific surface areas and high CEC are more effective in reducing the QAAC toxicities than 1:1 layer silicates, which confirms the postulated hypothesis by Mulder et al. (2018)[7] of interlayer sorption by smectites in environmental systems. Structurally, when compared to kaolinite, interlayer regions (which are not taken into account by the specific surface area) of smectites are of primary interest[31]. When QAACs enter interlayer galleries they can be sequestered from the surrounding medium, so that bacteria are sterically protected from a direct exposure or in other words, the bio-accessible QAAC concentration appears to be reduced in the presence of smectite.

It is remarkable that smectite increased the observed MIC value of DADMAC-C10 more than the MIC of BAC-C12, so we note a compound-specific interaction with clay and sequestration. In contrast to BAC-C12, DADMAC-C10 has two hydrophobic alkyl chains and thus exhibits stronger hydrophobic interaction compared to BAC-C12 with only one C12 alkyl chain (see also Table 3). Ismail et al.[32] studied sorption of several QAAC homologues including BAC-C12 to sewage sludge and found that sorption affinity correlated positively to hydrophobicity and negatively to critical micelle concentration. A stronger hydrophobic interaction of alkyl chains with clay mineral surfaces and between two layers of QAACs on the clay mineral surfaces could lead to lower accessibility of QAACs for the bacteria and a higher sorption affinity.

However, in our experiments the correlation of MIC to molecular adsorption to the surface was only moderate (Fig. SI-S4; $R^2 = 0.75$). Therefore, additional sorption sites should be considered as the cause of the MIC shifts. This is underlined by estimation of surface area occupancy (0.77 nm^2 and 0.88 nm^2) based on molar diameters.

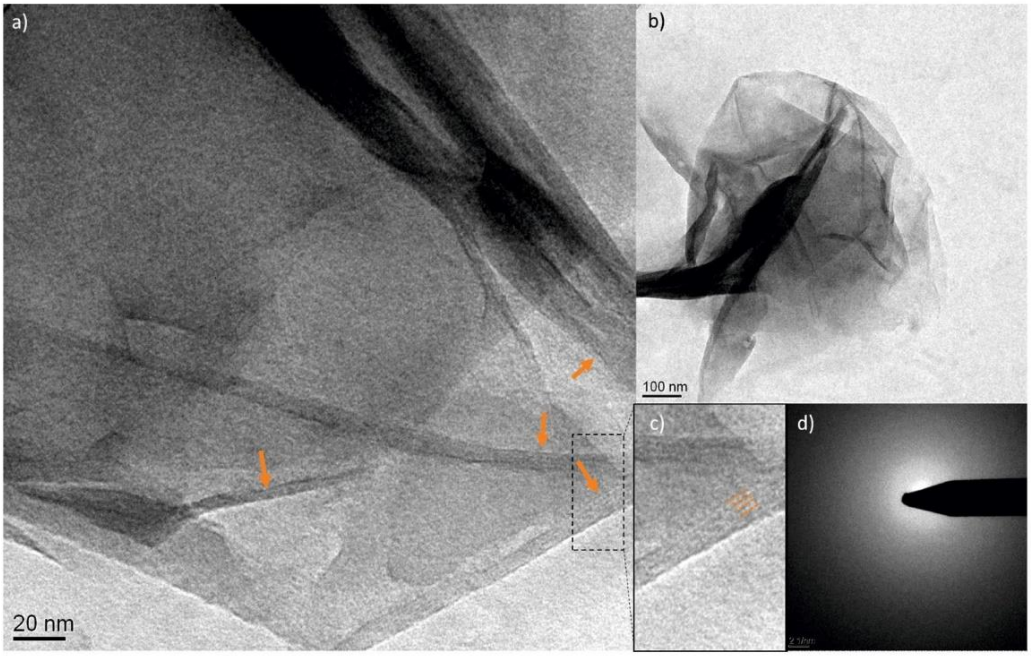


Figure 5. TEM images of a 50 µg BAC-C12 mL^{-1} saturated smectite. Orange arrows (**a**) indicate visible lattice fringes at 81,000 × magnification and crystallographic z-axis. (**b**) Overview of the larger particle or aggregate of particles with typically folded edges. (**c**) Detail demonstrating how three individual layers were identified (orange dashed line); space between lines = 1.99 ± 0.15 nm. (**d**) Diffractogram taken from particle as shown in (**b**) does not show crystallinity due to short-range order of the clay mineral.

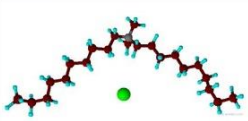
IUPAC name	*N*-Decyl-*N*,*N*-dimethyl-1-decanaminium chloride	*N*-Benzyl-*N*,*N*-dimethyl-1-dodecanaminium chloride
Molecular structure (schematically)		
CAS #	7173-51-5	139-07-1
Chemical formula	$C_{22}H_{48}NCl$	$C_{21}H_{38}NCl$
Acronym	DADMAC-C10	BAC-C12
CMC [mM]	1.78 ± 0.05	8.3 ± 0.1
Mol. Mass (g/mol)	362	339
mp [°C]	88	42
d_m [nm]	0.99	1.06

Table 3. Properties of the two QAAC-molecules used in the experiments. Both belong to the linear alkylammonium compounds. The equivalent molar diameter is given as d_m. The critical micelle concentration (CMCs) were determined in deionized water according to[50] using spectrofluormetry and pyren as fluorescens probe.

Giese and van Oss[33], who performed calculations by layer charge, found that individual QAACs molecules occupy an area between 0.23 nm^2 and 1.35 nm^2. Taking into account the clay concentrations used in the experiments, our calculations indicate that the clay surface was completely covered with QAAC molecules at dissolved QAAC concentrations of 0.77 µg mL^{-1} and 1.30 µg mL^{-1}. Since MIC shift occurred above those QAAC concentrations, additional mechanism and sorption spaces, besides a single layer sorption to clay surface, influence the shift of the MIC values.

Besides hydrophobic interactions with surfaces, a second sorption mechanism to the clay surface and interlayers of QAACs, is via cation exchange, which leads to a plateau around the CEC where all cations (mostly Mg^+ in this case) are replaced and thus saturation is achieved. At higher concentrations, QAAC molecules can be

adsorbed in an orthogonal arrangement to the mineral surfaces and they might form bi- and multilayer structures that are connected by hydrophobic interaction[34].

A closer look at nominal MIC values for the kaolinite-amended trials revealed that values for DADMAC-C10 were enhanced stronger than BAC-C12, which is explained by the higher initial QAAC concentration, thus the weak buffering effect of kaolinite steps out more strongly as the surface sorption is the leading process here.

The concentration of dissolved QAACs strongly increased in smectite experiments at sorbed concentrations exceeding 100% CEC (Fig. 4b, d). This was not observed in the kaolinite experiments. As the largest fraction of the CEC of smectite is localized in its interlayer space, this suggests that sorption in the interlayer space was much stronger compared to the outer surface. The results were in good agreement with our hypothesis that the sorption sites of smectites as provided by the CEC play an important role in QAAC sequestration and buffering.

The data of the sorption curves demonstrated that sorption occurred beyond the CEC, both in smectite and kaolinite experiments with BAC-C12 and DADMAC-C10. This is consistent with results of Kwolek et al.[35], who investigated the sorption of BAC to sodium smectite minerals, but not 1:1 layer silicates.

