A New Approach to Groundwater Remediation Treatability Studies - Moving Flow-through Column Experiments from Laboratory to *In Situ* Operation

By

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ABSTRACT

In situ remediation of contaminated aquifers, specifically *in situ* bioremediation (ISB), has gained popularity over pump-and-treat operations. It represents a more sustainable approach that can also achieve complete mineralization of contaminants in the subsurface. However, the subsurface reality is very complex, characterized by hydrodynamic groundwater movement, geological heterogeneity, and mass-transfer phenomena governing contaminant transport and bioavailability.

These phenomena cannot be properly studied using commonly conducted laboratory batch microcosms lacking realistic representation of the processes named above. Instead, relevant processes are better understood by using flow-through systems (sediment columns).

However, flow-through column studies are typically conducted without replicates. Due to additional sources of variability (e.g., flow rate variation between columns and over time), column studies are expected to be less reproducible than simple batch microcosms. This was assessed through a comprehensive statistical analysis of results from multiple batch and column studies. Anaerobic microbial biotransformations of trichloroethene and of perchlorate were chosen as case studies. Results revealed that no statistically significant differences were found between reproducibility of batch and column studies.

It has further been recognized that laboratory studies cannot accurately reproduce many phenomena encountered in the field. To overcome this limitation, a down-hole diagnostic device (*in situ* microcosm array – ISMA) was developed, that enables the autonomous operation of replicate flow-through sediment columns in a realistic aquifer setting. Computer-aided design (CAD), rapid prototyping, and computer numerical

control (CNC) machining were used to create a tubular device enabling practitioners to conduct conventional sediment column studies *in situ*.

A case study where two remediation strategies, monitored natural attenuation and bioaugmentation with concomitant biostimulation, were evaluated in the laboratory and *in situ* at a perchlorate-contaminated site. Findings demonstrate the feasibility of evaluating anaerobic bioremediation in a moderately aerobic aquifer. They further highlight the possibility of mimicking *in situ* remediation strategies on the small-scale *in situ*.

The ISMA is the first device offering autonomous *in situ* operation of conventional flow-through sediment microcosms and producing statistically significant data through the use of multiple replicates. With its sustainable approach to treatability testing and data gathering, the ISMA represents a versatile addition to the toolbox of scientists and engineers.

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ABBREVIATIONS

ASTM	American Society for Testing and Materials
BTEX	Benzene, toluene, ethylbenzene, xylenes
BTX	Benzene, toluene, xylenes
CAD	Computer-aided design
CE	Chlorinated ethenes
CFU	Colony-forming unit
CNC	Computer numerical control
cis-DCE	*cis*-Dichloroethene
1,1-DCE	1,1-Dichloroethene
1,1-DCA	1,1-Dichloroethane
DNA	Deoxyribonucleic acid
DNAPL	Dense non-aqueous phase liquid
DPRB	Dissimilatory perchlorate-reducing bacteria
FID	Flame-ionization detector
GC	Gas chromatography
HDPE	High-density polyethylene
HPLC	High-pressure liquid chromatography
ICS	Ion-chromatography system
ID	Inner diameter
ISB	*In situ* bioremediation
ISM	*In situ* microcosm
ISMA	*In situ* microcosm array
LLC	Limited liability company
MCL	Maximum contaminant level

MNA	Monitored natural attenuation
MTBE	Methyl *tert*-butyl ether
ND	Not detected
NIOSH	National Institute for Occupational Safety and Health
OD	Outer diameter
PCE	Perchloroethene
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
RSD	Relative standard deviation
RSE	Relative standard error
SE	Standard error
SPME	Solid-phase microextraction
TCE	Trichloroethene
TEAP	Terminal electron accepting process
U.S. EPA	United States Environmental Protection Agency
VC	Vinyl chloride

PREFACE

This dissertation is organized into five chapters, which I will outline below.

Chapter one is a critical review of the current use of treatability studies in lab and field and their ability to predict commonly observed failure modes when implementing *in situ* bioremediation. It is based on a survey among remediation professionals to supplement failure modes described in the (mostly academic) peer-reviewed literature. I conclude that failure of *in situ* bioremediation at the field scale could be studied and avoided if flow-through systems were used in either lab or field to assess and circumvent known potential failure sources prior to field implementation of cleanup technologies. Beyond *in situ* bioremediation, this conclusion also will hold true for other *in situ* remediation approaches governed by mass transport and delivery phenomena, e.g., *in situ* chemical oxidation/reduction or reactive barriers.

Chapter two is a comprehensive statistical analysis of the experimental reproducibility of batch and column microcosms routinely used to assess *in situ* remediation processes under static and continuous-flow conditions. The work was motivated by the present lack of information on variability between replicate batch microcosms, and the customary reporting in the peer-reviewed literature of flow-through sediment column experiments performed without replicates. Anaerobic microbial biotransformations of trichloroethene to dichloroethene, vinyl chloride and ethene, and of perchlorate were chosen as case studies, and different error sources characteristic of batch and flow-through microcosms were identified and quantified. Replicate small-scale column studies are identified as an attractive but currently under-utilized tool to study and parameterize phenomena occurring in flow-through environments such as aquifers. Miniaturized columns have reduced requirements for both sediment material and

laboratory space, yet enable one to conduct experiments with multiple replicates at experimental error rates comparable to those of batch microcosms.

Chapter three describes the design and capabilities of a novel device that enables the autonomous operation of replicate flow-through sediment columns in a realistic aquifer setting. When deployed in a well, the *in situ* microcosm array (ISMA) approximates *in situ* conditions by drawing groundwater directly from the aquifer and delivering it to replicate columns (up to 10) filled with site sediment. Storage of influent and effluent in the device affords *in situ* testing at ambient subsurface temperatures and conditions without causing release of either the chemicals or bacteria evaluated. The ISMA is the first device offering autonomous *in situ* operation of conventional flow-through sediment microcosms and produces statistically significant data through the use of multiple replicates.

Chapter four presents a case study for the ISMA. Two remediation strategies, monitored natural attenuation and bioaugmentation with concomitant biostimulation, were evaluated in the laboratory and *in situ* at a perchlorate-contaminated site. Field results qualitatively matched those of identical studies conducted in the laboratory; however, differences were found between first-order perchlorate degradation rates (0.55 hr^{-1} in the lab vs. 0.24 hr^{-1} *in situ*). Findings demonstrate the feasibility of evaluating anaerobic bioremediation in a moderately aerobic contaminated aquifer. They further highlight the possibility of mimicking *in situ* remediation strategies on the small-scale *in situ*. As demonstrated here, the ISMA enables analysis of effluent samples from each experiment for multiple parameters with statistical significance, as well as DNA analyses of both attached and suspended microbial communities.

Chapter five contains recommendations for hardware improvements as well as next steps to demonstrate use of the ISMA for remedy testing of other groundwater

contaminants. These are meant to aid moving the ISMA from a research stage to a technology that is available to environmental consultants and site managers in the U.S. and worldwide. With the creation of a startup company (*In Situ* Well Technologies, LLC.) and a commercialization agreement with an international environmental consulting firm in place, the ISMA is well on its way to be available for routine testing.

1. CRITICAL REVIEW OF FAILURE MODES AND OPPORTUNITIES FOR THEIR PREVENTION IN *IN SITU* BIOREMEDIATION

INTRODUCTION

In situ remediation of contaminated aquifers, specifically biological remediation, has gained popularity over pump-and-treat operations[1, 2] by representing a more sustainable approach that can also achieve complete mineralization of contaminants in the subsurface. In contrast, pump-and-treat operations merely transfer contaminants from water into another medium requiring subsequent disposal or treatment. However, the subsurface environment is very complex, characterized by groundwater movement, geochemical, physical and biological heterogeneity, zones with different terminal electron accepting processes, and mass-transfer phenomena governing contaminant transport and bioavailability. These lead to many challenges of *in situ* bioremediation (ISB). Remediation failures may be caused by inadequate site characterization, poor technology selection, and flawed design or implementation of a selected remedy. However, ISB is particularly prone to failure modes relating to microbiology; the peer-reviewed literature cites limited contaminant bioavailability[3-8] and survival of introduced microorganisms[5-10] as the main sources of failure. Further issues involving bioremediation discussed in the literature are incomplete contaminant degradation[5, 11], regulatory issues[4, 11], and injection-well clogging[8].

Failure Modes Survey

To supplement the failure sources identified by the mainly academic publications listed above, we conducted a limited survey (Figure 1-1.) among leading remediation practitioners that combined have worked on over 1400 bioremediation sites. Survey participants were asked to rank different failure modes on a scale of 1-5, and encouraged

to identify further failure modes not listed in the survey (survey provided in Appendix A).

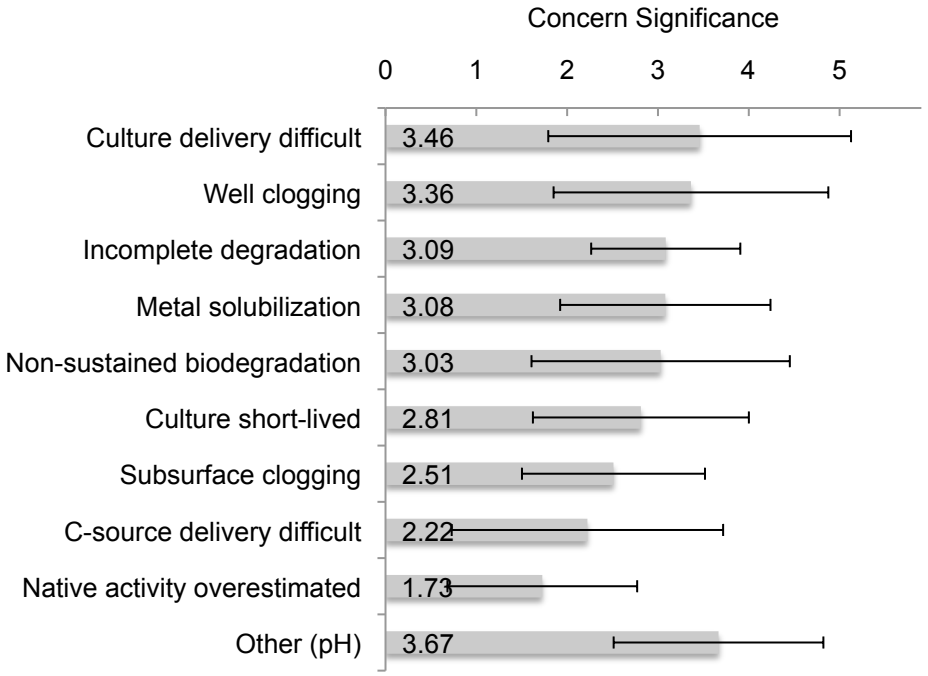

Figure 1-1. Results from limited survey among remediation practitioners on the relative importance of different failure modes encountered during *in situ* bioremediation. Surveyed individuals combined worked on a total of 1493 bioremediation sites. Only individuals with experience from >5 sites were considered. Captured under "other" are pH buffering issues pointed out by three survey participants. Failure modes were rated on a scale of 1 (minor concern) to 5 (frequent/important concern). Results shown are average responses, weighted by the number of bioremediation sites the individual has worked on. Error bars represent standard deviation (n=19).

Remediation practitioners identified ineffective delivery of bioaugmentation agents throughout the plume, clogging of injection-wells, and incomplete contaminant degradation and accumulation of toxic degradation products or metal solubilization from changing redox conditions as the main failure sources when implementing ISB, followed by unsustained biological activity and death/inactivity of bioaugmented microorganisms. Additional identified failure sources were clogging of the aquifer and ineffective delivery of nutrients, which often go hand-in-hand. Difficulty to control pH was identified as an

important failure mode by three individuals representing 750 bioremediation sites. The survey results reveal that many of these failure modes are equally important and no one predominant cause of bioremediation failure was identified.

In this critical review, we examine the fundamental processes leading to these failure modes, and discuss suitable methods to identify potential problems early on in the feasibility assessment phase during the treatability studies preceding ISB.

FUNDAMENTALS OF BATCH AND FLOW-THROUGH STUDIES

Because the prediction of issues encountered in the field are inextricably linked to fundamental differences between closed batch microcosms and flow-through treatability studies, we will highlight some fundamentals of these two systems before discussing different failure modes of ISB in detail.

Laboratory incubation of field samples is an accepted approach to studying subsurface processes, pinpointing potential failure modes, and devising alternative remedies to meet requirements at specific sites before full-scale remediation is implemented. For this purpose, samples assumed to be representative of the subsurface are brought to the laboratory and incubated under controlled conditions. While every investigator strives to replicate field conditions in the lab, the natural environments where microorganisms dwell are poorly understood[12]. Owing to this fact is our inability to culture many environmental microorganisms which thrive under natural conditions in the environment. It has been estimated that the fraction of culturable bacteria present in soil, sediment or freshwater is only 0.25 – 0.3% of total cell counts[13].

Typically, these laboratory incubations are conducted in batch microcosm (most often serum bottles ranging in volume from 100-250 mL[14-19]), filled with sediment and groundwater from the site under evaluation. Candidate amendments (carbon sources, electron donors, nutrients, microbial agents, etc.) are added to the batch microcosms,

and contaminant reduction is observed over time during long-term incubation (typically 50-100 days[14-16, 18, 20]). Laboratory microcosms are simple in design, offer good control over experimental conditions, facilitate complete mass balances, and allow for tests to be conducted in multiple replicates to distinguish between experimental reproducibility and distinct outcomes actually caused by the differing treatment scenarios simulated. They are therefore very popular to assess the feasibility of bioremediation, and are recommended by a number of guidance documents[21-25].

Although many limitations exist, laboratory batch microcosm studies can answer a range of questions, which are prerequisite for evaluating bioremediation at a contaminated site (Table 1-1):

1. Are microorganisms present at the site capable of degrading the contaminant(s) of concern without accumulation of harmful byproducts?[26]
2. Can augmentation of known degraders expedite and/or complete the degradation?[27-31]
3. Which amendments (carbon source, electron donor, and other co-factors) are effective and what dosing is needed?[32, 33]
4. What are important interactions between wanted degraders and competitive/synergistic microorganisms; and how can they be influenced to optimize contaminant removal?[34, 35]
5. Are toxic substances or inhibitors at the site interfering with complete removal?[36-40]
6. Is there a potential for metals leaching from sediment through changes in redox/pH conditions?[41]

Table 1-1. Suitability of different treatability test systems to assess fundamental processes relevant to implementation failure modes.

Batch Microcosm	Flow-through Microcosm	Field Test
Redox/pH changes[42, 43]	Small-scale mass transfer limitation[44-50]	Large-scale mass transfer limitation[51-55]
Presence of toxics/inhibitors in aquifer material[36-40]	Clogging/permeability reduction[56-58]	Geological/biological heterogeneity[59-64]
Evaluation of different nutrients for bioremediation[32, 33]	Long-term performance (1 yr[65], 6 mo[66], 5 mo[67], 3-4 mo[68])	Amendment delivery and distribution[69-72]
Presence/activity of degrading microorganisms / need for bioaugmentation[27-31]	Degradation rate under given flow conditions[73-76]	Bacterial survival[69, 77]

Beyond these, results from batch studies are often employed to inform full-scale system design and implementation of ISB, often without considering their inherent limitations in accurately predicting processes in the environment. However, it has been widely acknowledged that laboratory test results are often "quantitatively, even qualitatively different from the same determination *in situ*"[78]. This leads to failures during field-scale implementation that are not predicted by laboratory batch studies.

Feasibility studies conducted in batch bottles assess degradation under enhanced mass-transfer conditions. Sediment material is broken up, destroying the aggregate structures and eliminating air, water and nutrient gradients, which control microbial activity in intact sediment. A sediment slurry is prepared, reducing heterogeneity and allowing maximum contact between the sediment particles and the aqueous phase. Batch bottles can be incubated statically[18], but are often periodically shaken[20] or incubated under constant agitation on a shaker[15-17]. This agitation leads to enhanced diffusive and convective transport of contaminants from sorbed phase to dissolved phase (Figure 1-2) and enhances contaminant bioavailability[79]. In contrast, only some of the sediment in

the subsurface is in contact with the dynamic portion of the groundwater, whereas large portions of the sediment are in equilibrium with stagnant pore water, leading to only minimal diffusion of sorbed contaminants into the aqueous phase. This leads to significant mass-transfer limitations in the subsurface, which are described as limiting factors in numerous studies[3-8, 80, 81], but which cannot be adequately addressed by batch microcosm studies.

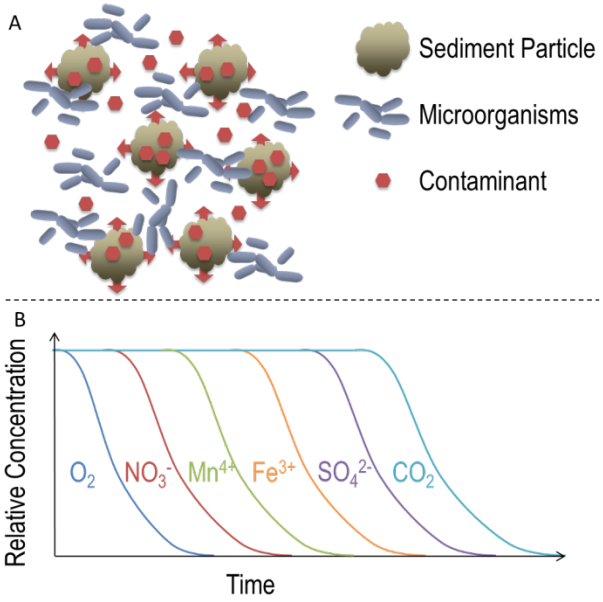

Figure 1-2. Dominating conditions in a laboratory batch microcosm: A - sediment slurry leading to maximized mass transfer between sediment and aqueous phase, and therefore maximized bioavailability of contaminant, nutrients, electron donor/acceptor; B - closed, hydrostatic system leading to a temporal sequence of terminal electron accepting processes, accumulation of degradation products, and transient microbial community composition caused by constantly changing conditions.

The subsurface contains distinct geochemical zones characterized by certain redox conditions along with the corresponding dominant terminal electron accepting process (TEAP; e.g., oxygen, nitrate, iron, sulfate reducing zones – see Figure 1-3). These zones have been found on different scales, ranging from centimeters to a few meters at the plume edge (perpendicular to groundwater flow)[62, 82-85] to several hundred meters to kilometers in length along the flow path of a plume[86, 87]. Associated with these TEAP

zones are microbial communities that utilize the dominant electron acceptor and are therefore responsible (along with abiotic redox reactions and groundwater movement) for the creation of zones with distinct microbiological and chemical conditions[88]. From the perspective of microorganisms living attached to the sediment, electron acceptor, electron donors (oxidized contaminant), and other nutrients are constantly supplied by groundwater flow or desorbing from sediment, while degradation products are carried away. Conditions at the scale of individual microorganisms are fairly constant. Although TEAP zones have been found to change over timeframes of multiple days to several months[87, 89], they are stable enough to accommodate the development of distinct microbial communities[83, 90, 91].

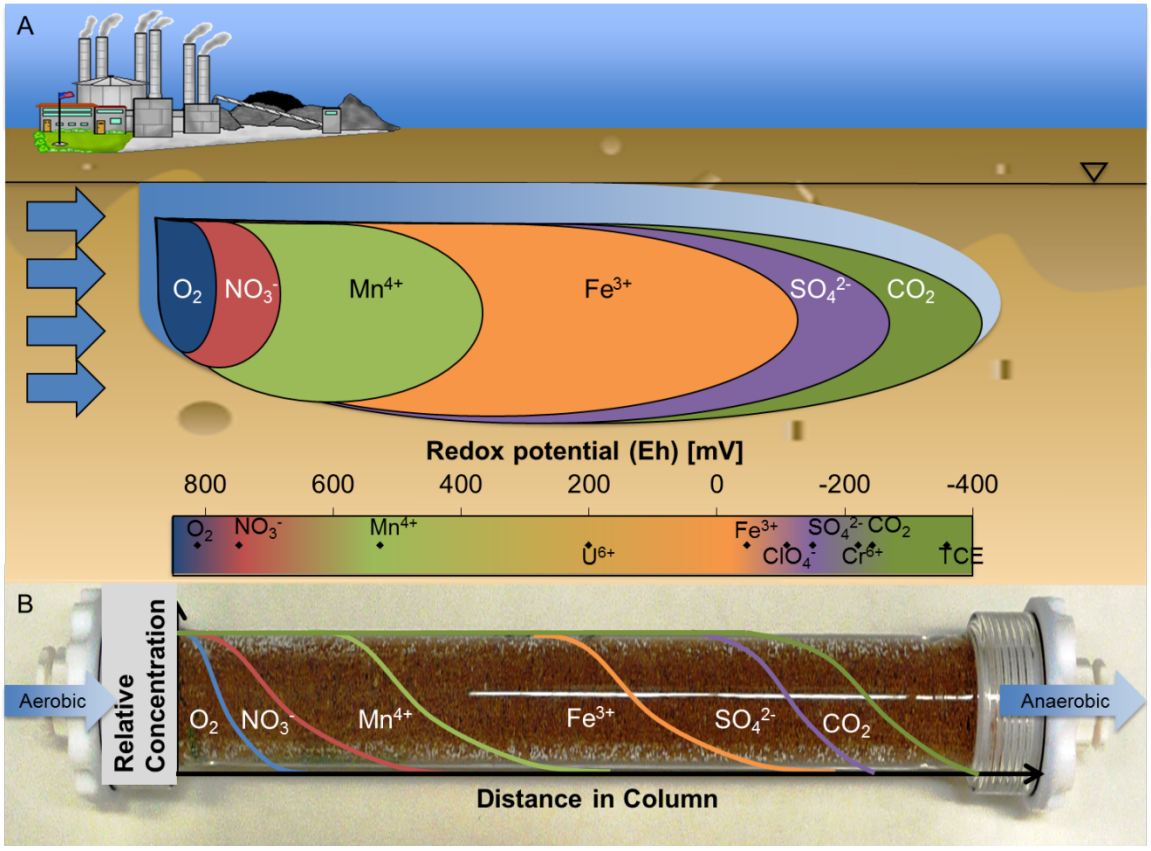

Figure 1-3. A – Idealized conceptual aquifer model showing sequential zones of dominant terminal electron acceptors along a redox gradient in a contaminant plume; B – Zones of terminal electron acceptors, which can be formed in a flow-through laboratory column simulating aquifer conditions.