QAAC adsorption at concentrations exceeding 100% CEC was possibly caused by hydrophobic interaction of the alkyl chains of multiple QAAC molecules. This interaction takes place on the outer surfaces of kaolinite and smectite, but is stronger on smectite due to its higher charge per formula unit, which is commonly 0.25–0.6 in smectite and 0 in kaolinite[36]. In contrast to ion-ion interaction, hydrophobic interaction is a very weak force[37] and hence bacteria may be able to access the second layer of QAACs at these spots even though the molecules are adsorbed. Zhu et al.[38] assumed that an alkyltrimethylammonium compound (ATMAC-C16) does not only form lateral mono- and bilayers, but also paraffin-type layers in the interlayer space of montmorillonite when the amount of QAACs exceeds the CEC. Altkyltrimethylammonium compounds are structurally related to BAC and DADMAC; the central nitrogen is surrounded by one alkylchain and three methyl groups. Thus, very low dissolved BAC-C12 concentrations at sorbed concentrations < 100% CEC, compared to DADMAC-C10 indicate that BAC-C12 can enter interlayers more easily. The two alkyl-chains of DADMAC-C10 might hinder entry into the interlayer space. This could also explain the higher MIC buffering potential of smectite in the BAC-C12 experiment (Fig. 4b) compared to DADMAC-C10 (Fig. 4d). Polubesova et al.[39] showed that adsorption of two short chain BACs with a methyl and the other with an ethyl group (sort of BAC-C1 and BAC-C2) to montmorillonite and illite is limited to 98% and 108% of CEC, respectively, which indicates that the formation of interlayer bilayers is driven by the length and amount of alkyl chains.

We assume that bacterial cells do not come in contact with BAC-C12 and DADMAC-C10 molecules that are sequestered into the interlayer galleries due to size exclusion of bacterial cells. For reference, the used QAACs are between 0.99 nm and 1.06 nm in diameter while bacterial cell sizes are in the order of 1 and 20 µm. This is supported by the fact that basal spacing of alkylammonium cation enriched smectite is between 1.48 nm and a maximum of 4.03 nm[38].

The observed aggregate formation in our experiments when clay minerals came in contact with QAAC solutions is in line with the results of Penner and Lagaly[40], who described this effect for alkyltrimethylammonium compounds and smectite, which are similar to BACs and DADMACs. Flocculation did (of course) not occur when clay was mixed with pure water and it was stronger for smectite suspensions in comparison to kaolinite suspensions. The presumable causes of this effect is on the one hand that QAACs carry a permanent cationic charge at the nitrogen that is attracted to the negatively charged surface of smectite particles. When adsorbed to the clay surfaces the positive charge might be still available for other negatively charged surfaces and thus further clay particles are attracted towards the QAAC molecule, which is holding the aggregates together. On the other hand, the repulsive forces of negatively charged clay surfaces is lowered by the absorbed QAAC. Thus, the attractive forces (e.g. van der Waals forces) prevail over repulsive forces and the net interaction potential leads to flocculation. Derjaguin, Landau, Verwey, and Overbeek described this so-called DLVO theory at first[41]. Especially the QAAC molecules inside those aggregates that are surrounded by smectite minerals might not be accessible for bacteria. This effect is less pronounced in kaolinite experiments as expected, since the charge of kaolinite surfaces is neutral.

The results confirm our hypothesis that 2:1 layer silicates like smectite are more effective in increasing the apparent MIC as non-expandable clay minerals. But we have to acknowledge that not only the CEC, but also the surface area of smectites as well as the charge density is greater than in kaolinites and may influence this MIC value shift.

TEM images document interlayer sorption and expansion of the basal spacings by QAACs to smectite. The imaging data are in good agreement with previous data based on X-ray diffraction by Kwolek et al.[35] that showed interlayer space of smectites increased twice as much by BACs with alkyl chain lengths greater than ten as with alkyl chain lengths smaller than ten. This provided strong visible evidence for the interlayer sorption of BAC-C12 to smectites as reported more generally for QAACs to 2:1 expandable layer silicates determined by x-ray diffraction[42].

Conclusions

We were able to confirm our hypothesis that there is a strain specific growth response to QAACs. This response differs between BAC-C12 and DADMAC-C10 by a factor of ten. Minimum inhibitory concentrations for the test strains were in a concentration range comparable to concentrations reported for wastewater. Considering that minimum selective concentrations are commonly several orders of magnitude smaller than MICs, this suggests that a selection of QAAC resistant bacterial strains could principally occur in wastewater, manure or soils receiving both as fertilizer. Since wastewater, manure and soils contain particles including clay minerals that can sorb QAACs, the magnitude of the effect of sorption on the inhibition of bacterial growth by QAACs is essential for assessing the likelihood of the selection of resistant bacteria. Sorption of QAACs to clay minerals

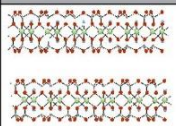
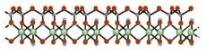
Name	Montmorillonite	Kaolinite
Molecular structure (schematically)		
Brand	Volclay	Pharmakaolin B860
Supplier	AMCOL Specialty Minerals (Cheshire, England)	ZIEGLER Mineralstoffe (Wunsiedel, Deutschland)
Chemical formula	$(Na,Ca)_{0.33}(Al_{1.67}Mg0.33)Si_4O_{10}(OH)_2 \cdot nH_2O$ [a]	$Al_2Si_2O_5(OH)_4$ [b]
BET surface area [$m^2\ g^{-1}$]	42	22
CEC [$cmol_c\ kg^{-1}$]	106.5	6.7

Table 4. Properties of the clay minerals used in the experiments. The cation exchange capacity (CEC) of the minerals was determined with the Cu-triethylenetetramine-complex-method. Specific surface areas were derived from BET sorption isotherms. [a51]. [b52].

can also explain how sensitive bacteria as the tested manure derived *Acinetobacter* strain can survive in a habitat containing concentrations of QAACs which are in the range of MICs.

Shifts of apparent MICs to larger total QAAC concentrations in combination with sorption curves prove that sorption of QAACs to 2:1 expandable clay minerals reduces their bio-accessibility and acute toxicity. Kaolinite was no efficient adsorbent. The magnitude of the MIC shift depends on the QAAC structure as well as the clay mineral structure. Our calculations confirmed that BET surfaces are not sufficient to explain amounts QAAC adsorbed and that interlayer sorption for smectite likely plays a dominant role.

Independent microscopic observations of increased interlayer distance of QAAC-treated smectite compared to untreated smectite together with the stronger reduction of toxicity in the presence of smectite compared to kaolinite point to the relevance of interlayer sorption of QAACs for the detoxification. In addition, the entrapment of QAACs in flocs and aggregates of clay mineral particles potentially causes a further reduction of bio-accessibility. Future experiments should clarify the effect of natural organic matter and micro-aggregation on the detoxification of an extended spectrum of different QAACs. Further research on the toxicity buffering effects of e.g. soil organic matter and micro aggregate formation is needed. In order to get a toxicity ranking expressed by MIC values, ATMACs should be tested in future works as well as QAACs with different chain length, since BAC-C12 and DADMAC-C10 are representing only two of the three main QAAC groups.

Materials and methods

Chemicals and materials. In view of the complexity of the system soil under study, it was our aim to address our central hypothesis by using model compounds. BAC-C12 and DADMAC-C10 were chosen as they belong to the most frequently encountered QAACs in the agricultural environment[2]. BAC-C12 consists of a benzyl, two methyl and one dodecyl group. DADMAC-C10 is composed of two methyl and two decyl groups. Both have a positively charged nitrogen cation, thus both are organic salts. Table 3 gives an overview of the structure and the main properties of these two QAACs.

High purity water (MQ) was prepared by a Milli-Q water purification system (Millipore) and used for all mineral and QAAC stock and working solutions, dilutions and HPLC (high pressure liquid chromatography)-solvent preparation. Samples and solutions were centrifuged either with a Micro Star 17R centrifuge (VWR) when the volume was below 2 mL or Rotanta 460R (Hettich) for volumes > 2 mL. BAC-C12 (> 98.0%) was obtained from TCI (Tokyo, Japan) and DADMAC-C10 (≥ 99.0%) purchased from Glentham Life Science (Wiltshire, UK).

The clay minerals used in the studies were the 2:1—layer silicate montmorillonite MX-80 as smectite (AMCOL, Cheshire, England) and the 1:1—layer silicate kaolinite pharmakaolin (ZIEGLER Minerals, Wunsiedel, Germany). The cation exchange capacity (CEC) was 106.54 $cmol_c\ kg^{-1}$ and 6.65 $cmol_c\ kg^{-1}$ for smectite and kaolinite, respectively. The Brunauer–Emmett–Teller specific surface area (BET) as determined via N_2-physisorption-isotherm was 42 $m^2\ g^{-1}$ and 22 $m^2\ g^{-1}$ for smectite and kaolinite, respectively (Table 4, with schematic mineral structures).

For the clay purification process, a 1 N sodium acetate buffer ($H_3CCOONa$, ≥ 99.5%, Merck; CH_3COOH 100%, Merck), sodium carbonate solution 0.125 g L^{-1} (Na_2CO_3, 99.9%, Merck), H_2O_2 (30%, Merck) and 1 M NaOH (Titrisol, Merck) were used. For the CEC determination, triethylenetetramine (≥ 97%, Sigma-Aldrich) and dehydrated copper(II) sulfate pentahydrate (Ph Eur, Merck) were used in order to prepare a 0.01 mol L^{-1} Cu-triethylenetetramine color complex solution.

Acetonitrile (100%, HiPerSolv Chromanorm, VWR), formic acid (≥ 98%, Rotipuran, Carl Roth) and ammonium formate (99%, Acros Organics) were used to prepare HPLC eluents as well as all QAAC stock and working solutions.

Bacterial strains. Eight bacterial strains (Table 5) were selected for the experiments, which represent taxa of pathogenic bacteria causing immense healthcare problems especially due to the development of antibiotic

Species	Strain	Isolation source	References
Escherichia coli	ESBL37B15_13_1E	Manure	*Schauss* et al. (2015)
Escherichia coli	ESBL232B15_13_2E	Manure	*Schauss* et al. (2015)
Escherichia coli	ESBL370B15_13_2A	Biogas plant digestate	*Schauss* et al. (2015)
Escherichia coli	ConF4	K12 variant	
Acinetobacter sp.	KPC-SM-21	Biogas plant digestate	Mishra (2014)
Pseudomonas fluorescens	DSM 50090^T	Biofilter	Migula (1900)
Enterococcus faecium	DSM 20477^T	–	Schleifer & Kilpper-Bälz (1984)
Enterococcus faecalis	DSM 20478^T	–	Schleifer & Kilpper-Bälz (1984)

Table 5. Overview of bacterial strains applied in this study. –, Source not known.

resistances. Those taxa are present in manure and wastewater and represent nosocomial pathogens which can survive in nature (soil) after their release.

Bacterial growth experiments were performed with four *E. coli* strains, the type strain of *P. fluorescens*, an *Acinetobacter* strain (all *Gammaproteobacteria*, Gram-negative bacteria), and the type strains of *E. faecalis* and *E. faecium* (Firmicutes, Gram-positive bacteria) (Table 5). Strains were isolated either from environmental samples in previous studies or obtained from type culture collections (Table 5). Strains were selected because genome sequences were available for all of them and they mostly originate from manure and soil. All strains were assigned to the species level and well characterized with respect to their antibiotic resistances and physiological properties. All strains were pre-cultured on Mueller–Hinton agar (MHA, Carl Roth) at 37 °C for 24 h. For long-term preservation fresh bacterial biomass was suspended in Gibro newborn calf serum (NBCS, ThermoFisher Scientific) and stored at − 20 °C.

Bacterial growth experiments and MIC value determination. Bacterial growth response to QAACs (BAC-C12 and DADMAC-C10) exposure and the determination of QAAC specific MIC values were performed in microdilution assays according to the standard procedure for antibiotic susceptibility testing given by the Clinical Laboratory Standards Institute CLSI[43] guidelines. All experiments were performed in Mueller–Hinton broth (MHB) in a total volume of 200 µl using transparent flat 96 well microtiter plates (Greiner Bio-One GmbH) covered with sterile transparent plastic lids. Each well was preloaded with 100 µL MHB (control wells without QAACs) or 50 µL double concentrated MHB mixed with 50 µL four-fold concentrated QAAC solutions. The used QAAC stock solutions were dissolved in autoclaved pure water and filtered using 0.45 µm sterile cellulose-acetate filter units (VWR). The stock solution was stored at 4 °C and refreshed monthly. Eleven different QAAC concentrations were tested in parallel with a growth control without QAAC addition. The final QAAC concentrations were in the range of 5 to 55 µg mL^{-1} for BAC-C12 and 0.5–5.5 µg mL^{-1} for DADMAC-C10 (with a gradual increase by 5 µg mL^{-1}). The applied concentration ranges for BAC-C12 and DADMAC-C10 were based on the range of the environmental BAC-C12 concentration (9–42 µg mL^{-1}) in wastewater since data for soils are currently not available[28]. In preliminary tests with a larger range of QAAC concentrations (data not shown) we found that the tested strains all had inhibited growth in concentrations ranges between 5 and 30 µg mL^{-1}. Further evaluation in preliminary tests had shown that DADMAC-C10 was ten times more toxic for bacteria as BAC-C12. Thus, DADMAC-C10 concentrations were selected accordingly. Immediately before the incubation experiments were started, 100 µL inoculated MHB was added to each well. The MHB cell suspensions were generated as follows: an inoculation loop full of fresh overnight cultured bacterial biomass was suspended in 6 mL of an autoclaved 0.9% (w/v) NaCl solution to a McFarland standard density of 0.5 which provides an optical density comparable to the density of a bacterial suspension of 1.5×10^8 colony forming units (CFU) mL^{-1}. For Gram-negative bacteria 109 µL and for Gram-positive bacteria 218 µL of the suspensions were used for the inoculation of 12 mL MHB. Microtiter plates were incubated for 48 h at 25 °C in an Infinite M200 or Infinite F200 spectrophotometer (Tecan; Germany). The bacterial growth was monitored during incubation by a continuous optical density (OD) measurement at a wavelength of 600 nm in 10 min intervals. Before each OD measurement the microtiter plates were shaken for 15 s with an amplitude set to 6. Growth curves were displayed in SigmaPlot 13.0 (Systat). The lowest concentration above the last concentration of the QAACs that showed bacterial growth represents the MIC.