In batch microcosms, spatial establishment of TEAP zones is not possible due to constant mixing and lack of directional flow of water. Instead, TEAPs come to pass sequentially over time. For the microorganisms in the system that means having to adjust to gradually, but constantly changing environmental conditions (e.g., facultative anaerobes adjusting metabolically from usage of oxygen to nitrogen as the terminal electron acceptor). When conditions change to a degree beyond their adaptive capability, microbial communities change completely with some groups becoming inactive and dying off, while others thrive under now more favorable conditions. This temporal sequence, vs. the evolution of spatial zones, is a fundamental difference between closed batch systems homogenized through agitation (as opposed to open, undisturbed Winogradsky columns) and flow-through systems such as sediment microcosms and natural aquifers.

DELIVERY AND DISTRIBUTION OF BIOREMEDIATION AGENTS

In a survey conducted among remediation practitioners, delivery and plume-wide distribution of bioaugmentation cultures was rated as the top concern when implementing ISB. It is often connected with inefficient delivery of nutrients. For remediation to be successful, carbon source, electron donor, and microorganisms should be evenly distributed throughout the plume to ensure contact with contaminants, which is the prerequisite for biodegradation, and presents one of the great challenges when implementing ISB[92]. Groundwater flow in the subsurface is characterized by uneven advection and dispersion, caused by low-permeability zones (clay lenses) and preferential flow channels (regions with coarse rock or sand). It is therefore challenging to distribute even soluble amendments throughout the plume. The challenge becomes even greater when microorganisms are amended to supplement intrinsic microbial communities[93]. Transport of bacteria through the subsurface is governed by sorption-

desorption reactions[70, 94, 95], filtration of microorganisms, as well as cell growth, death, predation and active attachment strategies by introduced and native microbes. A common approach to predicting and designing amendment delivery are numerical models based on the advection-dispersion equation[96, 97]. Models can examine the various scales relevant for aquifer-wide distribution of amendments through advection/dispersion of groundwater (across 10-1000s of meters in travel length), as well as for sorption/desorption and biotransformation processes occurring at the μm-scale[5, 98]. Models to predict microorganism distribution in the subsurface are also based on the advection-dispersion equation, with modifications to account for processes governing transport of bacteria outlined above[71, 99-104]. However, models have only a limited ability to predict microbial transport at a specific site[98], which is determined by sediment type and mineralogy[105, 106], pH and ionic strength of the water[107], as well as surface properties of the cells[108], predation, and motility[109]. Further, while numerical models are available to predict either micro-scale (biodegradation) or macro-scale processes (groundwater transport), major knowledge gaps exist at the interface between these scales[98]. Integrated models could potentially predict bioclogging of injection wells and interactions between sorption and biodegradation, and therefore provide more comprehensive data to inform design of bioremediation systems and predict long-term performance.

Until such models are available, and to provide field data for model validation, multi-well tracer tests that track specifically labeled microorganisms or surrogate particles throughout a portion of the plume can be employed[71, 72, 110]. These methods have been used in sandy sediments[71, 72] as well as fractured rock[110]. Tracer tests are also employed to characterize hydraulic conductivity, which is the main driver of amendment distribution. One study utilized tracer, flowmeter, and permeameter tests to characterize depth-

specific hydraulic conductivity in a heterogeneous aquifer[64]. Results yielded information on depths where solutions should be added to stimulate bioremediation. The study also showed that grain-size analyses of sediment cores was not a good indicator of hydraulic conductivity, and concluded that core-logging would not be sufficient to design remedial solution delivery systems[64].

Subsurface Heterogeneity

This leads us to another important consideration for delivery and distribution of bioaugmentation agents: subsurface heterogeneity. Heterogeneity of hydraulic and geologic features of the subsurface is one of the greatest challenges for *in situ* remediation. In the field, pilot studies seeking to demonstrate efficient *in situ* degradation may fail, contrary to results obtained in prior laboratory column tests[69, 111]. The stated reason for the failure was site heterogeneity, which prohibited establishing hydraulic control and effective delivery of amendments. Other experiments in heterogeneous media showed that variations in hydraulic conductivity impact availability of oxygen and substrate and therefore microbial growth in different regions[112]. Density effects impact reactive transport in heterogeneous matrices and are responsible for enhanced mixing of amendments[112]. Experiments with undisturbed sediment cores have shown that physical heterogeneities (aggregation, fracturing, stratification) control the transport of contaminants (in this case trichloroethene), and that mass-transfer limitations (sorption/desorption) are of secondary importance[50].

While the importance of heterogeneities for *in situ* remediation is undisputed, it is very difficult, if not impossible, to assess its impact prior to implementation. Thus, it is one of the driving factors to conduct experiments in the field rather than in the laboratory, where large-scale aquifer heterogeneities cannot be reproduced. Field tracer tests can

integrate the heterogeneity present along a given flow path and generate results that are more reflective of the transport behavior at aquifer scale[113], but even the larger area surveyed may not represent the full complexity of the site as a whole.

Multiple field studies have examined the transport behavior at heterogeneous sites for ISB[59-61, 63, 114, 115]. During a study conducted in preparation of enhanced ISB at a heterogeneous site, reactive and non-reactive tracers were injected to assess the infiltration of nutrients, mixing processes within the groundwater, and extend of microbial activity in the plume[60]. Samples were obtained from a multi-level sampling network to assess the effect of geological heterogeneities on the parameters mentioned previously. Results informed a site-specific numerical flow- and transport model, and showed which infiltration conditions are suitable to treat a large section of the aquifer.

SURVIVAL OF BIOAUGMENTATION CULTURES

Historically, there has been a discussion in the peer-reviewed literature over bioaugmentation vs. biostimulation, with augmentation proponents arguing for the necessity of adding known degraders when they are not naturally present or to speed up the degradation process, and bioaugmentation skeptics arguing that indigenous microorganisms are better adapted to prevailing environmental conditions and therefore will outperform introduced microorganisms in the long run.

While for most contaminants, biostimulation of indigenous microbes is a viable option, bioaugmentation is advantageous in some cases. For example, bacteria capable of anaerobic degradation of benzene are sparsely distributed in the environment; and bioaugmentation can initiate and accelerate benzene degradation[114, 116-118]. Further, complete dechlorination of chlorinated ethenes to innocuous ethene is only carried out by specialized bacteria of the genus *Dehalococcoides* spp., which may need to be augmented if not naturally present[119, 120]. The consent among remediation industry

practitioners and academics alike is that bioaugmentation is necessary where contaminant degraders do not naturally exist or can be enticed to grow to sufficient numbers[121].

Successful bioaugmentation is based on the long-term activity of the augmented microorganisms under environmental conditions they may not be well adapted to. Bacterial survival in soil depends on a range of factors[122], including bacterial movement[108, 123], substrate competition with indigenous bacteria[124], inoculum density[125, 126], and protozoa that actively graze on bacteria in aerobic environments. Results from numerous studies have shown that numbers of protozoa increase when large numbers of bacteria are introduced[127, 128] or stimulated to grow[129, 130], leading to a sharp decline in bacteria numbers[127]. In a laboratory batch study, protists present in aquifer material were found to inhibit reductive dechlorination by selectively grazing on bioaugmented *Dehalococcoides* spp., and potentially causing failure of bioaugmentation at the site[131]. Competition of introduced bacteria with the indigenous community was monitored in another laboratory batch study[132], which found that survival of introduced bacteria was dependent on the introduced strain, inoculum size, and physicochemical conditions imposed in the laboratory. Yet another study conducted with two different pure strains showed soil type and moisture content to influence bacterial survival[133]. The results of these studies point to specific parameters that can influence bacterial survival in the subsurface. However, all these studies were conducted in laboratory batch microcosms, sometimes with pre-sterilized sediment[133], and therefore have little predictive power for assessing the likely survival of bacteria introduced into the subsurface.

It has also been shown that bacteria attached to sediment and located in sediment-micropores exhibit the chief degradation activity *in situ*[134, 135], and are mostly protected from fluctuating environmental factors[136] and protozoan grazing[122, 126, 136-140], which

targets planktonic bacteria. However, most batch microcosms are constructed as sediment slurries, where bacteria may preferentially pursue a free-living lifestyle. One study examined protist predation and contaminant mineralization in soil slurries compared with intact soil[79]. The researchers concluded increased predation in soil slurries (soil:water ratio of 1:3 to 1:100) lead to slower mineralization of contaminants when compared to intact sediment. Therefore, and for our inability to accurately reproduce environmental conditions in the laboratory, batch microcosms may not correctly predict the survival of introduced microorganisms in the subsurface. To really assess if bioaugmentation can be successful in the subsurface, it needs to be tested *in situ*[77]. This problem is highlighted in a remediation project in Michigan. Laboratory column studies conducted with groundwater from the site showed complete degradation of carbon tetrachloride without accumulation of chloroform to be achievable only after bioaugmentation with a pure culture [111] capable of carbon tetrachloride degradation[111]. During the field pilot study[69] it became evident, however, that the augmented strain could not compete with the indigenous microflora, following a period of poor nutrient conditions. These feasibility studies were followed by successful site remediation using a biobarrier design with the previously tested bacterial strain[141], which remained active four years after installation.

SUSTAINED BIOLOGICAL ACTIVITY

Sustained biodegradation of contaminants over time was identified as an important failure mode when implementing ISB. This should come to no surprise when often results from batch microcosm studies are used to inform expected microbial behavior *in situ*. Batch microcosms are not designed to assess ongoing biological activity. Microorganisms in a closed system degrade a specific mass of contaminant and then go

into dormancy unless more contaminants (or alternative nutrients) are provided, e.g., by "re-spiking" of batch microcosms.

However, long-term performance can be tested in continuous-flow reactors such as flow-through columns[66-68, 142], or continuously stirred reactors with constant supply and waste. These tests should be conducted for an extended period of time (several months, if the remediation project is expected to take years).

For example, one laboratory column study examined the anaerobic bioremediation of benzene, toluene, ethylbenzene, and xylene (BTEX) through bioaugmentation with two different methanogenic communities. Significant benzene removal (up to 88%) could be sustained for one year[65], while toluene degradation was only observed after two years of acclimation. While this is an example of an extremely long study period, which likely would be cost-prohibitive to conduct on a routine basis, it highlights the potentially long timeframe that can be monitored with flow-through studies.

COMPLETENESS OF CONTAMINANT DEGRADATION

For ISB and other remedies considered, achieving cleanup goals is the most important measure of success. However, biological degradation faces specific challenges when very low contaminant levels need to be achieved to meet cleanup goals. The rate of biological degradation slows down with decreasing substrate concentrations. It has long been recognized[143, 144] that extended adaptation periods and high inoculum densities are needed at contaminant concentrations much smaller than the half-saturation constant (Monod kinetics) or Michaelis constant (Michaelis-Menten kinetics). Biological degradation is characterized by a minimum substrate concentration[145] below which microorganisms do not gain enough energy from a particular metabolic process, and therefore become inactive or switch to an alternative substrate[80, 146-148]. This limitation is especially significant at the plume edge and in the end phase of site cleanup when

significant reductions in contaminant concentrations have been achieved but levels are still hovering above cleanup goals. We surveyed published half-saturation constants (K_S) for priority contaminants in soil/groundwater systems (Figure 1-4). If contaminant concentrations are $<<K_S$, they may not be sufficient to induce enzyme expression and sustain active growth of contaminant degraders, or microorganisms utilize alternative substrates yielding more energy. This can lead to ineffective or lacking biodegradation of contaminants, even under stimulation. For contaminants with a maximum contaminant level (MCL) of <10 µg/L (e.g., chlorinated ethenes, benzene, polychlorinated biphenyls [PCB], polycyclic aromatic hydrocarbons [PAH], etc.), situations may arise where concentrations exceed the MCL, yet are still too low to support effective biological degradation of that contaminant. This poses a potentially significant problem for ISB.

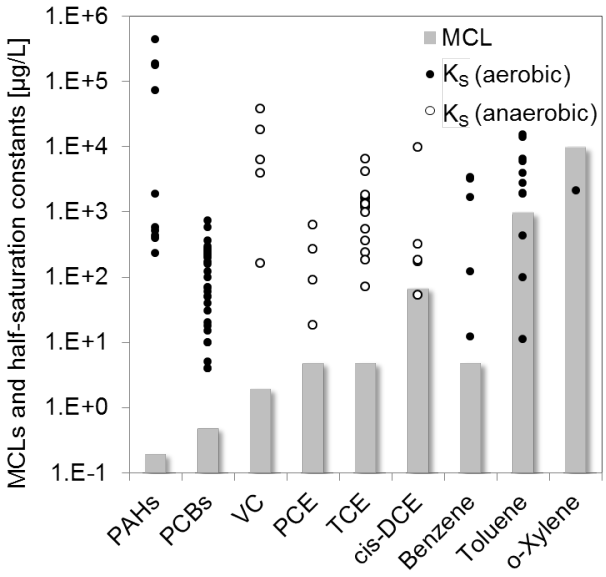

Figure 1-4. Published half-saturation constants for priority contaminants and their corresponding maximum contaminant level (MCL), which often serves as cleanup goal. Contaminant (Number of different cultures/species/strains assayed): PAHs (3); PCBs (5); VC (5); PCE (4); TCE (14); *cis*-DCE (5); Benzene (4); Toluene (10); o-Xylene (1).

To address the likelihood of this remediation failure mode prior to implementation, one needs to assess the ability of the microbial community to degrade even very low contaminant concentrations while sustaining growth. This can initially be done in batch bottle microcosms, where the endpoint of degradation is determined. However, as described earlier, batch bottle microcosms have fundamental differences to conditions in the aquifer, and results obtained cannot simply be extrapolated to the field. Metabolic enzymes may still be expressed and active because they were induced at higher contaminant concentrations at the beginning of the batch bottle test. This kind of setup does not investigate if biological degradation can be sustained over time by low contaminant concentrations. In the subsurface, although microorganisms can migrate significant distances within a contaminant plume, the chief degradative activity has been attributed to attached bacteria forming biofilms on particle surfaces[134, 135]. Therefore, from the perspective of the biofilm, conditions at the edge of a plume resemble a flow-through reactor with continuously low contaminant concentrations, rather than a batch system that starts out at higher contaminant concentrations and transitions to lower ones.

To gain an understanding if the contaminant can be degraded to cleanup levels at the edge of a plume within a given hydraulic residence time, flow-through studies should be conducted. These can be column studies operated in the laboratory, which simulate flow conditions found in the aquifer, and enable the formation of distinct zones along the flow path. These zones are characterized by distinct redox conditions, contaminant concentration, and characteristic microbial communities adapted to those specific conditions. They resemble the geochemical zones that are found in a subsurface plume[149, 150] (Figure 1-3).

For example, one study employed flow-through columns to simulate co-metabolic degradation of low levels of benzene, toluene and o-xylene (BTX) by adding co-substrates[151]. The results showed that benzoate, but not acetate, was able to stimulate BTX degraders, triggering a reduction of contaminant concentrations from ~190 µg/L each to below the regulatory limit of 5 µg/L within a hydraulic residence time of just over 2 hrs. The acclimation period of the columns was 2 days, and continued degradation of low BTX concentration was observed for 10 days.

Another aspect of the subsurface environment is the bioavailability of the contaminant. Several studies have shown that contaminants sorbed onto sediment are not available for biodegradation[152-164], and that this lack of bioavailability is caused by slow diffusion of contaminants from micropores in soil and sediment aggregates to the location of active microbial communities on particle surfaces[157, 165-167]. In fact, limited bioavailability has been listed as the number one limitation of ISB by a number of publications[3-8]. Due to physical limitations, bacteria only occupy about 50-70% of the pore spaces in the subsurface[80]. As a consequence, bacterial colonies can be quite distant from pollutants sorbed to the sediment matrix[80]. Therefore, mass-transfer limitations between sorbed and dissolved phase are the driver for bioavailability, because desorption from sediment and diffusion from micropores to larger pore spaces where microorganisms reside must occur before biodegradation can proceed. In batch microcosms containing soil slurries, this limitation is not accounted for. One study showed that slurrying of soil enhanced the rate of biodegradation for organic compounds compared to degradation in consolidated materials[81]. Diffusive transport of small organics in soils and sediments can be up to 12 orders of magnitude slower than in water[80], caused by physical barriers of sediment particles[168], and chemical or physical interactions with soil constituents. As described above, an evaluation of small-scale mass

transfer phenomena is possible with flow-through studies[44-49], which incorporate the porous structure of the subsurface and potential small-scale mass transfer limitations of the contaminant between sorbed phase, dissolved phase, and that fraction of the contaminant that is actually available to enzymatic degradation (*i.e.*, intra-cellular concentration). Large-scale mass transfer phenomena can only be assessed through field pilot tests[51-55] as discussed earlier.

Not achieving cleanup goals can also stem from accumulation of toxic breakdown products, e.g., vinyl chloride from dehalogenation of trichloroethene. This can be caused by inhibition or absence of vinyl chloride reducing bacteria (dehalococcoides type organisms), which have more fastidious metabolic needs and are less abundant[169] than perchloroethene and trichloroethene degraders such as dehalobacter, desulfitobacteria[170], or desulfuromonas[171].

The presence of key degraders at the site can be determined by employing samplers that trap resident microorganisms, e.g., "bio-traps"[172, 173]. These are passive samplers that are placed in the aquifer and serve as a solid matrix upon which microorganisms can settle and grow. Upon retrieval of the bio-trap, microorganisms can be identified and enumerated by quantitative PCR, and metabolic activities can be inferred from incorporation of isotopically-labeled contaminants into biomass captured on the sampler[172]. While bio-traps provide important information on presence and activity of key microorganisms, they are not designed to assess degradative rates under typical mass-transfer limiting conditions extant in the aquifer. Microorganisms grow directly on the substrate loaded with amendments and/or contaminants, and therefore experience maximum bioavailability of contaminants. In that sense, bio-traps provide a best-case scenario, but are not able to predict limitations regarding mass-transfer in the aquifer and completeness of contaminant degradation under flow conditions.

SECONDARY CONTAMINATION

A significant concern with *in situ* remediation, aside from not achieving cleanup goals, is to negatively impact groundwater quality during treatment of primary contaminants. One area of particular concern is the solubilization of redox-sensitive toxic metals (Cr, As, Cd, Sb, Pb, Cu, Se, U), a process readily induced by changes in pH and/or redox conditions (Eh)[174, 175]. All microbial reactions lead to changes in soil redox potential if the redox capacity (ability of the system to "buffer" the redox potential) is exceeded. Reduction and oxidation are the mechanism for all microorganisms to gain energy[176], and therefore impact redox conditions (the only exception being fermentation reactions, where the substrate is both oxidized and reduced, leading to a net zero change in redox potential, but which can still effect changes in pH).

In contrast, not all microbial reactions lead to changes in pH, but bioremediation can lead to acidic or alkaline conditions if the buffering capacity of the groundwater is exceeded, and no other steps are taken to control pH. Acidification can be caused by fermentation of carbon sources or electron acceptors with the concurrent release of hydrogen ions[177-181]; alkalization can be caused by bacterial sulfate reduction, for example[182, 183]. Low pH can also decrease or completely cease contaminant reduction, e.g., in the case of dehalogenation of chlorinated ethenes[179, 184, 185], and therefore has undesirable effects beyond solubilization of metals.

The potential for redox and pH changes, and associated behavior of redox sensitive metals, is routinely assessed in batch microcosms[42, 43]. This provides a "worst-case-scenario" based on maximum exposure of sediment surfaces participating in redox reactions. Effects of pH will also be maximized, as no additional buffer capacity (e.g., dissolved carbonate) enters the closed batch system. As pointed out by one of the survey

participants, metal solubilization is easily assessed, but unfortunately often not considered in the remedial design phase.

Another form of secondary contamination is the production of harmful degradation products. The prime example for this unwanted event is the accumulation of vinyl chloride from degradation of higher chlorinated ethenes[186-188]. Although significant degradation activity may be present, remediation is only successful if chlorinated ethenes are completely dechlorinated to the innocuous ethene. The potential for complete dechlorination may be assessed in batch microcosms[25], which can inform on the presence of site-specific inhibitors limiting dechlorination. However, even if complete dechlorination is possible in laboratory batch microcosms, the reaction may not be completed within the treatment zone given the flow conditions at the site. In a study employing batch bottle activity tests to supplement a meso-scale artificial aquifer experiment, perchloroethene dechlorination was limited in the flow-through aquifer (only 30% conversion to trichloroethene and dichloroethenes). Sediment cores were taken from the artificial aquifer and incubated in batch microcosms, where complete dechlorination of perchloroethene was observed[184]. As described in the prior section on 'completeness of contaminant degradation', flow-through studies, e.g., laboratory column studies, should be employed to assess degradation in a hydrodynamic environment within a given hydraulic residence time. This residence time in the column can easily be converted to its corresponding treatment zone in the aquifer at a given groundwater velocity.