Growth experiments elucidating the buffering effect of clay minerals on QAAC toxicity. The potential buffering effect of clay minerals on the susceptibility of bacterial cultures to the exposure of QAACs (MIC value shift) was tested as described above in 96-well plate test systems. Concentrated QAACs and clay mineral solutions were therefore pre-mixed and equilibrated for 30 min in 2 mL Eppendorf tubes before they were added to microtiter plates and inoculated with bacterial biomass. The nominal QAAC concentrations (uncorrected for sorption), that were chosen around the predetermined MIC values, were 6.25, 12.5, 25, 50, and 100 µg mL^{-1} for BAC-C12 and 0, 0.625, 1.125, 2.5, 5.0, 10, and 20 µg mL^{-1} for DADMAC-C10, respectively.

In a preliminary trial different clay mineral concentrations were tested in parallel. Subsequently, the clay mineral concentrations for the growth experiments were chosen in a way that the highest concentration of QAAC employed corresponded to the CEC value of 76.4 $cmol_c$ kg^{-1} for smectite[44]. As kaolinite is not capable of interlayer sorption, the amount of kaolinite in the incubation experiments were adjusted in order to provide

identical BET surface areas as present in the smectite experiments. The potential interlayer surfaces that can hypothetically be accessed by QAACs are only present in smectites and are not expressed by the BET surface area values of purified clays given in Table 4. The larger specific surface area for smectite compared to kaolinite is a result of their smaller crystallite size.

First tests included final smectite and kaolinite concentrations in the range of 0.016 to 0.064 mg mL^{-1} and 0.094 to 0.374 mg mL^{-1}, respectively. Finally, clay mineral concentrations of 0.03 mg mL^{-1} (smectite) and 0.09 mg mL^{-1} (kaolinite) were selected for the further experiments. Growth controls included bacterial growth in MHB without any additives and in the presence of clay minerals. Each control and each QAAC/clay ratio was tested in duplicate (Tab. SI-S1-S4).

Clay preparation and characterization. Before kaolinite and smectite were used in the experiments, a pretreatment was carried out in order to purify them; the associated components carbonate and organic matter were removed and a subsequent particle size fractionation allowed for a homogenous clay fraction (≤ 2 µm). The method applied was adapted from Tributh and Lagaly[45]. For carbonate removal, a 1 N sodium acetate buffer solution ($H_3CCOONa$, CH_3COOH) adjusted to pH = 5 was added to each clay type. The suspensions were stirred periodically while heating to 90 °C in a water bath until bubbling ceased. Suspensions were then centrifuged at 920 g for 30 min and the clear supernatant was discarded. This process was repeated twice, omitting the heating.

Organic matter and sulfide traces were removed by subsequently adding H_2O_2 in a pH = 5 sodium acetate buffer. The sample was then re-suspended and the suspension heated to 65 °C and H_2O_2 added every hour until bubbling ceased. In order to decompose the remaining H_2O_2, the suspension was brought to a slight boil just below 100 °C. The supernatant was poured off after centrifuging at 920 g for 30 min.

All clay samples were dispersed with pH = 10 sodium carbonate solution. The pH values were checked and if necessary adjusted to 8.5–9.5 with NaOH prior to particle size fractionation. Separation of particles < 2 µm was accomplished by centrifugation (Rotanta centrifuge, Hettich, Tuttlingen, Germany) for 6 min at 100 g according to Stokes law. The supernatant containing the < 2 µm fraction was decanted and collected. The sample dispersion and centrifugation step was repeated three times. To reduce the volume of the gained clay suspension, NaCl was added to flocculate the sample. After decanting the clear supernatant, the remaining suspension was washed with deionized water (DI water) until the electric conductivity was smaller than 20 µS cm^{-1}. The exact clay concentration of the suspension was determined gravimetrically by drying suspension aliquots at 105 °C.

Determination of the CEC was performed with copper triethylenetetramine $[Cu(trien)]^{2+}$ complex using the method of Meier and Kahr[46] as modified by Ammann[47]. The $[Cu(trien)]^{2+}$color complex solution was added to 100 mg of lyophilized smectite or kaolinite and the mixture was shaken for 30 min. The samples were centrifuged at 2950 g for 30 min. Three mL of the supernatant were transferred into cuvettes and its extinction measured with a photometer (T80 UV/Vis Spectrometer, PG Instruments Ltd) at 577 nm. The CEC was calculated from the concentrations of the $[Cu(trien)]^{2+}$ color complex according to Ammann[47]. Measurements were performed in triplicate.

The surface area of porous solids and fine powders such as dried clay minerals can be measured by gas adsorption[48]. The BET method was used to determine the specific surface area of the clay minerals. To this end, 100 mg of freeze-dried clay from the pretreated smectite and kaolinite suspensions were heated to 120 °C for 16 h in order to desorb air moisture. The adsorption and desorption of N_2 was measured with a Quadrasorb evo (Quantachrome, Boynton Beach, USA) and the BET sorption isotherm served to determine the specific surface area[48].

Transmission electron microscopy (CM30 Phillips TEM with EDAX 9900 EDX-Detektor, 300 kV) was used in order to observe fine structural changes of smectite particles upon QAAC treatment. Smectite suspensions containing 0.3 mg smectite mL^{-1} were mixed with 0, 30, 50, and 2000 µg mL^{-1} of BAC-C12 in polypropylene centrifuge tubes and allowed to equilibrate at 250 rpm on an orbital shaker for 1 h. Smectite samples were mounted onto a carbon-coated copper grid (Plano, Wetzlar, Germany) from suspension. For each sample we intended to investigate a minimum of four locations on the grid where we could record images in a magnification range of 10,500× to 110,000×. Control and 50 µg mL^{-1} BAC-C12 treated smectite grids were used for systematic analysis.

Characterization of QAACs. We used the equivalent molar diameter d_m to get an estimate of the size of QAAC molecules. This simplified model assumes a spherical structure but is considered to be adequate in order to get an idea of the molecule size[49].

Based on the molar volume $\left(V_m = M * \rho^{-1}\right) d_m$ is calculated as follows:

$$d_m = 2 * \left(\frac{3 * V_m}{4 * \pi * N}\right)^{\frac{1}{3}} \quad (1)$$

with N equal to Avogadro's number, ρ the compounds' density (g m^{-1}) and the molar mass M (g mol^{-1}). As an approximation, the area occupied by QAAC molecules is calculated with the following equation:

$$A_m = \frac{\pi * d_m^2}{4} \quad (2)$$

Additionally the absolute mass of the QAAC molecules was determined by dividing the molar mass by Avogadro's number. The maximum loading of the clay surface area is assumed by taking account of the absolute mass and the molecule size of the QAAC, as well as the BET surface area of clays.

The critical micelle concentration (CMC) is a parameter that is typically reported for surfactants and denotes the concentration, above which a compound self-assembles to form micelles with the polar (in our case cationic)

head groups pointing outwards in a polar solvent. Below the CMC, QAACs occur dissolved as single molecules, similar to other electrolytes, with a tendency to accumulate at surfaces and phase boundaries. Above the CMC, the micelles formed rather act as a second phase, comparable to hydrocarbon droplets, that could for example solubilize other hydrophobic compounds in the system. For our study, the CMCs of BAC-C12 and DADMAC-C10 were determined using spectrofluorometry and pyrene as sensor to the different solubilization caused by micellization. The peak intensity ratio was used to derive the CMC values according to Aguiar et al.[50].