PERMEABILITY REDUCTION

During *in situ* remediation, permeability of the subsurface can be reduced by biomass build-up (biofouling), precipitation of minerals, or accumulation of gases in pore spaces (gas clogging). All lead to a reduction in permeability, and therefore decrease hydraulic conductivity. This is of concern to remediation practitioners because it limits the ability to inject amendments, and can cause contaminated groundwater to circumvent a treatment zone, and therefore forego treatment.

In the field, amendment injection regimes that aim to prevent zones of very high biological activity can control biofouling and gas clogging. These measures include pulsed injection[189-191], adjusting amendment concentrations[192], or choosing the right amendment as nutrient source[193].

Permeability reduction is of particular concern in reactive barriers or funnel-and-gate systems, where the treatment zone is limited, and passing of all contaminated groundwater through that treatment zone is paramount. Failure of these systems if often caused by permeability reduction in the treatment zone and subsequent bypassing of groundwater[194-199]. Long-term performance of these *in situ* reactors should be tested in prior flow-through tests with relevant contaminant concentrations. Performance data for abiotic reactive barrier material[197, 199-211] (zero-valent iron, activated carbon, etc.) exist to inform hydrological models[212], and in general do not require site specific testing.

For aquifer-wide *in situ* remediation clogging can occur in the direct vicinity of the well, or in the aquifer itself. Well-clogging is often easier to prevent, and measures are available to clean a clogged well[213-216]. As a last resort, a different well can be used for amendment delivery. Permeability reduction in the aquifer is difficult to address, and is best avoided through predictive treatability studies using flow-through columns[56]. Laboratory column studies have been used to pinpoint causes of permeability reduction

in situ. In one study[57], the use of unfiltered groundwater from the site was essential, and colloids suspended in the groundwater were identified as the major cause for permeability reduction, besides mineral precipitation and gas clogging[57]. In biological systems, it is also important to use fresh groundwater for flow-through tests as protozoan grazing on bacteria can effect clogging[217]. In summary, different phenomena leading to permeability reduction are fairly well understood in principle. But, as a detailed review on biological clogging shows[58], flow-through studies are needed to quantify the extent of permeability reduction in a given situation, especially if they involve environmental factors such as predation or competition among microorganisms. Measures to prevent permeability reduction in treatability studies, as taken in some laboratory flow-through studies[184, 210, 218], should be avoided as they prohibit insights into potential clogging issues.

SUMMARY AND CONCLUSIONS

Failures of ISB do occur and delay cleanup of contaminated sites, as has been identified in the survey among remediation practitioners. Although not all potential failures can be predicted prior to implementation of ISB, some of the most frequently encountered failure sources can be addressed in suitable treatability studies. These include the inability to (i) deliver and distribute bacteria and/or amendments within the subsurface; (ii) ensure survival and metabolic activity of augmented microorganisms; (iii) sustain biological activity in time and space; (iv) reach cleanup goals within the treatment area; (v) prevent dissolution of heavy metals or accumulation of toxic byproducts; and (vi) prevent or manage clogging of injection wells and the surrounding aquifer.

While laboratory batch microcosms are useful tools to answer fundamental questions on the suitability of ISB at a given site, and can address some concerns of remediation

practitioners, e.g., secondary contamination through metal dissolution, they are not suited to predict many common failures of implementing ISB. Column experiments are suitable to pinpoint many of these issues early on and may therefore reduce the failure rate in the field, leading to cost savings and reducing the time to reach cleanup goals.

Mass-transfer limitations govern many *in situ* processes, including contaminant transport and bioavailability. On the small scale these process are simulated in continuous treatability studies such as flow-through columns, which can inform on continuous biological activity of contaminant-degrading microorganisms, extent of contaminant degradation within a given flow field, and bioavailability limits in a sediment dominated environment. Sustained biological activity is also intricately linked with the survival of augmented microorganisms in face of competition with indigenous microbes and other environmental factors. Because of fundamental limitations of laboratory studies to replicate environmental conditions, interaction of indigenous and introduced microorganisms is best assessed by introducing the bioaugmentation culture into the contaminated environment. Field experiments are also vital to test delivery systems for remediation agents. The distribution of any amendment throughout the site is strongly influenced by heterogeneity of site geochemistry, hydrology and biology, and can be assessed to some extent by fate and transport models. However, issues with amendment delivery are typically not encountered prior to full-scale treatment, and can only be addressed by designing a flexible remediation system.

TRANSITION 1

The first chapter presents compelling arguments for the use of flow-through systems to study processes occurring in groundwater aquifers. These can be multi-well studies in the field, using the natural or induced groundwater gradient. In the laboratory, sediment columns are typically used to study hydrodynamic processes. However, due to cost-, space-, and/or material limitations, column studies are often conducted without multiple replicates. While it is a paradigm of environmental science that differences between experiments can only be determined with some certainty if the data variability within the experiment is known (*i.e.* by using multiple replicates), this is often not extended to flow-through column studies.

The second chapter of this dissertation is the first study to systematically examine and quantify variability sources in batch and flow-through sediment microcosm studies commonly performed in the remediation industry and academia. I examine the reproducibility of column studies conducted in the laboratory, and compare the findings to the more commonly used batch microcosm approach. Based on analysis of 833 data points from triplicate batch and column microcosms, relative standard errors in batch and column studies were found to be statistically indistinguishable. Contributions to the overall standard error from analytical error sources were minor, and column-study specific error sources, *i.e.*, variability in pump flow-rate and packing of columns with site material, also contributed little. Biological variability was identified as the main contributor to overall standard error (up to 90%) for both batch and column microcosms. Replicate small-scale column studies are identified as an attractive but currently under-utilized tool to study and parameterize phenomena occurring in flow-through environments such as aquifers.

2. STATISTICAL ANALYSIS OF BATCH AND COLUMN MICROCOSM METHODOLOGIES EMPLOYED FOR THE IN SITU REMEDIATION OF CONTAMINATED AQUIFERS

INTRODUCTION

Flow-through column studies are regarded as more realistic than batch microcosms to simulate processes in a hydrodynamic environment, such as contaminated aquifers[219]. They are commonly used for treatability studies of *in situ* permeable reactive barriers for groundwater remediation[52, 68, 199, 209, 210], and are recommended in guidance documents for the design of reactive barrier systems[220, 221]. It has been recognized that batch microcosm tests are useful as "an initial screening tool", but that "column testing provides more reliable reaction rate parameters than batch testing" and it "provides information from dynamic flow conditions"[221] similar to conditions underground. Column studies afford the assessment of flow-through reaction kinetics, longevity of reactive material, and hydraulic properties of the barrier material[220]. Much of this information is valuable not only to reactive barrier design, but also to full-scale *in situ* remediation feasibility assessments.

Column treatability studies for *in situ* bioremediation are expensive when conducted by commercial laboratories, where one column experiment can cost on the order of $10-15K, as much as a complete batch microcosm study with 12 bottles (SiREM, personal communication). They are generally regarded as challenging to set up and operate and often are limited by the need for large amounts of site material to fill and operate multiple columns. Additionally, the space requirement of multiple large columns can be a significant cost factor for commercial laboratories. Consequently, column studies are not routinely conducted for bioremediation treatability studies, and typically lack replicates when performed. In the rare instances where replicate columns are used,

investigators may opt to not explicitly report on the variability between them[222-227] or show representative data from individual columns only[228]. Only few column studies regarding groundwater remediation were identified that reported individual data from duplicate columns, either graphically[229-234] or numerically[235-238]. The use of triplicate columns is even less common. A thorough literature search only identified eight column studies[211, 239-245] for groundwater remediation that reported results from more than two replicate columns, only one of which assessed an *in situ* biological remediation process extant in a permeable reactive barrier[211].

While multiple replicates and statistical significance testing of results is recognized as essential to environmental science in general, it appears this has not been extended to the design of column studies for *in situ* remediation, where single columns continue to be recommended even in guidance documents[23, 220].

Results from multiple nutrient amendments[246, 247], distinct bioaugmentation cultures[65], and different treatment approaches, are compared based on single columns per treatment, often without discussion of reproducibility or information on the margins of statistical significance between the various treatments. For example, one study looking at bioaugmentation to degrade carbon tetrachloride set up two identical columns[111]. Although the columns were operated almost identically, some parameters differed between the columns (*i.e.*, age of inoculum, duration of nutrient addition, pH adjustment). While in general performance was similar for both columns, some parameters varied quite significantly (specifically, chloroform production in only one column, different extent of colonization of column sediment by introduced bacteria, different location and extent of bioactive zone) and could have been caused by differing conditions or simply by real-world variability between replicates. To interpret such data,

it would be invaluable to understand in statistical terms the variability of biological microcosm experiments and the type and magnitude of error sources involved.

To address this knowledge gap, the objective of the current study was to examine the statistical variation of several data sets (two batch microcosm studies and four column studies) gathered from simulation of *in situ* biodegradation of trichloroethene and perchlorate, to assess the relevance of errors associated only with column studies, and to draw comparisons to statistical variation found in other column studies. At the onset of this work, we hypothesized that the data variability from column studies would be larger than from batch microcosms, because in addition to mutual error sources like biological activity, sampling and analytical error, column studies possess additional error sources (e.g., column fill material, flow rate, influent composition/feed source) that might lead to greater data variability.

EXPERIMENTAL SECTION

Batch Microcosm Studies

Culturing of a chloroethene-dechlorinating consortium (Dehalo R^2) was carried out as described previously[19]. Briefly, Dehalo R^2 was maintained in sediment-free anaerobic mineral medium supplemented with vitamins as well as sodium lactate and methanol as sources of electrons and carbon. Trichloroethene (TCE) was supplied as an electron acceptor and injected manually with a gas-tight syringe. Data suggest good reproducibility.

Batch Study I was carried out in 160-mL glass serum bottles capped with butyl rubber stoppers and aluminum caps. Bottles contained 10 g of dry sediment (<0.5 mm grain size), 10 mL Dehalo R^2 inoculum (contained about 1.54*10^9 and 1.25*10^10 gene copies of *Dehalococcoides* and total bacteria, respectively), and mineral medium or

groundwater was added to give a total liquid volume of 100 mL, leaving 60 mL headspace for sampling of volatilized chlorinated ethenes (CE). Batch microcosms were incubated at 30°C on an orbital shaker. Batch Study II was carried out in 250-mL glass serum bottles containing 50 g of sediment, 180 mL of anaerobic mineral media medium or groundwater, and 3 mL of KB-1 inoculum. Batch microcosms were incubated statically at room temperature. Sodium lactate was supplied when dechlorination stalled. An overview of the experiments conducted is provided in Table 2-1. All experiments were conducted in triplicate.

Table 2-1. Experimental design for batch microcosms and column studies. All medium/groundwater contain vitamins, unless otherwise specified.

	Inoculation	Medium composition	Electron donor/acceptor supplied [mg/L] (mM)
Batch Microcosm Study I:			
Control	10 mL Dehalo R^2	Adjusted to 100 mL with mineral medium	TCE [73] (0.55) Methanol [396] (12) Na lactate [560] (5)
Groundwater	10 mL Dehalo R^2	Adjusted to 100 mL with groundwater	TCE [73] (0.55) Methanol [396] (12) Na lactate [560] (5)
Sediment	10 mL Dehalo R^2	10 g dry sediment Adjusted to 100 mL with mineral medium	TCE [73] (0.55) Methanol [396] (12) Na lactate [560] (5)
Batch Microcosm Study II:			
Control	3 mL KB-1	180 mL mineral medium	TCE [16] (0.12) Na lactate [311] (2.8)
Bioaugmentation without Vitamins	3 mL KB-1	180 mL groundwater (without vitamins)	TCE [16] (0.12) Na lactate [311] (2.8)
Bioaugmentation	3 mL KB-1	180 mL groundwater	TCE [16] (0.12) Na lactate [311] (2.8)
Column Study I:			
No amendment	N/A	Groundwater (sediment in column)	TCE [33] (0.25) Methanol [179] (5.6)
Biostimulation	N/A	Groundwater (sediment in column)	TCE [33] (0.25) Methanol [179] (5.6) Na lactate [2000] (17.8)

Bioaugmentation	Dehalo R^2	Groundwater (sediment in column)	TCE [33] (0.25) Methanol [179] (5.6) Na lactate [2000] (17.8)
Column Study II:			
No amendment	N/A	Groundwater (sediment in column)	TCE [33-0.4] (0.02-0.003) Methanol [179-2.14] (5.6-0.067)
Biostimulation	N/A	Groundwater (sediment in column)	TCE [33-0.4] (0.02-0.003) Methanol [180-2.1] (5.6-0.1) Na lactate [2000] (17.8)
Bioaugmentation	Dehalo R^2	Groundwater (sediment in column)	TCE [33-0.4] (0.02-0.003) Methanol [180-2.1] (5.6-0.1) Na lactate [2000] (17.8)
Column Study III:			
No amendment	N/A	Groundwater (sediment in column)	ClO_4^- [0.5] (0.005)
Na acetate	Perchlorate reducers	Groundwater (sediment in column)	ClO_4^- [0.5] (0.005) Na acetate * 3H2O [1000] (7.3)
Ethyl lactate	Perchlorate reducers	Groundwater (sediment in column)	ClO_4^- [0.5] (0.005) Ethyl lactate [290] (2.5)
Column Study IV:			
No amendment	N/A	Groundwater (sediment in column)	ClO_4^- [0.5] (0.005)
Na acetate	Perchlorate reducers	Groundwater (sediment in column)	ClO_4^- [0.5] (0.005) Na acetate * 3H2O [1100] (8.1)
Ethyl lactate	Perchlorate reducers	Groundwater (sediment in column)	ClO_4^- [0.5] (0.005) Ethyl lactate [340] (2.9)

N/A – not applicable

Column Studies

All column experiments were conducted in triplicate using glass chromatography columns (25 cm long, 1.2 cm inner diameter, Chemglass Life Sciences, Vineland, NJ) filled with site sediment (0.5 – 1 mm grain size). The columns were packed with dried, sieved sediment and tapped vigorously on all sides to ensure homogenous packing. Columns were operated in upflow-mode. A schematic of the setup is provided in Figure

2-1. The column studies were conducted at 22°C and columns were covered with aluminum foil to prevent growth of photosynthetic organisms. Columns were fed from the same influent reservoir, ensuring minimal variability in influent composition between columns. A multi-channel peristaltic pump (Ismatec, IDEX Health and Science, Oak Harbor, WA) was used to provide groundwater flow to the columns, and a multi-syringe pump (New Era Pump Systems, Farmingdale, NY) was used to add nutrient amendments to the columns (added at 1% of groundwater flow). Experimental parameters for all column studies are listed in Table 2-1.

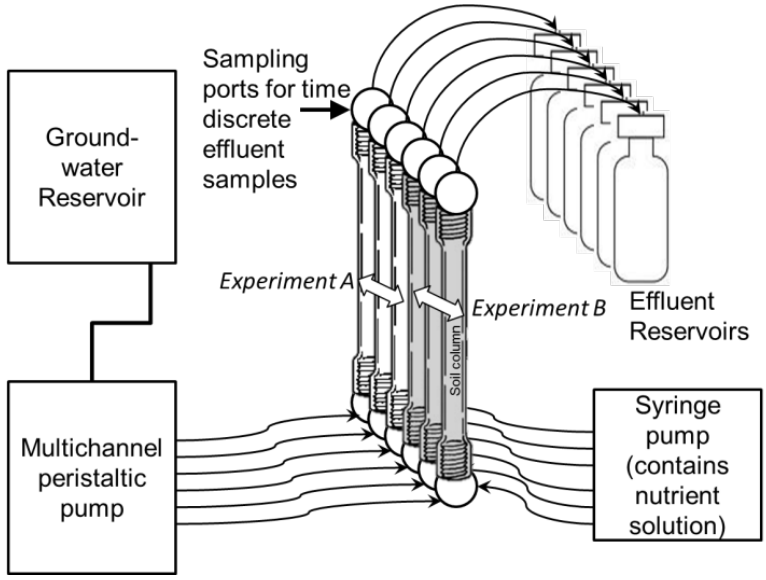

Figure 2-1. Experimental setup for flow-through column studies.

The first column study (Column Study I) was conducted with high TCE influent concentrations to ensure that TCE was not limiting dechlorination activity. Natural attenuation (no amendment), biostimulation (addition of sodium lactate) and bioaugmentation (sodium lactate and Dehalo R^2[19] inoculation) were tested simultaneously (each in triplicate). Site groundwater amended with TCE and methanol was used as the column influent for all nine columns. Groundwater was pumped through

the columns at a flow-rate of 5 µL/min, leading to an approximate column residence time of 30 hrs. Sodium lactate was supplied at 2 g/L effective concentration. This concentration was chosen to provide non-limiting carbon concentrations to reduce all electron acceptors present in the groundwater (oxygen, nitrate/nitrite, sulfate, manganese, iron, CEs, etc.).

The second column study (Column Study II) was conducted similar to the former. Throughout the study, several parameters (flow rate, column length, TCE concentration) were varied, and dechlorinating activity in all columns was monitored in response to parameter changes.

Column studies III and IV were conducted with perchlorate-contaminated groundwater. A facultative anaerobic microbial consortium (enriched from sewage sludge obtained from five different U.S. wastewater treatment plants) was utilized as a seed culture for bioaugmentation experiments. Sodium acetate trihydrate or ethyl lactate was added as carbon source and electron donor (see Table 2-1). All columns were operated at 15 µL/min flow, equivalent to residence time of 10 hours in the column (as determined in tests employing a conservative tracer, bromide).

Analytics

For batch microcosms, 200 µL headspace sample was withdrawn with a gastight glass syringe with Teflon plunger, and injected directly into a gas chromatography (GC) system with flame ionization detector (FID). Chlorinated ethenes (perchloroethene [PCE], TCE, dichloroethene [DCE] isomers, vinyl chloride [VC]) and ethene as well as methane were quantified. Method details have been previously described[19].

For Column Studies I and II, 1 mL liquid sample was taken from each column outlet using a gastight syringe and transferred to a 1.5-mL glass vial with crimp top. Liquid

samples were analyzed with a solid-phase microextraction GC/FID system using an autosampler. Chlorinated ethenes (PCE, TCE, DCE isomers, VC) and ethene were quantified. The method has been described in detail elsewhere[228].

For Column Studies III and IV, 1 mL liquid sample was taken from each column outlet and pH was measured before the sample was filtered through a 0.45-µm filter for further analysis. Perchlorate was analyzed using an ion chromatography system with conductivity detector following EPA method 300.0. Perchlorate standard solution was obtained from SPEX Certiprep (Metuchen, NJ). Details of the analytical methods have been published previously[248]. Sulfate was analyzed following EPA method 314.0. Standard solutions were obtained from Dionex (Thermo Scientific, Sunnyvale, CA; Combined Seven Anion Standard containing fluoride, chloride, nitrite, bromide, nitrate, phosphate, sulfate).

Statistical Data Analysis

Relative standard error (RSE), instead of standard deviation (σ), was used to express statistical uncertainty of analytical results (N – number of equivalent observations; y_i – individual observations; y_{av} – sample mean).

$$\sigma = \sqrt{\frac{\sum_{i=1}^{N}(y_i - y_{av})^2}{N-1}} \qquad (1)$$

$$RSE = \frac{\sigma}{\sqrt{N} * y_{av}} \qquad (2)$$

Standard error indicates the uncertainty around the estimate of the mean measurement, and is most useful as a means of calculating a confidence interval[249]. In contrast, standard deviation describes the variability of individual values, but does not describe the accuracy of the sample mean.

A thorough literature review was conducted using 'Web of Knowledge' and 'Google Scholar'. Peer-reviewed publications regarding *in situ* groundwater remediation were identified that conducted flow-through column studies with two or more replicates per experiment. Relative standard errors were calculated from numerically reported data in those manuscripts. To compare standard errors from different studies, values were transformed to RSE. Standard errors of one parameter (e.g., metal leachate concentration) measured for different experimental groups (e.g., control, treatment A, treatment B) were combined to give an average RSE. These mean values are reported along with the SE of the mean.

The following setting was used for all student *t*-tests conducted on multiple data sets: unpaired, 2-tailed, homoscedastic.

RESULTS AND DISCUSSION

To examine the reproducibility of results in commonly used batch microcosms compared to flow-through columns, two independent batch microcosm studies and four columns studies were conducted. Different scenarios of biological dehalogenation of chlorinated ethenes and biodegradation of perchlorate were investigated with respect to variability and reproducibility among replicates (triplicates). In total, 381 samples from triplicate batch microcosms and 452 samples from triplicate columns yielded a mean relative standard error (RSE) of 18 ± 1.1% and 26 ± 1.1%, respectively, which were statistically indistinguishable, as revealed by a student *t*-test ($p>0.1$).

Feasibility of Triplicate Column Studies

Most column studies for bioremediation are conducted using a single large column, constructed from stainless steel, glass or plastic with dimensions of 5-10 cm diameter and 1-2 m length[65, 111, 250, 251]. These dimensions require significant amounts of site material (2000 cm^3 – 16,000 cm^3) and groundwater (up to 40 L per week and column[251]). Columns for chemical treatment (e.g., reactive barriers) are often smaller, <5 cm in diameter and <50 cm long[68, 73]. However, both bioremediation and chemical remediation studies have been conducted with columns as small as 1 cm or less in diameter and as short as 10 cm in length[252-254]. To minimize wall effects using small columns, the ratio of column-to-particle diameter ideally should be 50 or greater[255], but greater than 10 at a minimum[256]. The ratio of column length-to-diameter should be small (<50) to minimize wall effects[257] and to incorporate vertical and longitudinal transport[258]. For this study, we used small glass columns (1 cm inner diameter, 25 cm long) filled with site material (~20 cm^3 per column; 0.5-1 mm grain size), resulting in a column-to-particle diameter ratio of 100 and a column length-to-diameter ratio of 25, well within the recommended range. Each experiment was conducted in triplicate. Due to the low flow-rate (5-15 µL/min) and the small column size, only moderate amounts of groundwater/liquid medium were needed to conduct the studies (50-150 mL per week and column). These moderate needs for site material, on par with the material needed for batch microcosms, enable the setup of replicate columns even for non-local sites, where site material has to be transported over long distances and shipping costs can be substantial. The small size of the columns also minimizes laboratory bench space requirements, which is another cost-driver for commercially conducted column studies (the bench space used for each column study as shown in Figure 2-1 – including pumps, influent/effluent reservoirs, columns, etc. - was about 1 m^2).

Mutual Error Sources for Batch and Column Studies

Variability between replicate microcosms/columns originates from biological, sampling and analytical parameters. Sampling errors can be avoided through good sampling protocols (using compatible materials, not storing samples for prolonged periods, etc.) and careful execution of these protocols by the investigator. Analytical errors are associated with the instrumentation and methods used for sample analysis. Standard analytical methods for CEs (gas chromatography with different detectors; listed in National Environmental Methods Index, www.nemi.gov) and perchlorate (ion or liquid chromatography with different detectors) list precisions between 0.78 – 25% and 10.6 – 14% relative standard deviation (RSD), respectively. The U.S. Food and Drug Administration guidelines for bioanalytical methods recommend an RSD of less than 20%[259]. In our study, the error associated with replicate measurements of performance samples (see Table 2-2 for individual RSEs) ranged from 1.8 to 6.8% RSD (1.0 – 3.8% RSE) for batch microcosms (GC-FID analysis of headspace samples). For column studies, analytical errors ranged from 1.1% RSD (0.7% RSE) for perchlorate (IC with ion suppression and conductivity detector) to 2.5 – 9.3% RSD (1.5 – 4.7% RSE) for CEs (SPME-GC-FID analysis of liquid samples), which are in the same range as those of other analytical methods.

Biological variability is typically the most significant of the three and is difficult to avoid, especially when using mixed microbial communities or environmental materials (groundwater, soil, sediment, etc.). It can be minimized through homogenization of the materials.

Table 2-2. Mean standard errors (± SE) [%] for dechlorination batch microcosm and column experiments over the monitoring period. Concentrations of chlorinated ethenes [mM] were measured over time in triplicate microcosms/columns. The number of individual SEs comprising the mean SE is given in parenthesis.

	TCE [%]	*cis*-DCE [%]	VC [%]	Ethene [%]	Total CEs [%]
Batch Microcosm Study I:					
Control	26±7 (17)	33±6 (19)	30±4 (36)	16±3 (36)	26±2 (108)
Sediment	8±2 (16)	18±3 (17)	8±1 (34)	7±1 (32)	10±1 (99)
Groundwater	8±1 (34)	56±5 (34)	16±2 (34)	ND	27±3 (102)
Batch Microcosm Study II:					
Control	5±1 (4)	18±8 (6)	15±3 (5)	4±1 (5)	11±3 (20)
Bioaugmentation w/o vitamins	6±1 (9)	11±8 (5)	19±101 (2)	ND	9±9 (16)
Bioaugmentation	10±2 (9)	10±2 (4)	ND	ND	10±2 (13)
Batch Analytical Error (RSE)[a]	3.4	3.9	1.0	3.6	3.0±0.7 (4)
Column Study I:					
No Amendment	14±3 (20)	2±1 (6)	6±1 (7)	ND	11±3 (33)
Biostimulation	8±2 (21)	9±3 (6)	28±12 (8)	ND	12±3 (35)
Bioaugmentation	27±4 (20)	15±5 (11)	31±4 (15)	47±4 (10)	29±2 (56)
Column Study II:					
No Amendment	20±3 (30)	18±4 (30)	35±7 (7)	ND	19±2 (67)
Biostimulation	41±3 (25)	27±3 (42)	40±6 (19)	ND	34±2 (86)
Bioaugmentation	38±7 (7)	41±3 (37)	36±4 (43)	36±3 (35)	38±2 (122)
Column Analytical Error (RSE)[b,c]	1.5 (1)	1.7 (1)	2.3 (1)	4.7 (1)	2.5±0.7 (4)

[a] determined from triplicate sample analysis
[b] determined from quadruplicate sample analysis
[c] modified from standard deviation of recovery in groundwater as listed in Table 1 in Ziv-El et al. 2013[228]
ND – analyte not detected

Error Sources Specific to Column Studies

Flow-through column studies have additional sources of variability associated, e.g., from the soil/sediment used to fill the columns, uneven packing causing preferential flow paths, variability in flow-rates between columns, and variable influent/amendment composition.

In this study, and most other column studies, the column fill material was homogenized prior to packing the columns. The reproducibility of the packing method was tested with conservative tracer tests. The RSE of tracer concentrations in the column effluent (2 sets of columns with different sediment grain sizes, each in triplicate) was 43±6%, and therefore represents a relevant source of variability (tracer curves are shown in Appendix B, Figure S1). However, the resulting residence times in triplicate columns only had an RSE of 3% and 7% for two different grain sizes, respectively.

The flow-rate RSE in our column study setup ranged from 2.5±1.3% to 1.4±0.4% for different flow rates tested (200, 100, 50, 20 µL/min, Figure S2), and therefore represents only a minor source of variability. The column feed came from one vessel, split into multiple channels through a manifold. This ensures that the influent composition for all columns is identical at all times. Amendments were supplied with a piston-driven multi-syringe pump, which is able to provide a very constant flow rate independent of pressure conditions in the column.

A thorough literature review identified twelve *in situ* remediation studies that reported numerical results from flow-through columns with two or more replicates. Published results were analyzed to identify RSEs for different parameters (listed in Table 2-3). Average RSEs for abiotic or process parameters are generally low, between 3% and 21%. However, biological processes were found to exhibit higher RSEs. The RSEs found for

two published studies examining biological phosphate and nitrate removal was 19% and 36±18%, respectively. These are on the high end of RSEs found for columns studies.

Table 2-3. Average standard errors [%] (± SE of the average) reported in peer-reviewed literature that conducted column studies with multiple replicates.

Parameter Observed	Average SE [%]	Number of SEs	Reference
Cr(VI) and As(V) removal capacity[a]	1.2 ± 0.4	6	[236]
Mass balances for different electron acceptors[a]	36 ± 18	39	[237]
Cr(VI) concentration in column soils[a]	37 ± 6.7	8	[238]
pH in column soils[a]	2.7 ± 1.2	8	[238]
Cr(VI) concentration in column soils[b]	28 ± 2.9	5	[238]
pH in column soils[b]	2.4 ± 0.4	5	[238]
Bacterial recovery[b]	7.4 ± 4.0	19	[242]
Steady state parameters and kinetic values[b]	5.0 ± 1.7	5	[243]
Metal concentration leached from different soils[b]	6.7 ± 2.6	10	[244]
Norfluorazon concentration leached from different soils[b]	3.8 ± 0.8	9	[245]
Biological sulfate removal rate[c]	19	2	[211]

[a] Column study conducted in duplicate
[b] Column study conducted in triplicate
[c] Column study conducted with 6 and 4 replicates

Total Variability in Batch Microcosms and Column Studies with Biological Activity

The reproducibility of triplicate batch microcosms was assessed for six sets of experiments assessing different scenarios of biological dehalogenation of chlorinated ethenes (CE). The reproducibility of dechlorination activity was found to be fairly good, with RSEs of measured CE concentrations ranging from 4 – 56% (Figure 2-2, Table 2-2), with an average RSE of 18 ± 1.1%. This includes the error sources discussed above (analytical, sampling, biological errors).

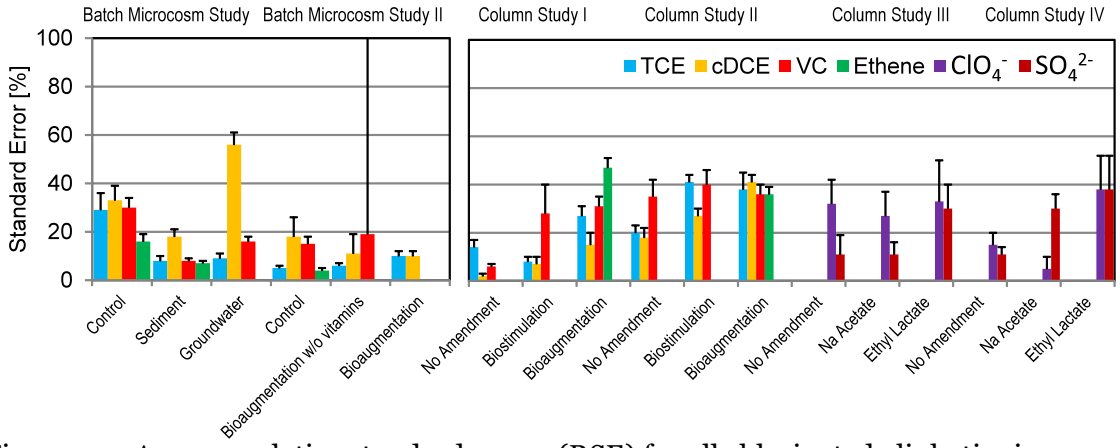

Figure 2-2. Average relative standard errors (RSE) for all chlorinated aliphatics in samples from batch microcosm/column studies. Error bars represent SE of the mean RSE.

The RSE for CE effluent concentrations in Column Studies I and II ranged from 6 – 41%, and from 5 – 38% for anions in column studies III and IV (Figure 2-2, Table 2-4). RSEs for all column studies were not significantly different from RSEs found for batch microcosms ($p>0.1$). This is despite the additional error sources characteristic for flow-through column studies, and underscores the significance of biological variation expected to be similar in batch and flow-through systems. Similar RSEs (Table 2-5) were found for published data from a bioaugmentation column study examining TCE degradation rates[235]. Analysis of 12 duplicate data points revealed that RSEs in that study ranged from 0–71%, with a mean RSE of 32±8 %. This is very similar to the average RSE for Column Studies I and II we conducted, which was 27±1.1%.

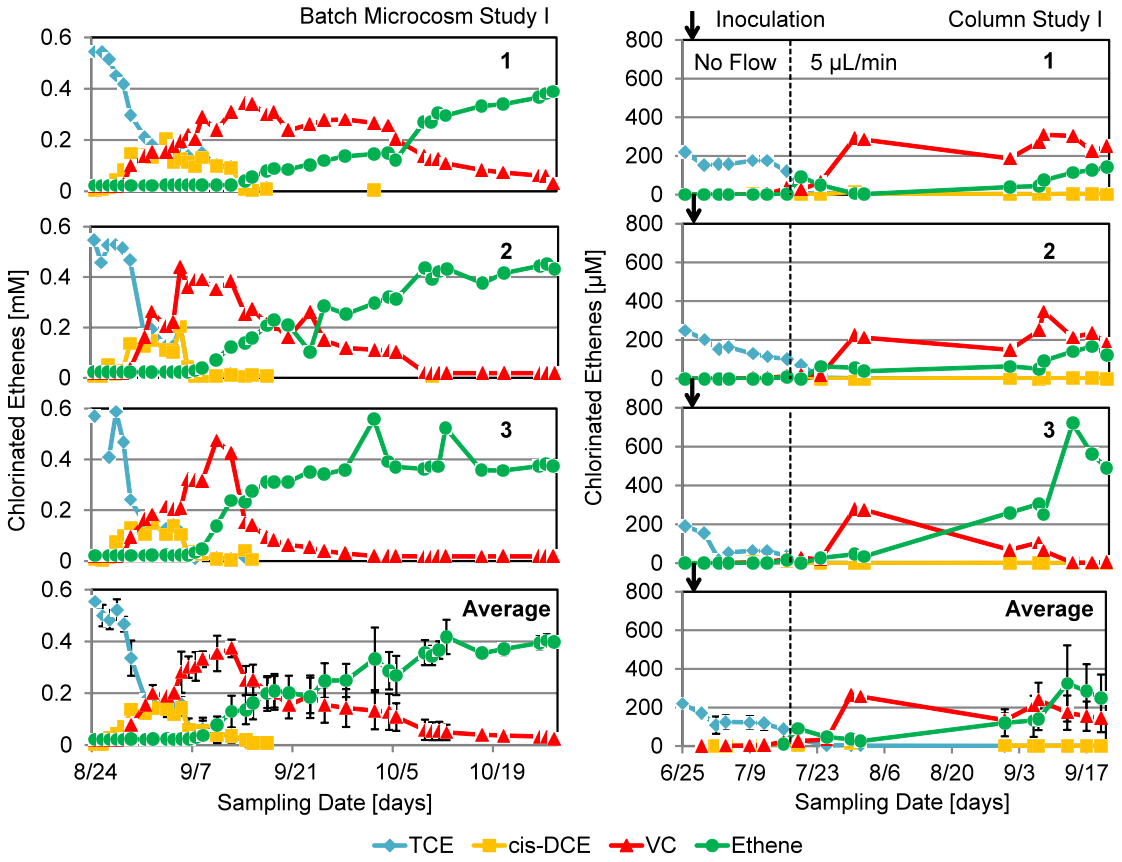

Figure 2-3. Concentrations of chlorinated aliphatics monitored in triplicate batch microcosms (left) and column effluent (right). Shown are control microcosms (mineral medium, augmented with dechlorinating culture), and bioaugmented flow-through columns. Data from individual microcosms/columns are labeled 1-3; average data (±SE), as typically presented, are shown in the bottom panels.

Table 2-4. Mean standard errors (± SE) [%] perchlorate-reduction column experiments over the monitoring period of 21 days. Concentrations of anions [µg/L] and pH were measured over time in triplicate columns. Nine individual SEs comprise the mean SE, except for (*) which consisted of 8 samples.

	ClO_4^- [%]	SO_4^{2-} [%]	Total Anions [%]	pH [%]
Column Study III:				
No Amendment	32 ± 10	11 ± 8*	22 ± 7	1.3 ± 0.5
Na Acetate	27 ± 10	11 ± 5	19 ± 6	1.2 ± 0.3
Ethyl Lactate	33 ± 17	30 ± 10	32 ± 9	1.1 ± 0.2
Column Study IV:				
No Amendment	15 ± 5	11 ± 3	13 ± 3	1.1 ± 0.6
Na Acetate	5 ± 5	30 ± 6	18 ± 5	2.0 ± 0.4
Ethyl Lactate	38 ± 14	38 ± 14	38 ± 10	1.7 ± 0.4
Analytical Error (RSE)	0.7 ± 0.3 (14)			

Table 2-5. Standard errors [%] for duplicate column experiments described in Haest et al. 2011235. Concentrations of chlorinated ethenes [mM] were measured in column effluent after 245 days of incubation. Total CEs represents the mean standard error (± SE) for all CEs listed.

	TCE [%]	*cis*-DCE [%]	VC [%]	Ethene [%]	Total CEs [%]
High flow	70	5.4	64	0	35 ± 19
Medium flow	13	12	63	71	40 ± 16
Low flow	71	0	6.4	6.5	21 ± 17

To further examine reproducibility of flow-through column studies, we conducted one study (Column Study II), where several parameters (flow-rate, column length, TCE influent concentration) were varied, and TCE dechlorination in response to these changes was observed. We hypothesized that RSE would increase following a change in operating conditions before conditions would stabilize again, accompanied by a decrease

in RSE. However, data showed that the RSE itself is not indicative of a change in operating conditions.

The presented data illustrate the feasibility of conducting flow-through column studies for *in situ* bioremediation on a small scale that does not require large amounts of site material or laboratory space. Reproducibility was found to be in a range similar to that of batch microcosms, with no statistically significant differences detectable. Biological variability was identified as the main error source (up to 90%, with sampling, analytical, and column specific errors making up the rest), as underlined by the very similar RSE (p=0.98; student t-test) found for column studies with perchlorate (non-volatile, non-sorbing salt) and CEs (volatile, sorptive), the latter of which are much more prone to sampling and analytical errors. This bodes well for the routine use of column studies (with multiple replicates) to produce results that are much more applicable to flow-through environments such as subsurface aquifers, and should therefore be preferred over the more common methodology of using batch microcosms.

TRANSITION 2

The first half of my dissertation identified flow-through studies (Chapter 1), and more specifically flow-through studies with multiple replicates (Chapter 2) as superior approaches over routinely used batch microcosms for the study of hydrodynamic environments, such as groundwater aquifers.

In addition to choosing the appropriate experimental setup, it has been widely recognized that laboratory artifacts lead to limitations when predicting field performance from laboratory findings. Many of these artifacts impact specifically the microbial community found in different environments. Owing to this fact is our inability to culture the majority of microorganisms in laboratory settings, while they thrive under environmental conditions. Artifacts are introduced by removing material (water or sediment) from its environment, potentially changing its pH, concentration of dissolved gases (carbonate, oxygen, etc.) and salts, as well as removing microorganisms from protective niches in the sediment matrix and modifying cultivation conditions (temperature, humidity, etc.).

The main part of my dissertation focuses on the development of a novel device that enables flow-through column studies to be conducted in contaminated aquifers, thereby preventing some of the artifacts described above. The *in situ* microcosm array (ISMA) approximates *in situ* conditions by drawing groundwater directly from the aquifer and delivering it to replicate flow-through columns filled with site sediment. Storage of column influent and effluent in the device affords *in situ* testing at ambient subsurface temperatures and conditions without causing release of either the chemicals or bacteria evaluated. Chapter 3 describes the design and capabilities of the ISMA.

3. *IN SITU* MICROCOSM ARRAY (ISMA) TECHNOLOGY FOR TREATABILITY STUDIES IN GROUNDWATER REMEDIATION – PART 1: DESIGN AND CAPABILITIES

INTRODUCTION

The National Research Council has estimated that in 2012 126,000 sites across the U.S. still have contaminated groundwater, and that their closure is expected to cost at least $110-127 billion[260]. To limit these costs, the remediation industry has largely moved away from energy-intensive pump-and-treat systems to clean up contaminated aquifers. It has been recognized that pump-and-treat systems on their own often do not achieve cleanup goals in a timely manner or at all[261, 262], but are effective in plume control. To address recognized limitations of groundwater extraction and ex situ treatment, there has been a movement in the last decade to more sustainable *in situ* remediation technologies, which ideally destroy contaminants permanently, while using less energy and producing less hazardous waste in the process. The percentage of National Priority List sites at which *in situ* remediation is implemented has increased from 6 to 64% during the years 1986 through 2005[2], and some form of *in situ* remedy was implemented at 81% of National Priority List sites between 2005 and 2008[1]. Research budgets to further these *in situ* technologies are limited, however. The U.S. government (Environmental Protection Agency, Department of Defense, Department of Energy) requested almost $9 billion for its environmental restoration obligations in 2012, but only $250 million (<3%) were requested towards research efforts[263-265]. This means that new remediation technologies will only come to market if they can be tested in a cost effective, yet scientifically defensible way.

Rigorous treatability studies need to be conducted to determine if *in situ* remediation at a given site will be effective in lowering contaminant concentrations to meet cleanup

goals[21]. Treatability studies are conducted to identify the appropriate reagent and its dosage. They also need to inform on the impact of the remedy on other groundwater constituents, such as dissolution/mobilization of metals, changes in pH, redox conditions, and the potential to create hazardous byproducts[266]. Above all, treatability studies need to be predictive of the processes and degradation rates that actually will prevail in place, i.e., *in situ*.

Current methodologies that are used for initial screening of accepted and experimental remediation strategies are laboratory batch microcosms[267], laboratory flow-through column studies[23], and *in situ* field studies conducted on the small scale[268]. Laboratory batch microcosms are most commonly used to assess a range of treatment options because they offer a fairly simplistic design and low implementation costs. Most guidance documents for *in situ* remediation call for the implementation of microcosm studies, typically in the form of batch microcosms[24, 25, 269-271]. The more elaborate approach[272] of using sediment columns is sparingly applied by remediation professionals and in many instances deemed to be cost-prohibitive. Yet, from a scientific perspective, flow-through columns are considered the gold standard approach for studying phenomena in saturated subsurface environments[273] because they can serve to study transport phenomena, which are key in the design and implementation of *in situ* remediation.