BAC-C12 and DADMAC-C10 sorption curves. In order to assess the dissolved concentration ($C_{dissolved}$) of BAC-C12 or DADMAC-C10 in equilibrium with clay minerals, the conditions of the experimental design in the MIC-incubation-experiments (section "TEM analysis and molecular considerations of sorption") were mimicked at a larger scale for sorption curves, thereby providing enough sample volume for the required HPLC-tandem mass spectrometry (MS/MS) measurement and analysis. QAAC concentration ranges were chosen to encompass the respective MIC values. Compared to the MIC-experiments, smaller concentration range steps (for a finer resolution) were selected. Concentrations for smectite (0.03 mg mL^{-1}) and kaolinite (0.09 mg mL^{-1}) added remained constant. Smectite or kaolinite were equilibrated with different concentrations of QAACs in 20 mL amber glass vials shaken overhead for 30 min, analogous to the QAAC and clay mixing in 2 mL Eppendorf tubes. Two mL of this solution were transferred again to 20 mL empty amber glass vials and mixed with 2 mL of MHB (Sigma Aldrich) (44 g L^{-1}) and 8 mL of bacterial blank solution, which is equivalent to the conditions in the well plates described in section "TEM analysis and molecular considerations of sorption". After shaking with a vortex shaker (VORTEX 3, IKA, Germany) and centrifuging at 710 g for 30 min, aliquots of 30–120 µL of the clear supernatants were transferred to 2 mL clear glass vials and made up to 1 mL total volume with acetonitrile for the HPLC–MS/MS measurement. The experiments were performed in triplicate. For each experiment, a seven point calibration in acetonitrile was prepared.

For separation of QAACs from other organic compounds, a Waters 2690 Separations Module, equipped with a Waters XSelect CSH Phenyl-Hexyl column (3.5 µm particle size, 2.1 mm inner diameter × 150 mm length) and a guard XSelect column of the same material (3.5 µm particle size, 2.1 mm inner diameter × 5 mm length) was used. Column temperature was set at 37 °C and an isocratic flow of 0.3 mL min^{-1} with 15% solvent A (MQ-water and 50 mM formic acid and 10 mM ammonium formate) and 85% solvent B (acetonitrile) and 20 µL injection volume were chosen. The matrix effects of MHB on separation and detection were tested and could be neglected at the applied concentrations of maximally 3.66% (v/v). A Micromass Quattro Micro triple quadrupole mass spectrometer operating in positive ion multiple reaction-monitoring mode was used for mass detection (further settings in SI-S7). Peak integration was performed with MassLynx Quanlynx (Waters), data analysis with Microsoft Excel 2010 and curve fitting with SigmaPlot 12 (Systat).

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Author contributions
BH, SG, LB, IM and JS designed the experiment and formulated the hypotheses. BH and LB carried out the experiment. RE performed BET measurement and KK acquired the TEM images. All authors contributed to the interpretation of the results and the writing of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Supplementary data

Smectite clay minerals reduce the acute toxicity of quaternary alkylammonium compounds towards potentially pathogenic bacterial taxa present in manure and soil

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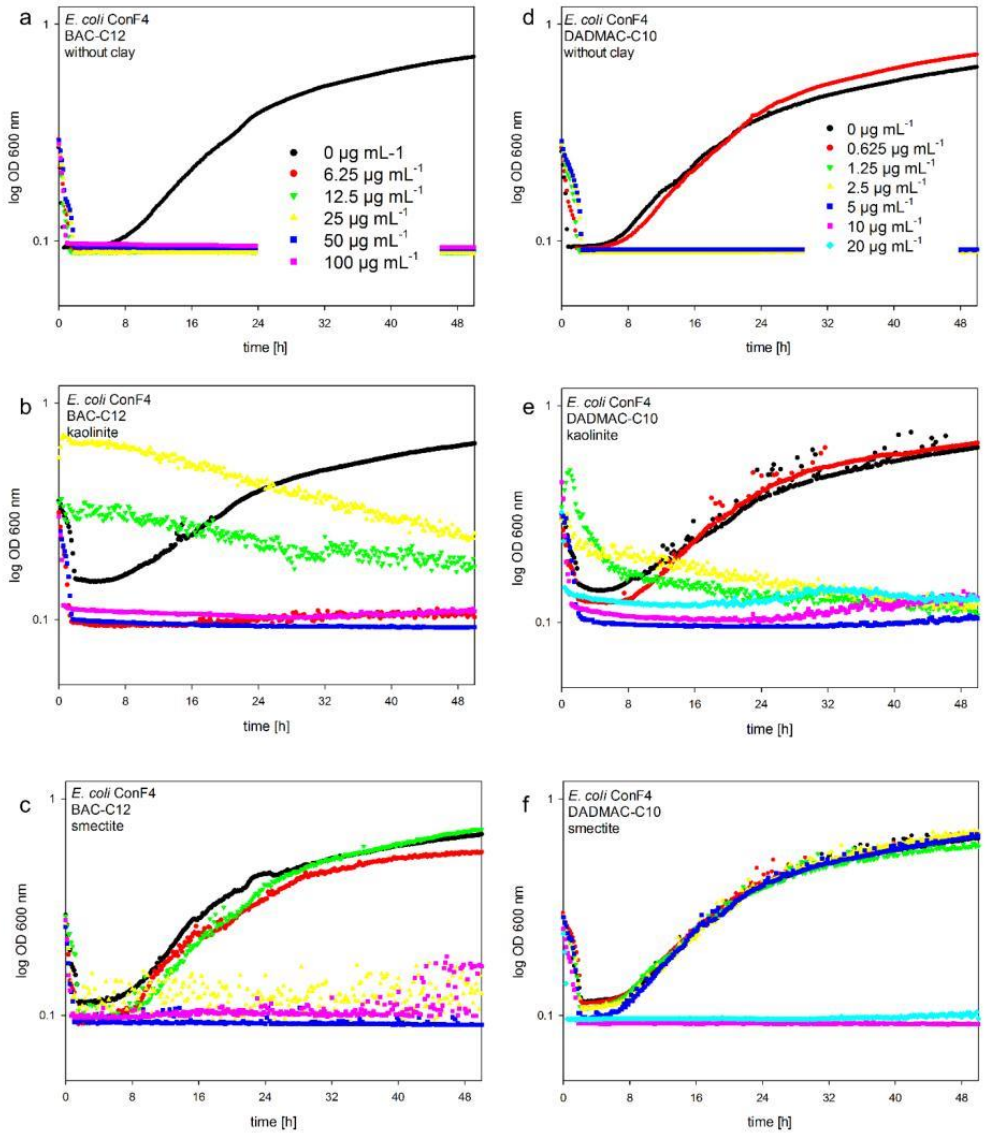

Figure S1 Growth curves of strain *E. coli* ConF4 in the presence of different BAC-C12 (a-c) and DADMAC-C10 (d-f) concentrations without clay mineral addition (a, d) and in the presence of kaolinite (b, e) and smectite (c, f). QAAC concentrations marked with dashed lines represent concentrations which were inhibitory in the absence of clay minerals and the presence of kaolinite while growth was non-inhibited in the presence of smectite.

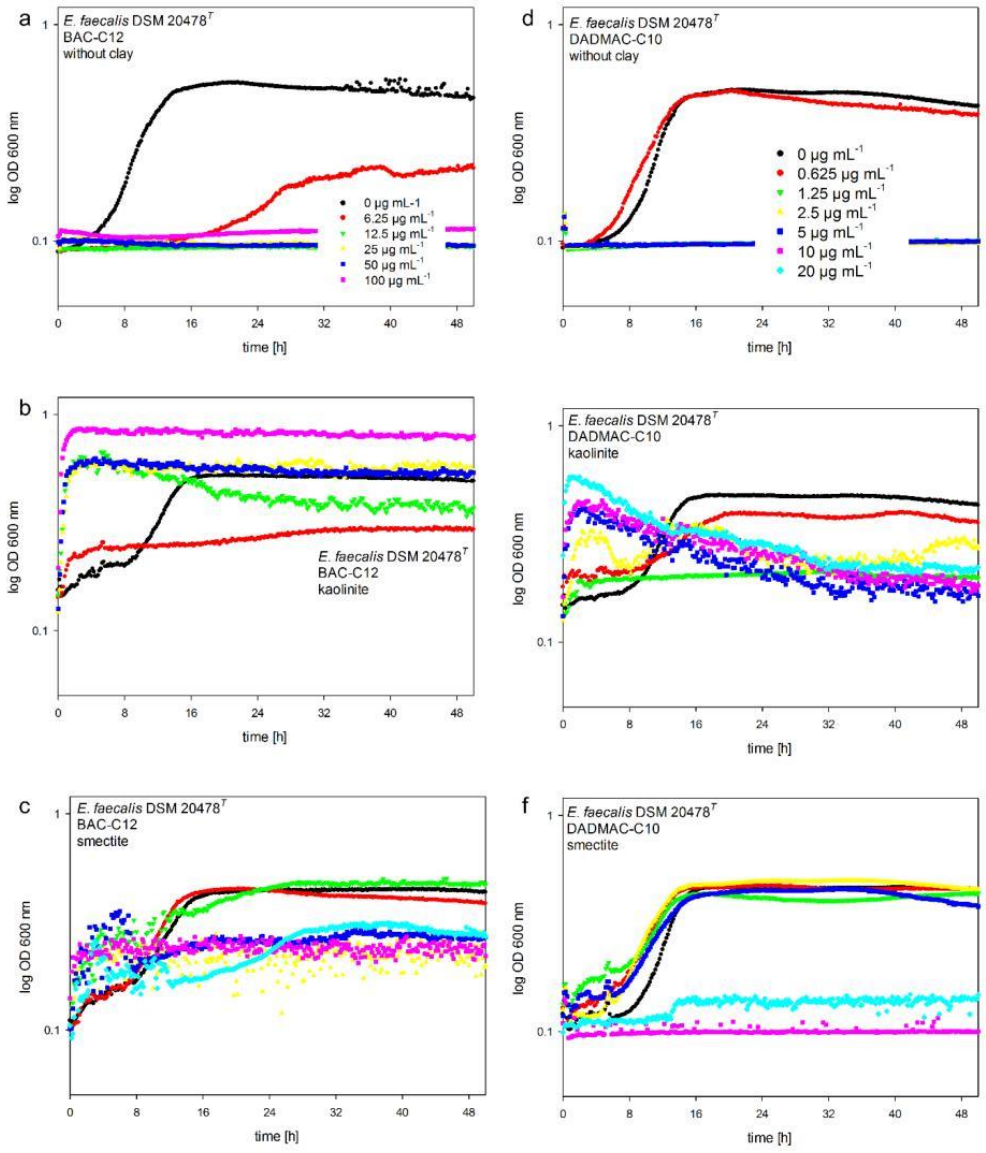

Figure S2 Growth curves of strain *E. faecalis* DSM 20478^T in the presence of different BAC-C12 (a-c) and DADMAC-C10 (d-f) concentrations without ton mineral addition (a, d) and in the presence of smectite (b, e) and kaolinite (c, f). QAAC concentrations marked with dashed lines represent concentrations which were inhibitory in the absence of ton minerals and the presence of kaolinite while growth was non-inhibited in the presence of smectite.