It has been acknowledged that lab and field results are expected "to be quantitatively, even qualitatively, different from the same determination if it could be done *in situ*"[274]. The shortcomings of laboratory microcosm studies have been recognized by many[261] and resulted in calls for the development of alternatives[24]. By removing site groundwater from the subsurface environment, changes in chemical and microbial parameters are introduced. These can include out-gassing of carbon dioxide, leading to changes in the

buffering capacity of the water and possibly its pH, which in turn can effect changes in the speciation and solubility of metals. Volatilization of organic compounds, e.g., halogenated aliphatics, and precipitation of metals and salts can change the concentration of contaminants of interest. Furthermore, during sampling and storage of groundwater, the microbial community is removed from its protective sediment environment and becomes susceptible to grazing by protozoa[136], exposure to oxygen and to other unfavorable conditions. These stressors can lead to significant changes in the microbial community[275], going as far as complete inactivation, death or removal of susceptible microorganisms that may be essential for bioremediation progress to occur (e.g., poisoning with oxygen of strictly anaerobic bacteria of the genus *Dehalococcoides*). All these factors play a significant role for *in situ* remediation[276] and can distort a legitimate extrapolation of results obtained from laboratory treatability studies[277-279].

To overcome the fundamental limitations of laboratory studies[78], *in situ* field studies can be conducted to obtain more realistic results. Treatability tests designed to compare multiple remediation approaches can be performed using amended passive samplers, for example Bio-Traps® [280] (sometimes referred to as BACTRAPs® [281]), that capture microorganisms which actively grow on a porous artificial medium (e.g., Bio-Sep® beads). They can provide qualitative information on the potential for *in situ* contaminant degradation[173] cost-effectively and in a relatively short amount of time, by showing the presence of specific degraders and/or incorporation of isotopically labeled contaminants into microbial biomass[282]. However, these samplers are neither meant to, nor suitable for, capturing the complex mass transfer processes that occur in the saturated sediment of the aquifer. Use of so-called *in situ* microcosms (ISMs)[283, 284] – stainless-steel cylinders that are pushed into the sediment to isolate a portion of the aquifer, thereby enabling withdrawal, amendment with different agents, reinjection and sampling of

groundwater over time[285] – offer the advantage of leaving microbial and geological features of the aquifer intact. However, this approach is labor intensive, requires on-site attendance and is applicable only to shallow subsurface sites[284] where direct-push methods can be used.

To overcome these limitations and to bridge the gap between laboratory tests, passive sampling technologies and pilot scale treatability tests, new approaches are needed that offer the benefits of realistic flow-through column studies and can sidestep many of the established artifacts associated with conducting experiments in the laboratory. We seek to address this need of conducting flow-through treatability studies in the field by introducing a novel down-hole remedial design tool, termed the *in situ* microcosm array (ISMA). This manuscript describes the design and capabilities of the ISMA, including how treatability tests can be conducted and what kind of data are produced when using the ISMA for *in situ* treatability studies.

EXPERIMENTAL SECTION

Design and Manufacturing of the In Situ Microcosm Array

The outer shell of the device and some internal components were designed using computer-aided design software (3DS SolidWorks, Dassault Systèmes SolidWorks Corp, Waltham, MA).

Pump Design

An off-the-shelf pump (Ismatec, Glattbrugg, Switzerland) was modified to fit within an 8.9 cm outer diameter shell, as required for the ISMA device to fit into a standard 10-cm (4-inch) inner diameter groundwater monitoring well. Pump cassettes that control flow in the pump tubing of the peristaltic pump were manufactured using rapid prototyping technology.

Performance of the customized pump was evaluated for long-term stability of delivered flow, accuracy, and inter-channel reproducibility of the flow volume. To test accuracy and inter-channel reproducibility, pumps were mounted in the laboratory and performance tests conducted in triplicate for 4.5 – 5 hours at flow rates set to 20, 50, 100, or 200 μL/min. Pumped water was collected and measured volumetrically to infer flow rates. A long-term pump-rate stability test was conducted in the same fashion operating 12 channels at a target flow rate of 15 μL/min. Flow rate was monitored daily for a period of 35 days.

Pump accuracy was also tested for an unmodified comparable pump (Ismatec Reglo Digital, Ismatec, Glattbrugg, Switzerland). The pump was operated in the laboratory with 24 channels at a target flow rate of 79.1 μL/min in duplicate experiments for 0.5 and 2.7 hours, respectively. Results were averaged over all 24 channels and both tests.

Sediment Column Tracer Tests

Six custom glass columns (1 cm ID, 25 cm long; Chemglass Life Sciences, Vineland, NJ) were packed with dried, sorted sediment of two different grain size fractions (each in triplicate) referred to as fine (<0.5 mm) and coarse (0.5 – 1 mm) sediment. Sediment was obtained from a site in Mesa, AZ and characterized as well graded sands, gravelly sands, containing little or no fines, but containing inorganic clays. Tracer tests were conducted by injecting a slug of bromide (40 μL of 5 g/L NaBr) into sediment columns and monitoring effluent bromide concentrations over time. Bromide was analyzed following EPA Method 314.0. Details of the analytical method have been previously published[248].

Preservative Efficacy Test

A preservative (Kathon GC/ICP, Sigma-Aldrich, St. Louis, MO) was used to inhibit continuation of biological reactions in the effluent once it had left the sediment columns. The effectiveness of mixing of preservative and column effluent was tested using fresh groundwater that was pumped into four effluent capture vessels at a rate of 136 µL/min. The preservative was added according to manufacturer's specifications at 0.01% final concentration to two of the vessels, while the other two served as a control with no added preservative. After 24 hours of pumping at room temperature, 100 µL of each effluent was plated in multiple dilutions onto Luria-Bertani agar plates. Plating was done in triplicate. Colonies formed on the plates were counted after incubating them at room temperature for two days.

Adsorptive Cartridge Test

One type of gas sampling cartridge (Anasorb CSC, SKC Inc., Fullerton, CA) containing activated carbon was tested with a mixture of chlorinated ethenes (trichloroethene, *cis*-dichloroethene, vinyl chloride) and ethene, which were pumped as aqueous solutions at a rate of 80 µL/min into effluent vessels equipped with a gas vent line that was furnished with the cartridges. The test was conducted in duplicate. Influent concentrations were monitored daily, and the liquid in the effluent vessels was sampled at the end of the experiment. The gas sampling cartridges were analyzed for chlorinated ethenes by a contracted laboratory (Columbia Analytical Services, Simi Valley, CA) following standard methods (NIOSH 1003/1007). Mass recovery of the chlorinated ethenes was determined by comparing the total mass of chlorinated ethenes entering the vessels compared to the mass contained in the vessels at the end of the experiment, and the mass that was captured by the activated carbon.

Groundwater Well Sampling

Groundwater was sampled at field tests before and after deployment of the ISMA device in the well. The water was analyzed at the site for field parameters, including temperature, pH, oxidation-reduction-potential, and dissolved oxygen using a pre-calibrated multi-parameter probe (YSI Inc., Yellow Springs, OH).

Data Analysis

Degradation Rate Calculation

To assess the performance of each treatment approach tested during an ISMA treatability study the column effluent was analyzed for the contaminant of interest, as well as for potential degradation products and secondary groundwater quality parameters, such as heavy metals, nitrate and nitrite concentration, etc.

To calculate the *in situ* rate of contaminant transformation, R, two approaches are considered:

a) Conservative rate estimation assuming zero-order contaminant transformation

$$R_i = k_{0i} = \frac{dC_i}{dt} = \frac{C_{i_{\text{Influent}}} - C_{i_{\text{Treatment}}}}{\Delta T_{\text{Column}}} \qquad (1)$$

where R_i is the transformation rate and k_{oi} is the zero-order rate constant of contaminant i, t is time, ΔT_{Column} is the residence time of the groundwater in the sediment columns, C_i is the concentration of contaminant i in the microcosm effluent of a specific treatment or in the influent groundwater collected as control.

b) Conservative rate estimation assuming first-order contaminant transformation

For experiments conducted using the ISMA, the first-order reaction rate constant k_{1i} is calculated from log-transformed data according to the following equation:

$$k_{1i} = \frac{\ln(C_{i_{\text{Influent}}}) - \ln(C_{i_{\text{Treatment}}})}{\Delta T_{\text{Column}}} \quad (2)$$

The contaminant degradation rate *R* is then calculated according to the following equation.

$$R_i = k_{1i} * C_i \quad (3)$$

Geophysical Parameters

In addition to contaminant transformation rates, it is important to obtain information on changes in physical properties of the subsurface caused by a given treatment approach. By conducting simple tests on the sediment microcosms before and after field deployment, the porosity Φ and effective porosity φ_e can be determined using equations 4 and 5, respectively:

$$\Phi = \frac{W_{wet} - W_{dry}}{V_{Total}} \quad (4)$$

$$\varphi_e = \frac{q}{RT} \quad (5)$$

where *W* is the weight of the sediment microcosm with wet or dry sediment, V_{Total} is the total volume of the sediment microcosm, *q* is the specific discharge derived from the groundwater flow rate divided by the cross section of the microcosm and *RT* is the retention time of a conservative tracer in the microcosm determined by a pulsed tracer test.

RESULTS AND DISCUSSION

Flow-through column studies are the current gold standard when it comes to laboratory treatability tests in saturated media[273]. However, their value for *in situ* remediation could be much improved by conducting the experiments in a more realistic setting. We therefore set out to design and build a tool that can perform flow-through column studies *in situ* to eliminate laboratory artifacts. To make this tool practical for field application, a number of challenges had to be overcome, including autonomous operation of flow-through microcosms during incubation, and incorporation of all system components into a leak-proof shell. The resulting device, called *in situ* microcosm array (ISMA), contains all components of a laboratory column study in dual-containment design that was chosen to preclude the potential risk of releasing chemicals into the aquifer during treatability tests. During device deployment, fresh groundwater is pumped directly from the aquifer, which eliminates the need for transport and storage of groundwater from the contaminated site to the laboratory, and thereby avoids out-gassing of volatile compounds and carbon dioxide, potential introduction of oxygen and the associated adverse impact on groundwater chemistry and microbial community composition and activity in the groundwater.

Design and Manufacturing of In Situ Microcosm Array

To fit within the constraints of common 10-cm (4-in) diameter groundwater wells, many components of a standard laboratory column study needed to be miniaturized. Design restrictions included an 8.9-cm outer diameter (OD) of the device, a modular design limiting the length of each module to no more than 2.5 m, and the ability for quick assembly of the device in the field, while ensuring reliable functionality of all of its components. All materials needed to be compatible with a range of chemicals potentially

extant in contaminated aquifers. The materials used to manufacture reusable components (outer shell and connectors, sediment columns, pumps, pump cassettes, electronics, etc) include stainless steel (ASTM A312), Viton®, Teflon®, and glass.

Different components of the device are housed in tubular stainless-steel sections, which are connected sequentially during field deployment of the device (Figure 3-1, left panel). The connections between modules are load bearing, waterproof and transmit all necessary fluid and electrical lines. The device is suspended on a steel cable to the desired depth and electrical power is supplied from an array of batteries and solar panels in remote locations or from a standard electrical outlet (110 V or more) where available. This enables autonomous operation for the duration of the treatability test.

At the time of submission of this manuscript, the ISMA had been successfully deployed five times. To ascertain that no chemicals are released during *in situ* deployment, well water was sampled for multiple field parameters and chemicals before and after deployment of the ISMA. Results revealed no differences between groundwater chemistry before and after field deployments (data shown in Appendix C Table S1).

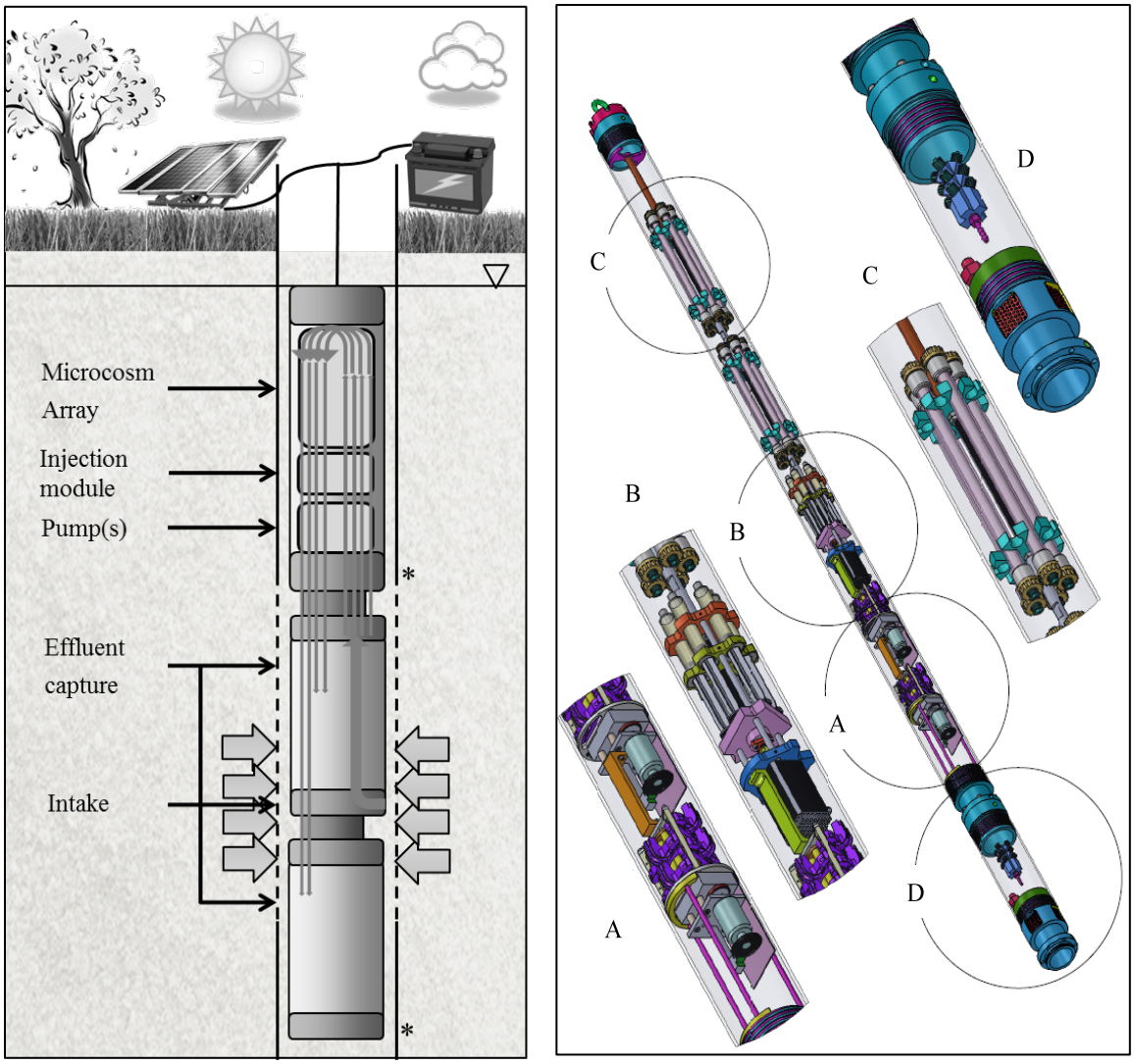

Figure 3-1. Left: Schematic of the *In situ* Microcosm Array in a groundwater well. *Denotes potential intake locations. Right: Detailed schematic of the ISMA device; Detail A - peristaltic pump; Detail B - injection module; Detail C - microcosm array; Detail D - 360° intake and multi-channel manifold.

Groundwater Delivery

Peristaltic pumps were chosen to achieve continuous low flow rates required for simulating slow groundwater movement through the sediment microcosms. The pump design chosen affords control and uniform flow of water through the multiple parallel channels regardless of differences in conductivity and headloss across the various microcosms. Additionally, none of the reusable parts of the pump hardware come into contact with contaminated groundwater with the chosen design. Selection of other types of pumps (piston pumps, gear pumps) would have increased the risk of chemical and bacterial cross-contamination when sequentially using the tool in different wells or at different sites.

To accommodate the stringent size limitations, an off-the-shelf pump (Ismatec, Glattbrugg, Switzerland) was modified to fit into the 8.9-cm OD stainless steel shell. Customizations included re-design of the motor mounting plate as well as the cassettes holding the tubing. The cassette material Ultem® (polyetherimide) was chosen for its low surface friction to eliminate rubbing of the tubing material, as well as its rigidity to provide even pressure across the pump tubing. Physical properties of the cassette material are listed in Appendix C Table S2. Drawings of the customized pump assembly are shown in Figure 3-1, right panel, Detail A. Performance of the customized multi-channel pumps was assessed in multiple flow tests. Results shown in Figure 3-2A and 2B demonstrate that flow rates are accurate (<30% standard deviation) and reproducible between multiple channels over a range of 20 – 200 μL/min flow.

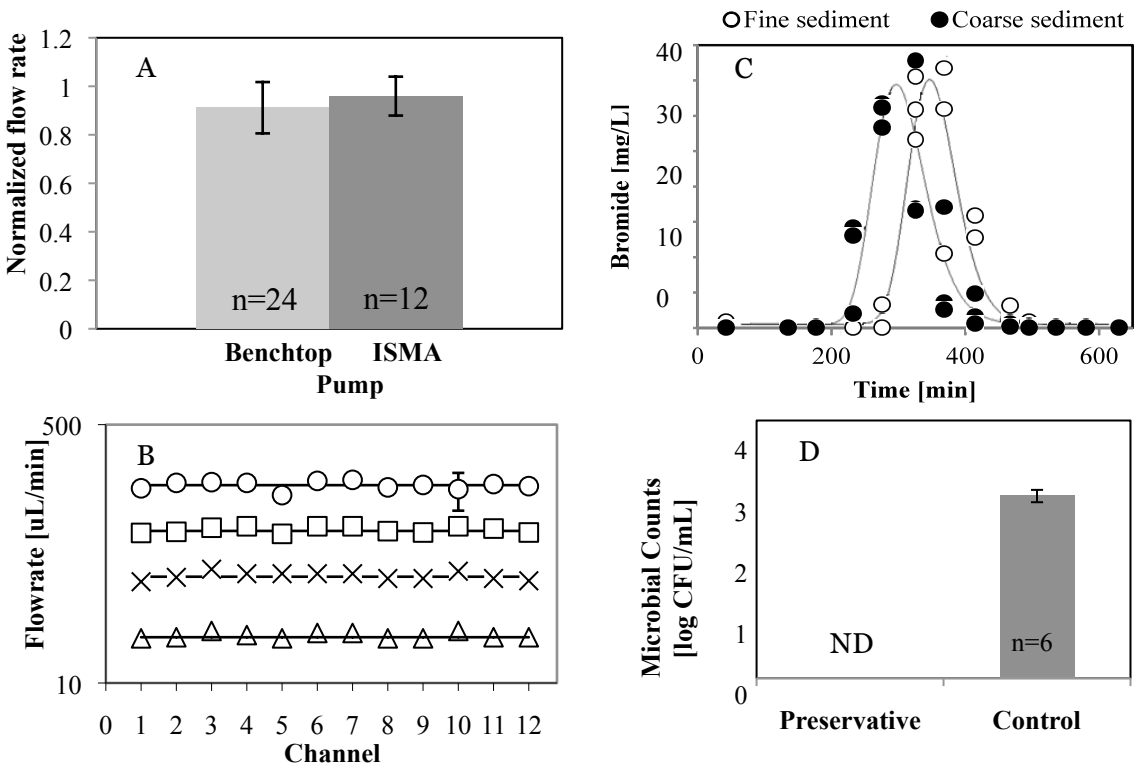

Figure 3-2. Performance control experiments: A - Pump flow rate accuracy for peristaltic benchtop pump (Ismatec Reglo Digital) and customized peristaltic pump used inside the *in situ* microcosm array (ISMA). Tests were conducted for 24 or 12 channels, respectively. B - Flow rate reproducibility between 12 channels for customized pump in the ISMA. Flow rates [µL/min] were set to 20 (Δ), 50 (x), 100 (□) and 200 (○) as indicated by the solid lines. Shown is the average of three measurements. C - Conservative tracer curves showing the bromide concentration in column effluent over time after a one-time injection of bromide. Sediment columns were filled with fine (<0.5 mm) or coarse (0.5 – 1 mm) grains. Experiments were conducted in triplicate. D - Results from preservative test showing plate counts of column effluent treated with preservative and without (control). Experiments were conducted in duplicate, plating was done in triplicate. ND = non-detect (<300 CFU/mL).

During the *in situ* test groundwater is pumped directly from the subsurface formation through a screened intake (100 µm pore size nylon mesh), which is aligned with the well's screened interval at target deployment depth. Groundwater entering the ISMA is split into twelve individual lines by a custom manifold (Figure 3-1, right panel, Detail D) and fed through two six-channel peristaltic pumps which push groundwater through columns in up-flow mode to ensure sediment saturation and enable escape of gas

bubbles. Flow rates can be adjusted to achieve column residence times representative of the linear velocity of groundwater at the deployment site.

Delivery of Treatment Agent

The ISMA was designed to enable assessment of both natural and enhanced contaminant removal rates. To deliver a treatment agent (e.g., chemical or biological agent) to the columns the ISMA device contains a customized syringe pump as an injection module (Figure 3-1, right panel, Detail B) that uses a single drive shaft to actuate multiple syringes. Different agents can be supplied to each microcosm. Pump rate and amendment concentration are adjustable to simulate different dosing regimens and treatment approaches. Injection agents include carbon sources and electron donors to simulate biostimulation, nonindigenous bacteria for bioaugmentation, or a chemical oxidizer/reducing agent to simulate *in situ* chemical treatment.