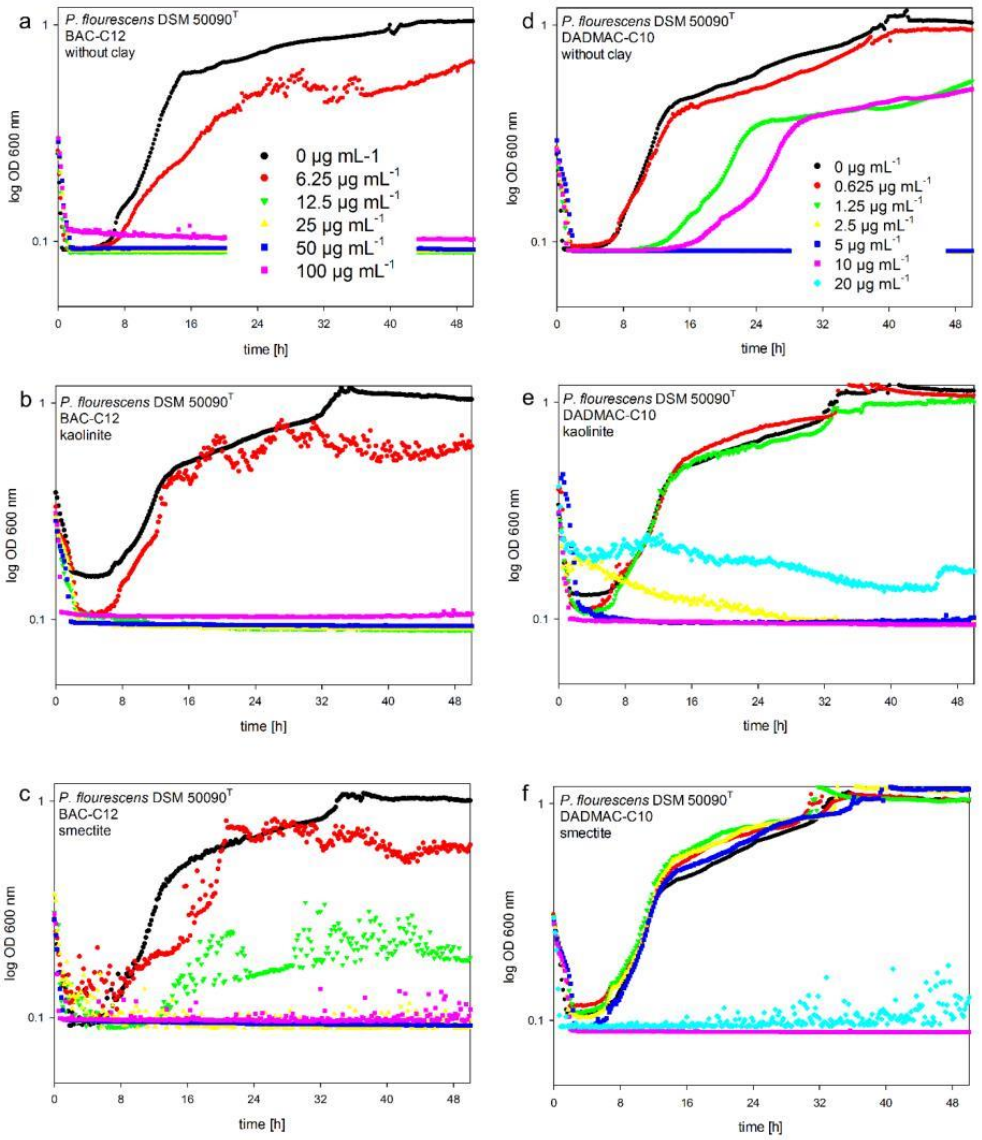

Figure S3 Growth curves of strain *P. fluorescens* DSM 50090^T in the presence of different BAC-C12 (a-c) and DADMAC-C10 (d-f) concentrations without ton mineral addition (a, d) and in the presence of smectite (b, e) and smectite (c, f). QAAC concentrations marked with dashed lines represent concentrations which were inhibitory in the absence of ton minerals and the presence of kaolinite while growth was non-inhibited in the presence of smectite.

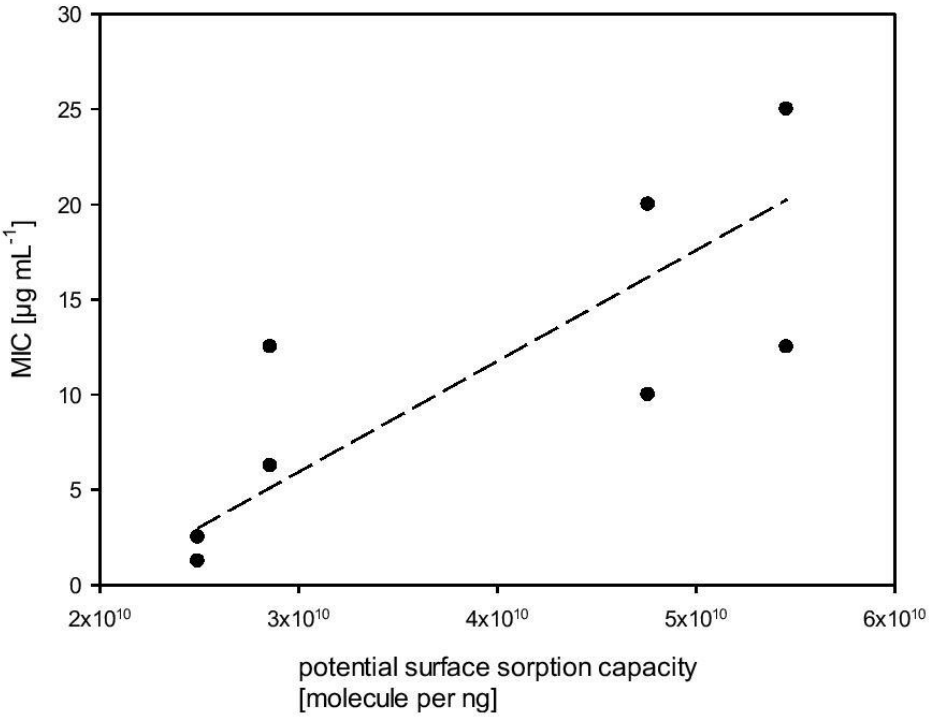

Figure S4 Correlation of all MIC values vs potential surface sorption capacity of BAC-C12 or DADMAC-C10 on kaolinite or smectite surface ($R^2 = 0.75$). Four treatments are BAC-C12 / kaolinite, BAC-C12 / smectite, DADMAC-C10 / kaolinite and DADMAC-C10 smectite. MIC values of *E. coli* ESBL 37 B15_13_1E, *E. coli* ConF4, *P. fluorescens* DSM 50090^T and *E. faecalis* DSM 20478^T are shown and some overlap others

Table S1 Schematic structure of the 96-well plates as used in the final tests with kaolinite and BAC-C12 0 – 100 µg mL^{-1}

	Without clay						Kaolinite [93.5 µg mL^{-1}]					
BAC-C12 [µg mL^{-1}]	0	6.25	12.5	25	50	100	0	6.25	12.5	25	50	100
E. coli ESBL37B15_13_1E	+ +	+ +					+ +	+ +				
P. fluorescens DSM 50090^{T}	+ +	+ +					+ +	+ +				
E. coli ConF4	+ +						+ +					
Without bacteria												

Table S2 Schematic structure of the 96- well plates as used in the final tests with smectite and BAC-C12 [0 – 100 µg mL^{-1}]

	Without clay						Smectite [32.17 µg mL^{-1}]					
BAC-C12 [µg mL^{-1}]	0	6.25	12.5	25	50	100	0	6.25	12.5	25	50	100
E. coli ESBL37B15_13_1E	+ +	+ +					+ +	+ +	+ +			
P. fluorescens DSM 50090^{T}	+ +	+ +					+ +	+ +	+ +			
E. coli ConF4	+ +						+ +	+ +	+ +			
Without bacteria												

Table S3 Schematic structure of the 96- well plates as used in the final tests with kaolinite and DADMAC-C10 [0 – 20 µg mL^{-1}]

	Without clay						Kaolinite [93.5 µg mL^{-1}]					
DADMAC-C10 [µg mL^{-1}]	0	0.625	1.25	2.5	5.0	0	0.625	1.250	2.5	5	10	20
E. coli ESBL37B15_13_1E	+ +	+ +	+ +			+ +	+ +	+ +				
P. fluorescens DSM 50090^{T}	+ +	+ +	+ +			+ +	+ +	+ +				
E. coli ConF4	+ +	+ +	+			+ +	+ +	+ +				
Without bacteria												

Table S4 Schematic structure of the 96- well plates as used in the final tests with smectite and DADMAC-C10 [0 – 20 µg mL^{-1}]

	Without clay					Kaolinite [93.5 µg mL^{-1}]						
DADMAC-C10 [µg mL^{-1}]	0	0.625	1.25	2.5	5.0	0	0.625	1.250	2.5	5	10	20
E. coli ESBL37B15_13_1E	+ +	+ +	+ +			+ +	+ +	+ +	+ +	+ +	+ +	
P. fluorescens DSM 50090^{T}	+ +	+ +	+ +			+ +	+ +	+ +	+ +	+ +	+ +	
E. coli ConF4	+ +	+ +				+ +	+ +	+ +	+ +	+ +		
Without bacteria												

Table S5 Schematic structure of the 96-Well Plates as used in the sensitivity tests of MO (*E. coli* ESBL37B15_13_1E; *E. coli* ESBL232B15_13_2E; *E. coli* ESBL370B15_13_2A; *E. coli* ConF4; *E. faecium* DSM 20477^{T}; *E. faecium* DSM 20478^{T}; *Acinetobacter* sp. KPC-SM-21; *P. fluorescens* DSM 50090^{T}; *P. fluorescens* DSM 50090^{T} BAC-C12 (F6); *P. fluorescens* DSM 50090^{T} BAC-C12 (E11)) to QAAC (BAC-C12 and DADMAC-C10)