Sediment Columns

In the configuration shown, up to ten flow-through columns can be operated concurrently (Figure 3-1). Custom glass columns (250 mm length, 14 mm ID) are furnished with Teflon® screw caps and Viton® O-rings that provide a waterproof seal (Figure 3-1; right panel, Detail C).

Columns are ideally filled with fresh site sediment where available. If intact cores are not available, archived sediment, representative of the subsurface stratum of interest, can be used. Alternative packing materials include quartz sand, activated carbon, sediment mixed with iron filings, etc.

The reproducibility of manually packing the sediment columns was tested by injecting a conservative tracer (bromide) and monitoring its concentration in the column effluent

over time. Figure 3-2C shows the tracer curves of two tests using different grain sizes of sediment in the columns. Both tests were carried out in triplicate. The tracer curves show that the replicate columns performed very similarly, proving the reproducibility of the packing method. The data also show that no preferential flow occurred in the columns as indicated by the tracer showing a retention time consistent with the pore volume of the column. Lastly, obtained data show that, as expected, the residence time in the column is dependent on the grain size of the sediment, due to the lower effective porosity of the smaller vs. the larger grains, which is inversely related to residence time (equation 5).

Effluent Capturing

The ISMA device is completely self-contained, which guarantees no impact on the well where the treatability test is conducted. All groundwater pumped through columns as well as an influent control (untreated groundwater) is stored inside the device in custom-made Teflon® sample capture vessels (Figure 3-1; left panel). To ensure that the degradation activity measured occurred in the columns, these vessels are loaded with a preservative/quenching agent designed to stop all unwanted biological or chemical activity once the effluent enters the sample capture vessel. Design criteria for the microbial preservative were that it needed to be fairly benign to humans upon accidental contact and provide broad-spectrum inhibition of bacteria, fungi, and yeasts. The preservative chosen (Kathon® CG/ICP) contains 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one as active ingredients. It is very stable, compatible with most analyses and is frequently used for abiotic control experiments[142, 286-289]. Passive mixing of the preservative with column effluent was demonstrated to be effective in inhibiting unwanted microbial growth (Figure 3-2D), and therefore further contaminant degradation.

The column effluent collected as a time-integrated sample is used for chemical and microbial analysis upon retrieval of the ISMA device. In addition to the effluent, the sediment inside the columns may be analyzed for sorbed or precipitated chemicals, presence and quantity of key microbial agents or geological sediment characteristics (permeability, porosity, etc.).

Through microbial activity or chemical reactions, significant amounts of gas (e.g., CO_2, N_2, H_2S) can be produced in the columns. The gas is allowed to vent from the sample capture vessels through vent lines, after passing through sorbent cartridges designed to capture volatile analytes of interest. Losses of volatile organics in escaped gas can thus be inferred from analysis of the sorptive material inside the cartridge. One type of cartridge containing activated carbon was tested with a mixture of chlorinated ethenes (trichloroethene – ethene), which resulted in a mass recovery by the cartridge (in percent of the total mass entering the vessel) of 1%, 3-7%, and 24-51%, for trichloroethene, *cis*-dichloroethene, and vinyl chloride respectively (data shown in Appendix C Figure S 3). Ethene, due to its small molecular weight and limited interaction with activated carbon, was not analyzed and was likely only captured in trace quantities. While this result suggests that the use of sorptive cartridges adds relatively little value to trichloroethene or *cis*-dichloroethene recovery, the capture of a significant portion of vinyl chloride, a known human carcinogen, justifies the use of sorptive cartridges to supplement chemical analysis of the liquid effluent.

Feasibility Testing Using the ISMA

For a mechanistic understanding of contaminant degradation processes, it is vital to discern biological degradation from abiotic processes, such as dilution, sorption, and abiotic degradation[78]. The National Research Council calls for cause-and-effect linkages to assess *in situ* degradation processes[270].

The ISMA device is uniquely able to answer these questions. Since multiple experiments can be carried out simultaneously in a single well, it is possible to suppress biological activity in one set of columns through addition of a biological inhibitor or sterilizing agent.

To evaluate natural attenuation processes of dilution, sorption or intrinsic degradation, columns in the ISMA can be operated with no addition of biological or chemical agents.

The possibility to simulate both reducing and oxidizing conditions in the same *in situ* treatability study without impacting the groundwater in the deployment well is another unique feature of the ISMA. An electron donor, e.g., lactic acid, or reducing agent, such as zero valent iron, can be added to a set of columns leading to reducing conditions, while an oxygen donor, e.g., hydrogen peroxide, may be added to a different set of columns leading to oxidizing conditions. These conditions can be simulated in the ISMA independent of the redox state of the aquifer and can simulate different biological or chemical *in situ* remedies.

To determine if bioaugmentation (addition of microorganisms and nutrients) is necessary, or if biostimulation (addition of nutrients only) of the intrinsic microorganisms may be sufficient, both approaches can be tested side-by-side. The columns can be inoculated with a bioaugmentation agent during the *in situ* test, or a

microbial community can be established in the sediment columns prior to field deployment.

Data Analysis

Degradation Rate

For the ISMA, a comparison of the primary contaminant concentration between influent control and the different treatment approaches serves as an indicator of the overall qualitative and quantitative success of each simulated treatment. All samples in the ISMA are collected as composite samples over the duration of the experiment. To determine degradation rates, the contaminant concentration in the composite influent (representing conditions at the start of the experiment) are compared to the composite samples of each treatment (representing conditions after treatment simulated in the different columns). These two data points in conjunction with the residence time in the sediment columns (reaction time) enables determination of zero-order (equation 1) or first-order degradation kinetics (equation 2 and 3) *in situ*.

Geophysical Parameters

In addition to contaminant transformation rates, it is important to obtain information on changes in physical properties of the subsurface caused by a treatment approach (Table 3-1). Conservative tracer tests can inform on porosity and effective porosity of the column sediment according to equations 4 and 5. The ISMA thus enables a determination of how different treatments affect sediment permeability, which has important implications for field implementation of a given treatment. Clogging of sediment pores and associated reduced permeability would limit the delivery of treatment agents, and can even lead to the contaminant plume bypassing the treatment zone by traveling through subsurface areas of higher conductivity.

Table 3-1. Overview of relevant parameters that are of interest in treatability studies and how they can be assessed using the ISMA device.

Parameter	Assessment Capability of ISMA
In situ rate of contaminant transformation	Zero-order or first-order transformation rate can be determined (see Experimental Section)
Release of secondary contamination / completeness of degradation	Secondary contaminants and degradation products may be analyzed from column effluent
	Precipitate formation can be analyzed from column sediment
Changes in geophysical parameters	Assessment through standard sediment column tests before and after field deployment (see Experimental Section)
Aquifer heterogeneity	Small-scale heterogeneity on the order of cm can be assessed by using intact sediment cores;
	No assessment of large scale heterogeneity
Long-term testing	Experiment duration from three to six weeks, longer if needed
Outcome variability	All experiments can be conducted in triplicate simultaneously
	Determination of statistically significant differences between treatment groups
Multiple treatment approaches	Multiple treatment strategies can be tested simultaneously in the same well allowing direct comparison
Mass transfer limitation of carbon and electron donor/acceptor	Can be assessed if added directly to the sediment, e.g., pure phase DNAPL, pure vegetable oil as carbon source, etc.
	No assessment of field-scale mass transfer limitations related to delivery of carbon source or electron donor
Sampling of attached microbial community	Microbial DNA and protein can be extracted from sediment material after field deployment
Sampling of suspended microbial community	Microbial DNA and protein can be extracted from column effluent after field deployment
Proof of biological activity	Direct comparison of microbially active (biostimulated or bioaugmented) columns with non-amended or poisoned control in the same experiment enables distinction of biological and abiotic processes

Microbiological Analyses

The success of *in situ* bioremediation hinges on the presence and successful stimulation of microorganisms that can degrade the contaminants at a site. It is well known that the majority of microorganisms reside attached to the subsurface sediment[135], and that representative biomass sampling should consist of both aqueous and solid phase samples[290]. By way of its design, treatability studies conducted with the ISMA afford the opportunity to obtain information on both attached microorganisms (present in the sediment columns) and planktonic microorganisms (suspended in column effluent). At the end of a field deployment the soil contained in the columns can be sampled and DNA extracted. Similarly, DNA can be extracted from the composite effluent collected from each column. Performing quantitative PCR on the genes of interest in both types of samples can inform on the presence and quantity of microorganisms critical to remedial success, such as *Dehalococcoides* spp. for dechlorination of halogenated ethenes[169, 267, 291-293]. Additional molecular tools can be employed to characterize the microbial community[294], such as pyrosequencing, clone libraries[295], terminal restriction fragment length polymorphism, etc.

Limitations of the In Situ Microcosm Array

Due to the physical size of the ISMA, it can only be deployed in wells with a diameter of 10-cm ID or larger. A survey of groundwater wells in five states (Arizona, Texas, Minnesota, Pennsyvania and Illinois) showed that the majority of wells have dimensions of 10 cm in diameter or larger. (Only wells up to 15.2 cm were considered in the survey, since larger wells are mostly used for water pumping purposes; so the numbers provided here are conservative.) If the whole device is to be submerged in water, a saturated thickness of 8.3 m or more is desirable so as to fully submerse the unit during incubation

in the well. However, because the location of the groundwater intake in the ISMA is flexible, shallower water levels can be accommodated, too.

The ISMA is also limited by the amount of column effluent that can be stored in the sample capture vessels (8.4 L total), which correspondingly determines the maximum deployment duration at a given pump rate. This total effluent volume can be allocated to different experiments accommodating individual needs regarding sample volume.

The physical size of the columns limits the residence time that can be achieved while simulating realistic groundwater flow velocity. This can mean that slow biological reactions do not go to completion, or that natural attenuation happening on a larger scale cannot be detected. Also related to the column size, the microcosms in the ISMA only incorporate soil heterogeneities at the cm-scale, similar to other small-scale *in situ* tests (ISM, single well push-pull tests). It is well known that geological heterogeneities, e.g., clay lenses and preferential flow channels, can have a significant impact on the success of *in situ* remediation[112, 296]. These factors can be assessed to some degree during thorough site investigation and in pilot scale treatability studies involving multiple wells, but may not be fully understood even during full-scale treatment.

The *in situ* microcosm array is a novel tool for remedial design and decision-making. It overcomes limitations of current approaches by offering the ability to test multiple remediation strategies side-by-side in a comparable manner in the same location using the same groundwater as influent. It also combines the benefits of laboratory studies (tight system control, more complete mass balances, and no release of agents into the aquifer) with the greater realism of studies conducted *in situ*. It utilizes site groundwater coming directly from the aquifer and site sediment, thereby ensuring minimal disturbance of the indigenous microbial community. Nothing is released into the environment during a treatability study using the ISMA, ensuring low risk to the aquifer

and on-site personnel. By way of these characteristics, the ISMA may be characterized as a sustainable approach to treatability testing for *in situ* groundwater remediation, for scientific studies, bioprospecting and risk assessment of novel treatment technologies.

TRANSITION 3

Chapter 3 of this dissertation described the challenges and solutions involved with miniaturizing all components of a flow-through column study to fit in a device that can be deployed in groundwater wells. In Chapter 4, a case study is presented for the use of the ISMA at a perchlorate-contaminated site. As demonstrated here, the ISMA enables the *in situ* operation of flow-through column experiments with multiple replicates for statistical evaluation. Analysis of effluent samples from each experiment for chemical constituents, hydrogeological analysis on sediment columns, as well as DNA analyses of both attached and suspended microbial communities were conducted. Thereby, the ISMA provides multiple lines of evidence on the performance of tested in situ remedies, as called for in guidance documents used by remediation practitioners and regulators. The concurrent evaluation of several *in situ* remedies side-by-side in the same well, with no impact on the deployment aquifer and well is demonstrated. As such, the ISMA technology provides a unique and novel capability for remedial design of *in situ* treatment by strategies including monitored natural attenuation, biostimulation and bioaugmentation.

4. *IN SITU* MICROCOSM ARRAY (ISMA) TECHNOLOGY FOR TREATABILITY STUDIES IN GROUNDWATER REMEDIATION – PART 2: FIELD APPLICATION IN A PERCHLORATE-CONTAMINATED AQUIFER

INTRODUCTION

In situ remediation of contaminated aquifers (in the subsurface) has become increasingly popular in the past two decades and has partly replaced or supplemented *ex situ* (i.e., pump-and-treat) approaches to aquifer remediation[297, 298]. Reasons for this shift are numerous. *In situ* treatment, specifically biological treatment, can decrease cleanup times and lower the cost when compared to pump and treat[2], creates little or no hazardous waste, and often irreversibly destroys or mineralizes the contaminants to benign end products. In contrast, *ex situ* systems often transfer contaminants from one medium to another (e.g., transfer of volatiles dissolved in groundwater to the sorbed state, immobilized on sorption media that require further treatment or disposal)[2].

However, as with any other treatment approach *in situ* remediation also carries inherent risks[6]. The challenge is to find the right treatment agent for success, and to inject it in the proper dosage into the subsurface to effect contaminant removal without clogging the aquifer. Therefore, it is necessary to conduct feasibility studies to determine outcomes, predict reaction rates and identify any potential adverse outcomes of subsurface manipulation[21, 261]. Unwanted side-effects of *in situ* remediation include production of harmful contaminant breakdown products (e.g., vinyl chloride generation from the reduction of di-, tri- and tetrachlorinated ethenes[299]), mobilization of heavy metals (e.g., arsenic solubilization)[41, 300], or increased iron and manganese concentrations[301] that can lead to exceeding regulatory limits for these ions, any of which would limit or prohibit beneficial use of the groundwater for irrigation or potable water.

To address these uncertainties, *in situ* remediation approaches are usually tested in laboratory systems (batch microcosms or flow-through columns), and in a variety of field experiments[261]. Batch microcosm tests offer a simple design and typically show greater reproducibility than field experiments. However, their sediment/water ratio and associated mass-transfer phenomena are very different from the subsurface, both of which have been recognized to govern biodegradation processes *in situ*[7]. In contrast, flow-through columns simulate a hydrodynamic environment and associated processes which cannot be observed in batch microcosms[272]. Therefore, compared to batch microcosms, flow-through column experiments are generally considered to yield qualitative and quantitative results of higher predictive value concerning what later will be observed during cleanup at the field scale. However, because column studies can be cost-prohibitive, most treatability studies are conducted using batch microcosms. Degradation rates generated under optimal conditions in the lab frequently misrepresent those later observed in the field[302-306].

To bridge the knowledge gap between laboratory treatability studies and full-scale pilot studies, *in situ* tests should be conducted. Commonly used field technologies include multi-well tracer tests[307], Bio-Traps®[280], single well push-pull tests[308], and *in situ* microcosms[284]. With the exception of (non-baited) Bio-Traps®, these technologies all impact the host formation in such a way that the wells can no longer be used for compliance monitoring.

As a hybrid approach between laboratory and field-scale treatability testing, we have created the *in situ* microcosm array (ISMA)[309], a novel technology that enables the study of multiple remediation strategies autonomously *in situ* on a small-scale with no risk of releasing chemical and biological agents into the deployment well and surrounding aquifer.

The ISMA relocates flow-through laboratory column studies to the aquifer, where multiple tests are conducted *in situ*. The ISMA contains all components of a traditional laboratory column study in a watertight shell measuring 8.9 cm in diameter, thereby being small enough for deployment in conventional 10-cm (4-inch) groundwater monitoring wells. Multiple flow-through sediment column tests can be performed over a period of several days or weeks, while the device is incubated in the well below the water table, and undisturbed groundwater is pumped through the columns. This technology is described in detail elsewhere[309].

To demonstrate the capabilities of this new technology, we report parallel results from laboratory and field tests at a perchlorate-contaminated site in the southwestern US. The perchlorate anion (ClO_4^-) is a common groundwater contaminant in the southwestern United States, originating primarily from explosives manufacturing sites, research facilities and military sites[310], where perchlorate is used as an oxygen carrier in rocket propellants and other explosives to speed up the combustion process. The U.S. Environmental Protection Agency (EPA) added this oxyanion to its Contaminant Candidate List in 1998 and estimates that it impacts the drinking water of more than 15 million people[311]. Perchlorate is highly water soluble and therefore very mobile in the subsurface. It is also stable under most environmental conditions and can travel significant distances with the groundwater flow, leading to extensive groundwater plumes.

Perchlorate is biologically reduced under anoxic conditions to benign chloride and oxygen with perchlorate acting as the electron acceptor. Dissimilatory perchlorate-reducing bacteria (DPRB) have been studied intensively[312-318] and are considered ubiquitous[319]. Addition of a carbon source and electron donor is typically sufficient to stimulate biodegradation[316]. However, some environments may lack adequate numbers

of perchlorate reducers to achieve the desired reduction. In such cases, it may be beneficial to shorten the lag time to onset of perchlorate biodegradation and therefore optimize the kinetics of perchlorate removal via introduction of DPRB into site groundwater in a process termed bioaugmentation.

Remediation strategies evaluated in the present study included: (A) monitored natural attenuation (MNA); and (*B*) bioaugmentation with a perchlorate-reducing microbial consortium enriched from wastewater with concomitant addition of a carbon source consisting of either sodium acetate (B_1) or ethyl lactate (B_2). Batch and flow-through column studies were conducted in the laboratory in preparation of a field column study. The objectives of this study were to: (*i*) demonstrate the usefulness of the ISMA for conducting *in situ* treatability studies, (*ii*) determine *in situ* degradation rates, (*iii*) compare these with rates determined *ex situ*, and (*iv*) demonstrate the utility of data generated by the ISMA technology.

EXPERIMENTAL SECTION

Chemicals

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO), and were of 99% purity or higher, except for ethyl lactate (>98% purity) which was obtained from SAFC (St. Louis, MO). Quantitative PCR kits were obtained from TAKARA BIO Inc. (Shiga, Japan), qPCR primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA).

Site Description

The field demonstration was conducted at a small explosives-manufacturing facility located in the arid southwest of the United States (Figure 4-1). Legacy disposal practices at the site (since the 1960s) released ammonium perchlorate into sediment and groundwater, resulting in groundwater contamination above regulatory limits. The contaminant source area features several monitoring wells. The sediment in the area is characterized by low organic carbon content and mostly consists of silty sands and gravels, poorly and well graded sands, clayey sands and clayey gravels. The groundwater table is approximately 53 m below ground surface (bgs). Groundwater flow is generally to the southeast and has been induced by a groundwater recharge project northwest of the site. The groundwater is moderately aerobic with dissolved oxygen concentrations between 0.6 – 4.5 mg/L and around 12 mg/L nitrate, as measured in grab samples from wells HPA-1 and MW-8.

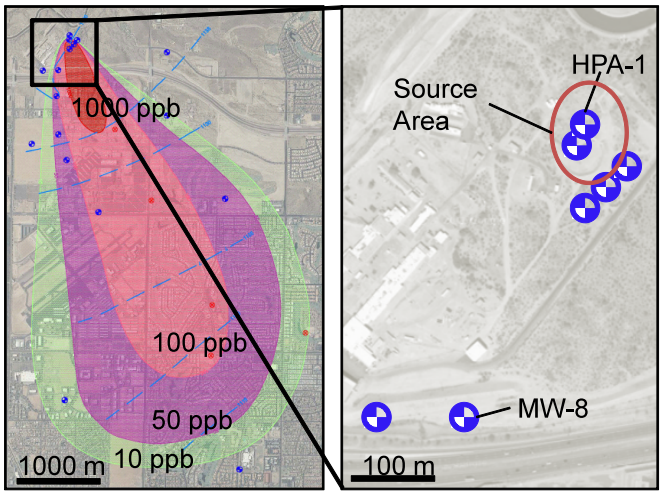

Figure 4-1. Left panel – Site overview including plume map of perchlorate in groundwater; Right panel – Source area of perchlorate contamination and location of wells used for *in situ* experiments and site materials obtained.
---- Groundwater elevation contours (5-ft steps between lines)
Groundwater monitoring well

Site Material

Both groundwater and sediments were obtained from well HPA-1 in 2010. Sediments had been stored at ambient temperatures in a closed barrel since the installation of the well in 2009. The sediment used for all experiments originated from 25 m bgs and contained about 500 mg/kg dry weight perchlorate . It further was composed of 64% sand, 22% silt, 14% clay, and 0.06% total organic carbon, and had a pH of 7.24.

All sediment was dried prior to sieving to obtain several grain size fractions (>1 mm; 1 – 0.5 mm; <0.5 mm). Since the sediment contained much higher concentrations of perchlorate contamination than the currently saturated zone at the site, it was washed with site groundwater until the perchlorate concentration in the wash water was below 3 µg/L.

Groundwater was obtained from well HPA-1 with a polypropylene bailer every few months and stored at 4°C until use in laboratory experiments.

Flow-through Experiments – Hardware

Flow-through experiments in the lab and *in situ* were conducted using the same sediment and groundwater sources. For the lab experiments, groundwater containing about 500 µg/L perchlorate was stored in a vented carboy at room temperature during the experiment. A manifold was used to split the influent into 12 individual lines. A multi-channel peristaltic pump (ISMATEC, IDEX Health & Science, Oak Harbor, WA), located downstream of the manifold, provided flow for the columns. Glass columns (25 cm long, 1.2 cm inner diameter, Chemglass Life Sciences, Vineland, NJ) with Teflon® caps were used. Sampling ports for time-discrete sampling were installed at the outlet of each column. Effluent from each column was collected in individual containers made

from Teflon®. Viton® tubing was used throughout. All other fittings were made from either Teflon® or nylon.