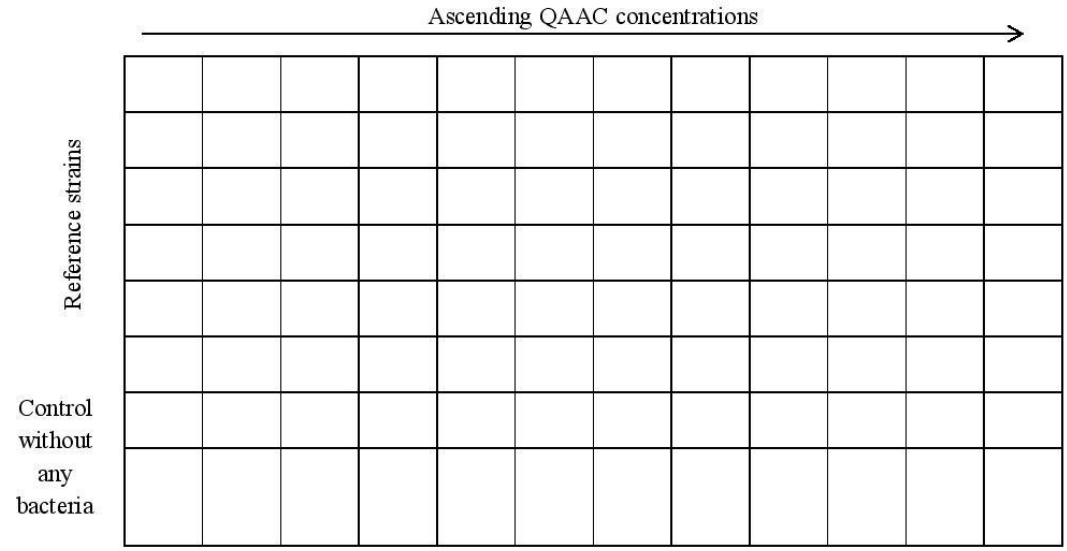

Table S6 Schematic structure of the 96-Well Plates as used in clay concentration test for MIC of *E. coli* ConF4 to QAAC (BAC-C12 and DADMAC-C10)

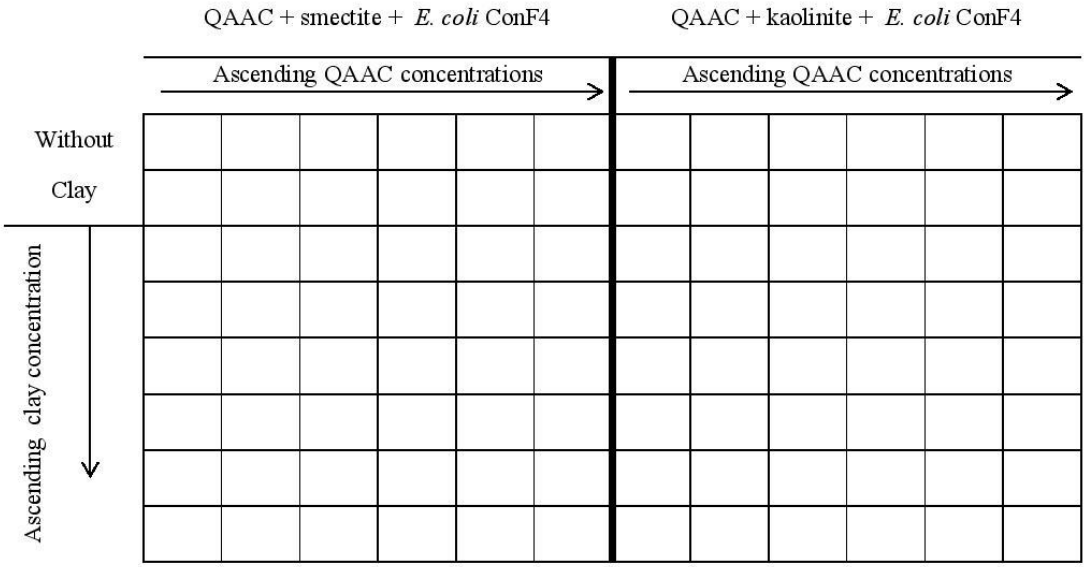

Table S7 Schematic structure of the 96-Well Plates as used in the final tests to determine MIC for *E. coli* ESBL37B15_13_1E, P. *fluorescens* DSM 50090^T, and *E. coli* ConF4 under influence of BAC-C12 and DADMAC-C10 and clay minerals

Without clay						Smectite or kaolinite						
0	6.25	12.5	25	50	100	0	6.25	12.5	25	50	100	BAC µg mL^{-1}
												E. coli ESBL37B15_13_1E
												P. fluorescens DSM 50090^T
												E. coli ConF4
												Control (without MO)

Table S8 MS/MS Instrument Parameters for positive ion multiple reaction-monitoring mode and ESI.

	Ion Transitions	Con Voltage [V]	Collision Energy [V]
DADMAC-C10	326.4 > 186.2	50	29
BAC-C12	304.2 > 90.6	40	25
MS/MS Parameters		Capillary [kV] = 3.50 Cone [V] = 43 Source Temperature [°C] = 130 Desolvation Temperatuer [°C] = 450 Desolvation Gas Flow [L h^{-1}] = 600 Cone Gas Flow [L h^{-1}] = 50 Collision Gas = Argon	

List of peer-reviewed publications

Below are listed the publications that were prepared during the doctoral phase.

Heyde B.J, Glaeser S.P, Bisping L, Siemens J, Mulder I (2020).
Smectite clay minerals reduce the acute toxicity of quaternary alkylammonium compounds towards potentially pathogenic bacterial taxa present in manure and soil. *Scientific Reports 10*, 15397, DOI: 10.1038/s41598-020-71720-5; Shared first Author

Heyde BJ, Barthel A, Siemens J, Mulder I (2020).
A fast and robust method for the extraction and analysis of quaternary alkyl ammonium compounds from soil and sewage sludge. *PLoS ONE 15(8),* DOI: 10.1371/journal.pone.0237020

Heyde BJ, Anders A, Siebe C, Siemens J, Mulder I (2021).
Quaternary alkylammonium disinfectant concentrations in soils rise exponentially after long-term wastewater irrigation. *Environ. Res. Lett,* DOI: https://doi.org/10.1088/1748-9326/abf0cf

Steckenmesser D, Vogel C, Böhm L, **Heyde B**, Adam C (2018).
Fate of heavy metals and polycyclic aromatic hydrocarbons (PAH) in sewage sludge carbonisates and ashes - A risk assessment to a thermochemical phosphorus-recycling process*. Waste Management (78)*, DOI: 10.1016/j.wasman.2018.06.027

List of Conference Contributions as first author

Heyde B.J, Mulder I, Siemens J. Gehalte, Dissipation und Akkumulation Quartärer Alkylammoniumverbindungen im Boden. DBG Tagung 2017, Göttingen, Poster

Heyde B.J, Mulder I, Glaeser S.P, Bisping L, Siemens J. Der Verbleib Quartärer Ammoniumverbindungen im Boden – Ergebnisse aus Studien mit Modellsubstanzen. SETAC Tagung 2018, Münster, Poster

Heyde B.J, Mulder I, Barthel A, Siemens J. Detektion von quartären Alkylammoniumverbindungen aus Böden, Klärschlämmen und Jauche. DBG Tagung 2019, Bern, Schweiz, Poster

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“in carrying out the investigations described in the dissertation.

Benjamin Justus Heyde