For field experiments, the same peristaltic pumps, glass columns, effluent containers, fittings and tubing as for the lab experiment was used.

Experimental Setup - Laboratory Experiments

Batch bottle experiments were conducted in 200-mL serum bottles capped with butyl rubber stoppers. Five replicate bottles were filled with 150 mL site groundwater and 5 g dried, well graded, washed sediment (<0.5 mm grain size) from the site. Each bottle was spiked with ethyl lactate (1000 mg/L; 8.5 mM) and potassium perchlorate (1000 µg/L perchlorate; 10 µM). No attempts were made to remove oxygen from the bottles at the beginning of the experiments. However, once capped, bottles were sampled periodically using gas-tight techniques to prevent oxygen from getting into the bottles, thereby enabling the development of anoxic conditions through microbial activity. Samples were analyzed for perchlorate concentration.

All flow-through sediment column laboratory experiments were conducted using the hardware described above (glass columns, peristaltic pumps, Teflon® vessels, Viton® tubing). Columns were packed with well graded sediment (0.5 - 1 mm grain size) obtained from drill cuttings from well HPA-1. Site groundwater containing about 500 µg/L (5 µM) perchlorate was used as the column influent for laboratory flow-through experiments. All lab experiments were conducted at room temperature, which is similar to the groundwater temperature of ~23°C at the deployment site.

Perchlorate is readily biodegradable[316] if sufficient perchlorate-reducing bacteria, nutrients, and appropriate redox conditions are present. Previous tests had shown a very low population of facultative anaerobic microbes in native sediment from the site[320].

Therefore, our experiments focused on bioaugmentation tests. The following experiments were conducted using flow-through columns in the laboratory: (*A*) site sediment without amendments, simulating monitored natural attenuation (MNA), (B_1) bioaugmentation with a seed culture containing perchlorate reducing bacteria with concomitant addition of sodium acetate as a supplemental carbon source and electron donor, and (B_2) bioaugmentation with concurrent addition of ethyl lactate. All experiments were conducted in triplicate. As a control, influent groundwater was collected in the same fashion as column effluent over the duration of the experiment without passing through sediment columns. All experiments were conducted simultaneously using the same source of site groundwater.

A facultative anaerobic microbial consortium enriched from sewage sludge obtained from five different U.S. wastewater treatment plants was utilized as a seed culture for bioaugmentation experiments to accelerate the onset and rates of perchlorate reduction. Each bioaugmentation column received 1 mL of seed culture at the beginning of the experiment by injection of the liquid culture at the influent (bottom) of each column. Sodium acetate trihydrate was added at 1100 mg/L (8.1 mM) influent concentration in experiment (B_1), and ethyl lactate at 340 mg/L (2.9 mM) in experiment (B_2). To compare bioaugmentation to the effects of natural attenuation, three columns were operated without addition of carbon source or biomass (Experiment *A*). All columns were operated in up-flow mode at 15 µL/min flow, equivalent to residence time of 10 hours in the column (as determined in conservative tracer tests).

The effluent of all columns was collected as a composite sample throughout the duration of the experiment to have effluent measurements comparable to the ones collected in the field *in-situ*. Effluent was stored at room temperature in individual Teflon® vessels containing a microbial preservative (Kathon® [5-chloro-2-methyl-4-

isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one], minimum concentration 0.5 mL/L effluent). In addition, time discrete samples of the effluent were collected periodically, filtered through a 0.45 μm polyvinylidene difluoride (PVDF) filter (PALL Life Sciences, Port Washington, NY), and analyzed for pH as well as concentration of perchlorate, nitrate, nitrite and sulfate using established techniques as described below.

Experiments were conducted for a period of 3 weeks. After termination of the experiments, composite effluent samples were analyzed for the same parameters as time-discrete samples. In addition, DNA was extracted from column effluent as well as the column sediment.

Experimental Setup - Field Experiment

The field experiments conducted in well MW-8 consisted of experimental scenarios (*A*) and (*B*), and were conducted in the same way as the laboratory experiments using identical equipment, including glass columns, peristaltic pumps, Teflon® vessels, Viton® tubing, etc. Field experiments were conducted in four replicates. The columns were operated in up-flow mode at an effective flow rate of 15 μL/min. The duration of the field experiments was 3 weeks, matching that of the lab experiments.

To ensure no impact on the deployment well had occurred, grab samples were taken from the well before and after deployment. The grab samples were analyzed for perchlorate concentration, DNA and a suite of other anions to assure no contamination had occurred.

Analytical Methods

Anion Analysis

A suite of anions (nitrate, nitrite, sulfate) was analyzed using an ion chromatography system with conductivity detector following EPA method 314.0. Standard solutions were obtained from Dionex (Thermo Scientific, Sunnyvale, CA; Combined Seven Anion Standard containing fluoride, chloride, nitrite, bromide, nitrate, phosphate, sulfate). Perchlorate was analyzed following EPA method 300.0. Perchlorate standard solution was obtained from SPEX Certiprep (Metuchen, NJ). Details of the analytical methods have been published previously[248]. To remove particles all samples were filtered through a 0.45 μm filter prior to analysis.

DNA Analysis

At the end of the experiments, DNA was sampled from 3 sections of the sediment column columns (inlet, middle, and outlet). Around 0.25 g of sediment removed from each section was extracted using the PowerSoil DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA) in combination with the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA). A detailed protocol is provided in Appendix D.

DNA was also extracted from the composite effluent from each column and from well grab samples. A known volume of effluent water was filtered through a 0.2-μm filter and DNA was extracted according to the manufacturer's protocol using the UltraClean Water DNA kit (MoBio Laboratories, Inc., Carlsbad, CA).

Extracted DNA was quantified spectrophotometrically using a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE) as per the manufacturer's instructions.

DNA from composite effluent and from sediments was characterized using quantitative polymerase chain reaction (qPCR). DNA targets included the 16S rRNA

gene as a measure of general bacteria, and the perchlorate reductase gene (*pcrA*) as a proxy of perchlorate reducing bacteria in each sample[321-323]. Plasmids containing the target DNA fragments used here were the same as those developed for previous studies[324]. PCR primer sequences are listed in Appendix D in Table S 3.

Residence Time, Porosity, Effective Porosity in Sediment Columns

Residence time (RT) in the sediment columns was determined by injecting a conservative tracer (40 µL slug of 5 g/L sodium bromide) into each column and monitoring its concentration in the effluent over time. This was done before the start of the lab experiment and after termination to observe potential effects from biomass growth, e.g., reduced porosity from biofouling leading to preferred flow paths and reduced residence times.

Porosity (Φ) was determined by comparing dry and wet weight (W) of the columns with the total volume (V) of each glass column.

$$\Phi = W_{wet} - W_{dry} / V_{Total} \quad (1)$$

Effective porosity (φ_e) was calculated from column residence time and specific discharge (q). Specific discharge is equal to the volume of flow per time divided by the cross section of the flow path (radial area of the glass column).

$$\varphi_e = q / RT \quad (2)$$

Perchlorate Degradation Rate

The first-order degradation rate of perchlorate was calculated for flow-through experiments in the lab and in the field. For laboratory experiments, time-discrete monitoring of the column effluent provided time-resolved data for calculation of a first-order degradation rate constant ($k_{Discrete}$) using the log transformed perchlorate

concentration of the influent (C_{in}) and effluent (C_{out}) grab samples for each experiment, as well as the residence time in the sediment column (RT).

$$k_{Discrete} = \frac{\ln(C_{in}) - \ln(C_{out})}{\Delta T_{\text{Column}}} \quad (3)$$

The first-order degradation rate R is then calculated according to the following equation:

$$R_{Discrete} = k_{Discrete} * C_i \quad (4)$$

where C_i is the mean contaminant concentration.

For the field experiments where time-discrete samples were unavailable, a composite sample collected over the duration of the experiment served to calculate a time-averaged first-order degradation rate.

$$k_{Composite} = \frac{\ln(C_{i_{\text{Influent}}}) - \ln(C_{i_{\text{Effluent}}})}{\Delta T_{\text{Column}}} \quad (5)$$

$$R_{Composite} = k_{Composite} * C_i \quad (6)$$

$R_{Composite}$ is the composite degradation rate, $C_{Influent}$ and $C_{Effluent}$ are the composite perchlorate concentration in the influent and effluent of each column, respectively.

RESULTS AND DISCUSSION

The main objective of this study was to demonstrate the possibility of conducting treatability studies *in situ* using the ISMA, with a perchlorate-contaminated field site serving as the deployment location of this proof-of-concept study. In preparation, laboratory batch and column studies were conducted prior to field column studies. The goal was to show the possibility of simulating anaerobic degradation in a moderately aerobic aquifer, without changing the groundwater in the demonstration well. Further,

we determined *in situ* degradation rates, and demonstrate the utility of the data generated during use of the ISMA.

Laboratory Experiments - Batch

Preliminary screening experiments were conducted in batch microcosms using groundwater and sediment from a perchlorate contaminated site. Biostimulation with ethyl lactate led to complete perchlorate reduction from 1000 µg/L to less than 3 µg/L within 4.5 to 23 days caused by lag times up to 10 days (data shown in Figure S 4). These long lag times likely are due to the low number of active microorganisms and low organic carbon content (0.06% total organic carbon) of the sediment. To overcome potential long lag times, a seed culture containing perchlorate-reducing bacteria was used in subsequent column experiments.

Laboratory Experiments – Flow-through Columns

Multiple transport parameters for the sediment columns were determined prior to the start of flow-through experiments. The hydraulic residence time in the columns was 10 ± 0.6 hrs and the mean effective porosity was 0.35. The hydraulic residence time was also determined after the experiments to detect effects caused by microbial growth, leading to clogged pore space and potentially shorter residence time. After the experiment, average hydraulic residence time decreased significantly ($p<0.05$) to 9 ± 0.3 hrs, and mean effective porosity was 0.33.

After bioaugmentation with a seed culture and both carbon amendments (experiment *B1* and *B2*), perchlorate was reduced consistently after an adaptation period of two days (Figure 4-2), while monitored natural attenuation (MNA - experiment *A* in Figure 4-2) did not lead to perchlorate reduction over the course of the experiment (Table 4-1). Both

carbon amendments led to similar perchlorate reduction rates (Table 4-1) determined from time-discrete sampling data as well as composite samples.

Table 4-1. Overview of perchlorate concentrations and first-order reduction rates (mean ± standard error). Results from lab and field flow-through experiments are listed.

		Influent [µg/L]	Composite Effluent [µg/L]	Time-discrete Concentration at End of Experiment [µg/L]	Composite Reduction Rate [hr-1]	Time-discrete Reduction Rate [hr-1]
LAB	Natural Attenuation	448 ± 44	525 ± 53	594 ± 60	0.02 ± 0.006	n/a
	Bioaug Sodium Acetate	448 ± 44	6 ± 3	<0.53	0.55 ± 0.12	0.74 ± 0
	Bioaug Ethyl Lactate	448 ± 44	22 ± 9	<0.53	0.35 ± 0.03	0.74 ± 0
FIELD	Natural Attenuation	228 ± 1	227 ± 2	n/a	<0.003*	n/a
	Bioaug Sodium Acetate	228 ± 1	30 ± 21	n/a	0.24 ± 0.09	n/a

n/a = data not available;
* A derivation of the minimum detectable reduction rate is provided in Appendix D.

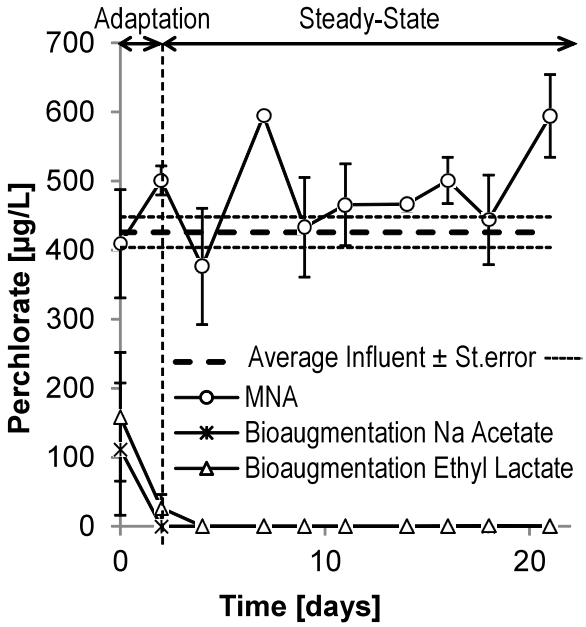

Figure 4-2. Concentration of perchlorate in effluent of continuous-flow sediment columns operated for 3 weeks. All experiments were conducted in triplicate, except for the influent concentration, which was measured from one sample at a time. Error bars represent standard error.

The groundwater also contained around 5 mg/L sulfate and traces of nitrate (<1 mg/L), both of which could serve as electron acceptors for the microbial community. Nitrate was reduced to <0.01 mg/L for both carbon amendments in less than two days, and no nitrate was detected for the remainder of the experiment. Nitrite was not detected at any point in the experiment. Sulfate was completely reduced to <0.01 mg/L in the columns with ethyl lactate amendment (*B2*) after an adaptation period of 16 days. During the adaptation period, sulfate concentrations decreased steadily. In sodium acetate amended columns (*B1*) sulfate concentrations started decreasing after 18 days, but only some sulfate was being reduced at the end of the experiment after 21 days. In MNA columns (*A*), neither nitrate nor sulfate was reduced throughout the experiment.

While nitrate is typically reduced before the onset of perchlorate reduction[312] or simultaneously with perchlorate reduction[325], it can be beneficial to perchlorate

reduction given that many perchlorate reducing microorganisms are also able to reduce nitrate. Therefore, the presence of small amounts of nitrate can serve as an alternate electron acceptor for perchlorate reducers stimulating their growth. The presence of sulfate has not been shown to directly affect the ability of bacteria to reduce perchlorate. Therefore, reduction of sulfate is not desirable for *in situ* remediation of perchlorate[326], as it consumes valuable carbon source and may produce hydrogen sulfide, which is toxic to many organisms.

DNA analysis of the column effluent and sediment revealed that sodium acetate stimulated the growth of bacteria and specifically of perchlorate-reducing bacteria much more effectively than ethyl lactate. This was evident from copy numbers for 16S rRNA genes and perchlorate reductase (*pcrA*) genes in both effluent and sediment, which were on average 31±17 times higher when sodium acetate rather than ethyl lactate was supplied as a carbon source (Figure 4-3).

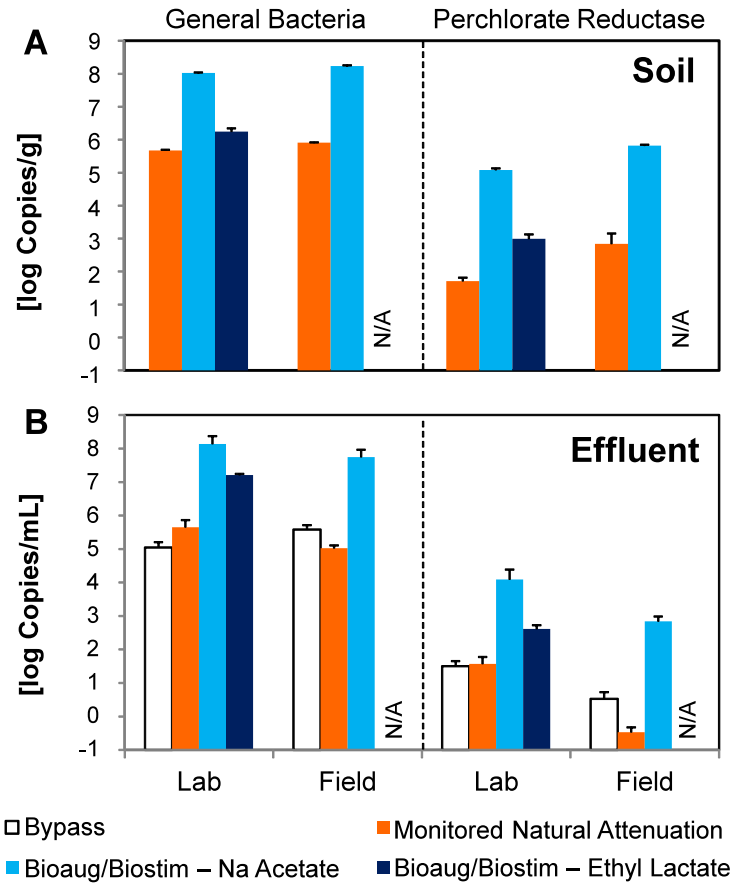

Figure 4-3. Results of quantitative PCR targeting the 16S rRNA gene of general bacteria and perchlorate reductase (pcrA). Shown are sediment results for the influent section of each column, which contained the highest numbers of bacteria compared to mid and effluent section. N/A = not tested in field experiment. Error bars represent standard error.

Field Experiments using the In Situ Microcosm Array (ISMA)

A flow-through field experiment was conducted with the ISMA testing MNA (experiment *A*) and bioaugmentation with a seed culture and sodium acetate (experiment *B*). In addition, an influent control was included in the experiment. Sodium acetate was chosen over ethyl lactate because in lab experiments its addition resulted in higher numbers of perchlorate-reducing bacteria and did not lead to unwanted sulfate reduction.

To ensure that the deployment well (MW-8) was not impacted by the deployment event, grab samples taken before and after deployment of the ISMA were analyzed for DNA concentration and a range of anions. Results (Appendix D, Table S4) revealed no impact by the deployment of the ISMA.

Perchlorate was reduced over a period of 21 days from 228±1 µg/L to 30±37 µg/L in the columns with the bioaugmentation treatment (*B*), while perchlorate concentrations did not decline in the MNA experiments (*A*), compared to the influent control (Figure 4-4). Sulfate was not reduced significantly in any of the samples (data not shown).

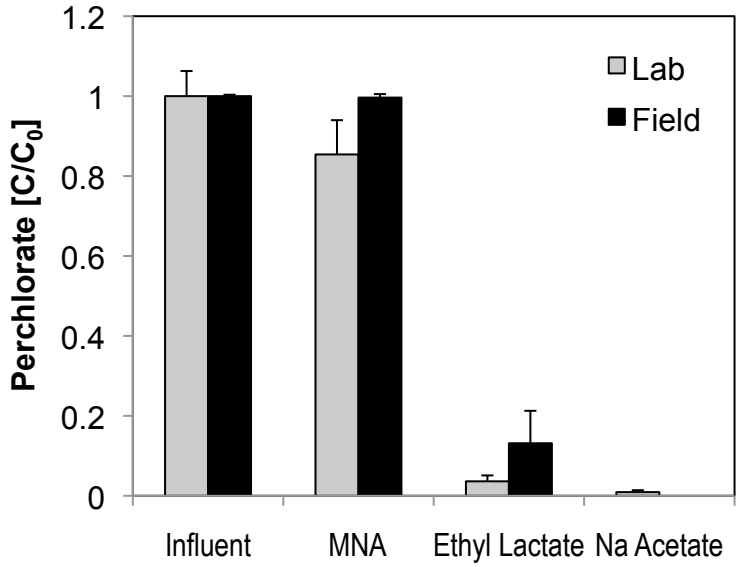

Figure 4-4. Concentration of perchlorate in different experimental groups normalized to influent. Data represent composite samples collected over 21 days, representing the whole duration of the experiments. N/A = not tested in field experiment. Error bars represent standard error.

By its design, the ISMA allows analysis of microbial communities in column effluent and sediment and examination of their spatial distribution across the columns. Sampling of both habitats has been recognized as essential to provide a complete picture of the microbial community[135, 327]. DNA analysis of effluent and sediment showed that perchlorate reducers mainly settled onto column sediment (concentration 2 - 3 orders of magnitude higher in sediment [copies/g] than in aqueous phase [copies/mL] – Figure 4-

3), while general bacteria were found at similar levels on sediment and suspended in the aqueous phase. Results further show that bioaugmentation with nutrient addition led to an increase in gene copy numbers of 16S rRNA (180-fold on average) and perchlorate reductase (*pcrA*) (690-fold on average), indicators for general and perchlorate-reducing bacteria, respectively (Figure 4-3). The column sediment was sectioned in three equal sections (inlet, middle, and outlet) and DNA copy numbers were analyzed. Results show that the majority of bacteria in all columns (lab and *in situ*) resided in the inlet portion of the sediment columns, which harbored 77±10 % of general bacteria (data shown in Appendix D Figure S 5). This was even more pronounced for the columns that were bioaugmented, where around 90±5% of general bacteria was found in the inlet portion of the sediment columns. The reasons for this likely are two-fold: in MNA columns to which no nutrients were added, different sediment filtration mechanisms[328, 329] straining the bacteria from the incoming groundwater most likely caused the high DNA copy numbers found near the inlet. In addition to sediment filtration, nutrient concentrations (carbon source and electron acceptors) in bioaugmented columns are highest at the inlet of the columns, and therefore provide ideal growth conditions for bacteria leading to higher numbers near the inlet. This has been found in several flow-through column studies[330-333].

Whereas concentrations of general bacteria were similar between lab and field experiments, the concentrations of perchlorate-reducing bacteria in the effluent and influent samples were about one order of magnitude lower *in situ* than in the lab experiment. Similar observations have been reported previously, where bacteria introduced through bioaugmentation were not able to compete with the indigenous community as effectively as predicted by lab studies[334, 335] or were subject to grazing by protozoa[122, 125, 336]. These effects play a large role *in situ* and are one reason why *in situ*

experiments are more valuable than similarly performed laboratory tests. At the same time, perchlorate-reducing bacteria were found in similar concentrations in the sediment for both lab and *in situ* experiments. This finding, which is in contrast to the differences found in effluent concentrations, is supported by previous findings that bacteria attached to surfaces (sediment) or residing in small pore spaces are generally better protected from adverse environmental conditions and attack by grazers[122, 140, 337]. Overall, the lower total number of perchlorate-reducing bacteria *in situ* is in accordance with the lower perchlorate reduction rate found in the field.

Degradation Rate Calculation

Biological degradation processes generally follow Monod kinetics[338] that describe the utilization of a single, rate-limiting substrate (in this case perchlorate) and resulting microbial growth. Half-saturation constants (K_S) for growth of perchlorate-reducers have been determined for a mixed community of autotrophic bacteria to be 14.9 ± 6.0 mg/L[339], and for two heterotrophic pure strain bacteria to be 470 and 45 mg/L, respectively[340]. These values for K_S are one to two orders of magnitude larger than the perchlorate concentrations in this study (~0.2 – 1 mg/L), suggesting that a first-order approximation should be used to describe perchlorate degradation in this study. To assert this, we used a graphical evaluation of the perchlorate degradation data for 5 batch bottle microcosms containing site groundwater, sediment, 1000 mg/L ethyl lactate and 1000 μg/L perchlorate. Log-transformed concentrations were plotted against time. We fitted all data sets with a linear regression revealing a first-order degradation rate of 0.05 ± 0.009 hr^{-1} with a correlation coefficient R^2 between 0.62 – 0.97 for five replicates (Figure S4). Overall, using a first-order approximation of Monod kinetics was found

reasonable for the perchlorate concentration range of the experiments conducted in this study.

In the configuration used for the field column study, the ISMA allowed collection of only a single composite sample per column, which was used to estimate the degradation rate from triplicate measurements by employing equations 5 and 6. To determine the magnitude of the impact caused by determining rates by this composite approach, we used the lab flow-through experiment to calculate degradation rates from both time-discrete and composite sampling. On a conceptual level, composite samples will yield inherently conservative rates, since they represent an average of the adaptation phase (when the contaminant is not reduced) and steady state conditions (stable contaminant degradation – see Figure 4-2). The extent of underestimation of the "true" degradation rate depends on the relative duration of adaptation vs. the period of steady state examined.

In the lab flow-through experiment, the time-discrete rates were on average 39% (±14%) higher than those calculated with composite concentrations (Table 4-1– comparing time-discrete degradation rate for bioaugmentation columns in lab [sodium acetate, ethyl lactate] to composite degradation rate for the same columns). The adaptation phase before steady-state contaminant reduction was only two days (or 10%) of the total duration of the experiment (Figure 4-2). We therefore expect the composite degradation rate determined *in situ* to underestimate the "true" rate by approximately 39%. In contrast, laboratory batch microcosms more commonly used by the remediation industry often overestimate field performance[302, 303], sometimes by one or two orders of magnitude (1,000 – 10,000 %)[277, 304, 305]. Thus, the discrepancy between rates observed in lab and field can be reduced from the hundreds of percent to the tens of percent by using *in situ* feasibility studies employing composite sampling. Further refinement of

estimates may be achieved by fractionating the effluent from individual ISMA channels to obtain time-resolved *in situ* data.

Perchlorate reduction rate determined *in situ* was half of that determined in the lab flow-through experiment (both from composite effluent) for bioaugmentation with sodium acetate. This difference is in line with overestimated laboratory-derived degradation rates found by other studies[302-306], and may be due to perchlorate concentrations fluctuating *in situ*[341], different numbers of perchlorate reducers in lab and field (Figure 4-3), as well as inherent differences between lab and *in situ* conditions[78]. These considerations again highlight the need to conduct treatability studies *in situ* rather than in the lab.

The data presented demonstrate that it is feasible to conduct multiple treatability tests *in situ* at the same time, through the use of the ISMA. This new tool enables analysis of effluent samples from each experiment for multiple parameters with statistical significance, as well as DNA analysis of both attached and suspended microbial community. The ISMA provides *in situ* generated degradation rates for contaminants in hydrodynamic sediment columns, which addresses a major shortcoming of the current standard (batch microcosms). The data presented here constitute experimental proof of the feasibility of evaluating multiple, mutually exclusive *in situ* remedies side-by-side in the same well, at the same time, without impacting in any way the groundwater well selected for remediation technology screening. As demonstrated here, this includes the capability of conducting anaerobic tests in aerobic wells without changing redox conditions in the deployment well itself. As such, the ISMA technology provides a unique and novel capability for the remedial design of contaminated, saturated subsurface environments.

TRANSITION 4

In Chapter 4 a case study using the ISMA to test biological perchlorate reduction is presented. The data demonstrate that several lines of evidence can be collected, including chemical concentrations, information on presence and abundance of key microbial species, and hydrogeological parameters. In addition to this case study, the ISMA has been used to study anaerobic reduction of hexavalent chromium and dehalogenation of chlorinated ethenes.

The goal of my work was to develop the ISMA and to make this tool available for groundwater remediation research and design. To this end, chapter 5 contains recommendations regarding hardware development and demonstration of the ISMA technology in other contaminated groundwater settings.

5. CONCLUSIONS AND RECOMMENDATIONS

The ISMA is intended as a tool to guide remedial design at sites with contaminated groundwater. As such, the goal was to develop a technology that can be made available to environmental consultants and site managers in the U.S. and worldwide. With the creation of a startup company (*In Situ* Well Technologies, LLC.) and a commercialization agreement with an international environmental consulting firm in place, the ISMA is well on its way to be available for routine testing. To support this process, I have outlined a few recommendations and opportunities for improvement as I see them. Many of these suggestions stem from past discussions with ISMA team members, first and foremost Tomasz Kalinowski.

IMPROVEMENTS TO ISMA HARDWARE

Several modifications can be made to make the ISMA more user-friendly, and thereby reduce chances of failure during field deployments.

Flexible Viton tubing is currently used to transport groundwater throughout the ISMA. Although easy to use, it is prone to getting pinched between the internal components and outer shell, and barbed connections can come apart if not connected properly. The use of permanent stainless steel or rigid Teflon tubing with compression fittings would minimize those sources of failure. In addition, use of permanent tubing would lower the cost of consumables (~150 ft of Viton tubing is needed per deployment, which costs ~US$300). The disadvantage of using permanent tubing is the added effort required for decontamination/sterilization of the device between deployments, and the risk or cross-contamination between different field sites.

Currently, the ISMA can be safely deployed at depths up to 6 m below the water table, which limits is usefulness at many sites with deep wells, or screened intervals farther below the groundwater table. This limitation is inherent to the peristaltic pump

technology used in the ISMA, which can control a differential pressure up to 1 bar (corresponds to a depth of 10 m). To address this limitation, a pressure reducer can be incorporated in the intake section of the ISMA. Available pressure reducers (e.g., Series P60-M5 Miniature Plastic Water Pressure Regulators, Watts, North Andover, MA) can reduce pressures up to 20 bar (corresponds to 210 m of below water) down to 0-1 bar. The pressure reducer should be situated after the groundwater is screened through the 100-μm filter of the intake to avoid large particles entering the pressure reducer, but before the peristaltic pumps.

On-line sensing capability has been in development, and it is mentioned here only for the purpose of completeness. The purpose of this capability is to monitor column effluent in real time for parameters that indicate biological activity in the columns. These parameters can be pH, dissolved oxygen, redox potential, concentration of certain anions (nitrate, nitrite, etc.), all of which can be measure using commercial electrodes. The latter three indicate a progression of oxidizing to more reducing conditions, while pH should be monitored to determine if favorable conditions prevail (around neutral pH for most biological processes). Decreasing or increasing pH can also indicate potential secondary effects, such as dissolution of metals. The monitoring of redox conditions can inform on the period of deployment when contaminant degradation likely occurs, as most biodegradation processes occur preferentially under aerobic (e.g., benzene degradation) or anaerobic conditions (e.g., dehalogenation of chlorinated solvents). Knowledge of the degradation period will make degradation rate calculations (as outlined in Chapter 4) more exact.

CONTAMINANTS TO TARGET

In addition to hardware improvements, demonstrations of the ISMA technology with further groundwater contaminants should be a priority. Currently, field data have been generated for the anaerobic biodegradation of perchlorate, perchloroethene, trichloroethene, dichloroethene isomers, vinyl chloride and hexavalent chromium. Although efforts were made to capture the very volatile chloroethene species (see "adsorptive cartridge tests" in chapter 3), it remains challenging to achieve a complete mass balance. A closed mass balance enhances the confidence of results and is therefore highly desirable. Without a closed mass balance, results still inform on degradation processes and rates, but in are considered less reliable, specifically in regards to measured degradation rates. Complete mass balances were achieved for perchlorate and hexavalent chromium, facilitated by their lack of volatility.

Therefore, initial applications should focus on non-volatile groundwater contaminants, such as metals (chromium, arsenic, uranium, etc.) and non-metal ions (perchlorate, nitrate, selenate, ammonium, etc.). Further applications should be done with gasoline components (mainly benzene, ethylbenzene, toluene, xylenes [BTEX], and methyl-*tert*-butyl ether [MTBE]), which are less volatile than chlorinated solvents (or non-volatile in the case of MTBE) and can therefore be captured more easily.

Another application that should be explored with the ISMA is the testing of combined remedies. These can be sequential aerobic and anaerobic processes, chemical and biological treatment, or concurrent treatment with chemical and biological amendments (e.g., addition of zero-valent iron and dechlorinating consortia to reduce halogenated contaminants).

One area where the ISMA has distinct advantages over other *in situ* tests is the exploration of unproven treatment technologies. The ISMA is self-contained, and

releases nothing into the aquifer. This allows for no-risk testing of experimental treatment approaches for emerging groundwater contaminants, e.g., 1,4-dioxane or perfluorinated organic compounds.

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APPENDIX A

SUPPORTING INFORMATION

CHAPTER 1

Failure Modes Survey conducted among remediation practitioners:

Please rate the importance of the following failure modes encountered during field implementation of *in situ* bioremediation:

(1 – minor, 5 – frequent/major concern)

Concern: Rating (1-5):

1. Clogging of injection well
2. Clogging of subsurface formation surrounding injection well
3. Biological activity cannot be sustained over time
4. Difficulty of delivering bioaugmentation culture throughout the plume
5. Difficulty of delivering biostimulation agent throughout the plume
6. Limited survival/activity of bioaugmentation culture (competition with indigenous bacteria/protozoa)
7. Limited activity of native microorganisms when compared to feasibility study outcomes
8. Solubilization of heavy metals and other sediment constituents as an unwanted side-effect
9. Incomplete degradation/detoxification (namely problematic species such as vinyl chloride formation from PCE/TCE)
10. Other (please specify)

What is the approximate number of bioremediation projects you have worked on?

APPENDIX B

SUPPORTING INFORMATION

CHAPTER 2

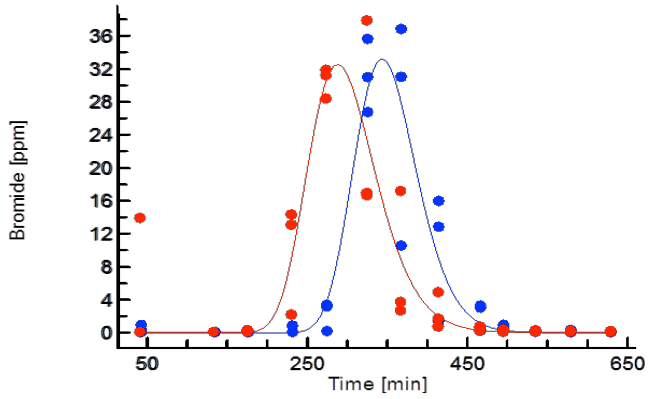

Figure S1. Conservative tracer curves showing the bromide concentration in column effluent over time after a one-time injection of bromide. Sediment columns were filled with fine (<0.5 mm – blue curve) or coarse (0.5 – 1 mm – red curve) grains. Experiments were conducted in triplicate.

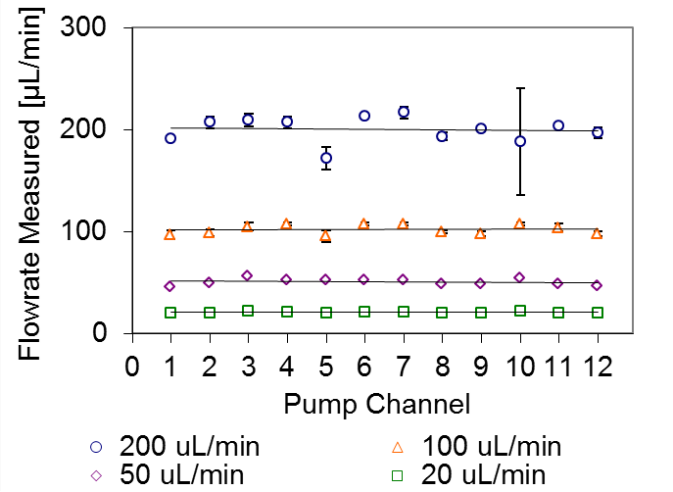

Figure S2. Flow rate reproducibility between 12 channels for customized pump in the ISMA. Flow rates [µL/min] were set to 20 (□), 50 (◇), 100 (Δ) and 200 (○) as indicated by the solid lines. Shown is the average of three measurements.

APPENDIX C

SUPPORTING INFORMATION

CHAPTER 3

Table S 1. Field parameters recorded at deployment wells before and after ISMA deployments. Parameters were measured from grab samples using an YSI 650 MDS meter. N/A – parameter not measured

Property		Prior to deployment	Post-deployment
Dissolved oxygen [mg/L]	Site 1	0.6	0.6
	Site 2	5.16	5.27
Temperature [°C]	Site 1	N/A	N/A
	Site 2	23.21	22.88
Oxidation-reduction potential [mV]	Site 1	-47	-36.3
	Site 2	264.3	260.6
pH	Site 1	8.2	8.14
	Site 2	7.44	7.71
Conductivity [µS/cm]	Site 1	N/A	N/A
	Site 2	1.039	1.670

Table S 2. Physical properties of pump cassette material

Property	Pump cassettes
Tensile strength	$1.14 * 10^8$ N/m^2
Flexural strength	$1.38 * 10^8$ N/m^2
Compressive strength	$1.52 * 10^8$ N/m^2
Maximum operating temperature	170° C

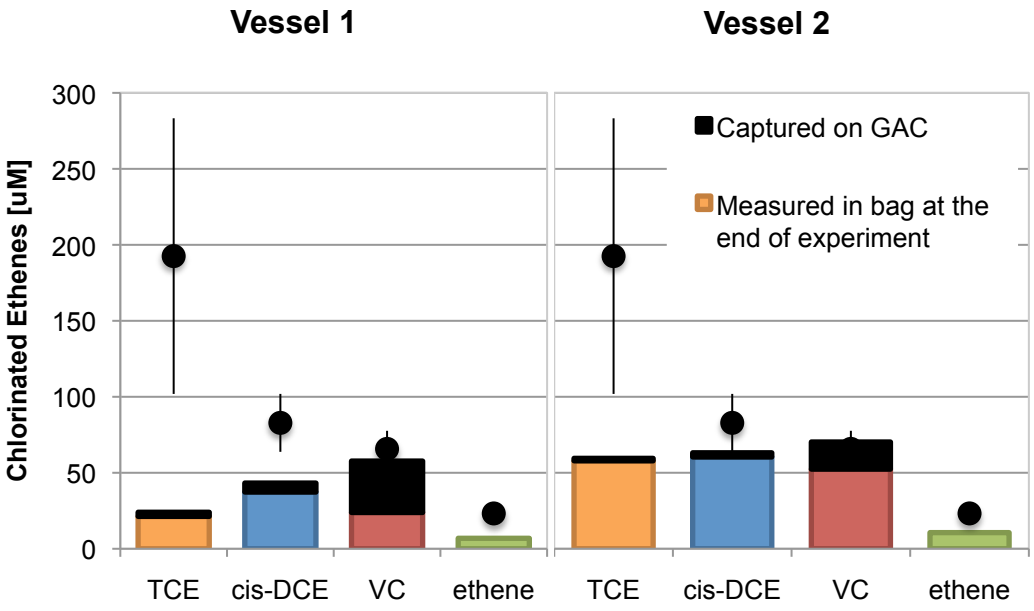

Figure S 3. Performance of activated carbon cartridge in capturing chlorinated ethenes. Test was conducted in duplicate; results from both tests are shown in comparison. Error bars represent standard deviation of six measurements. – Figure provided by T. Kalinowski

APPENDIX D

SUPPORTING INFORMATION

CHAPTER 4

Protocol for DNA extraction from sediment

Materials needed: PowerSoil DNA Isolation Kit from MoBio

DNeasy Blood & Tissue Kit from Qiagen

Sterile 2mL centrifuge tubes

Vortexer with MoBio tube adapter

Ethanol for sterilization

Use the following materials from the PowerSoil DNA kit:

1. To the **PowerBead Tubes** provided, 0.25 grams of soil sample. (sterilize spatula in flame between each sample)
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 **ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).

14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

From here use materials from the DNeasy Blood & Tissue kit:

15. Pipet the mixture from step 14 into the **DNeasy Mini spin column** placed in a 2 ml collection tube (provided). Centrifuge at _6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

    Repeat this step until you have loaded all the solution from step 14 onto the spin column. This can be up to 3 times.

16. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl **Buffer AW1**, and centrifuge for 1 min at _6000 x g (8000 rpm). Discard flow-through and collection tube.

17. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl **Buffer AW2**, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

    It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

18. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

19. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl **Buffer AE** directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

    Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

    Recommended: For maximum DNA yield, repeat elution once as described in step 18. This step leads to increased overall DNA yield.

    Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

Table S 3. Quantitative PCR Primers

Target	Primer name and sequence	Reference
Perchlorate reductase pcrA	320F 5'GCGCCCACCACTACATGTAYGGNCC-3' 598R 5'-GGTGGTCGCCGTACCARTCRAA-3'	[322]
General bacteria 16S rDNA	1055YF 5'-ATGGTGTCGTCAGCT-3' 1392R 5'-ACGGGCGGTGTGTAC-3'	[321] [323]

Table S 4. Deployment well chemistry pre- and post-deployment of ISMA

	Pre-deployment	Post-deployment
Perchlorate [µg/L]	234	228
Nitrate [mg/L]	12.6	12.1
Sulfate [mg/L]	58.2	57.8
Ammonium [mg/L]	532	531
Total DNA [ng/mL]	26	18

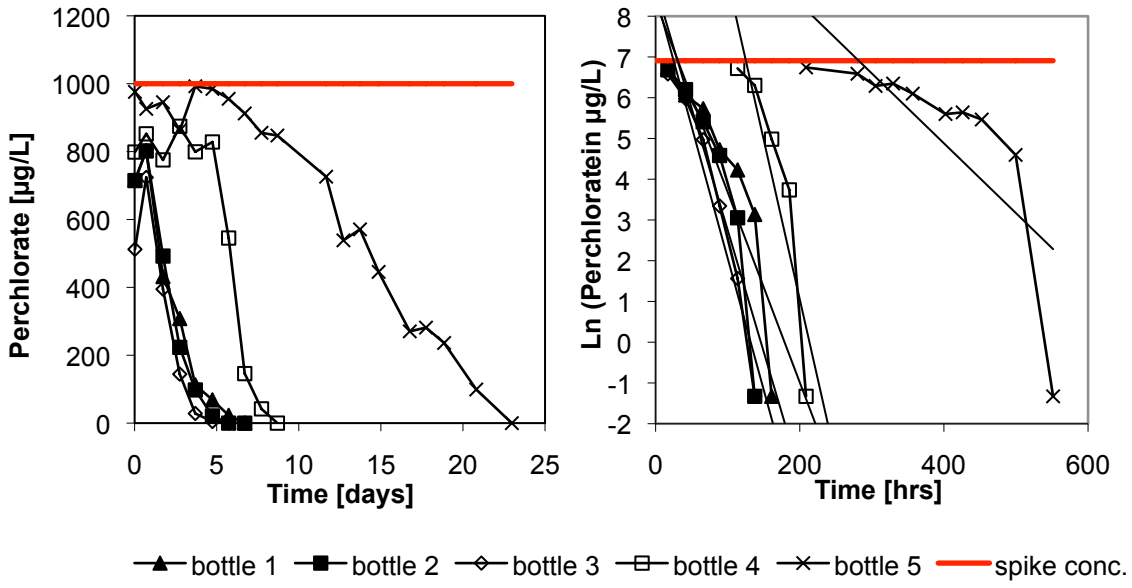

Figure S 4. Perchlorate Degradation in Batch Microcosms. Shown on the left are perchlorate concentrations monitored in batch microcosms over time. Shown on the right is the natural logarithm of the perchlorate concentrations and linear fits used to determine first-order degradation rates for batch microcosms.

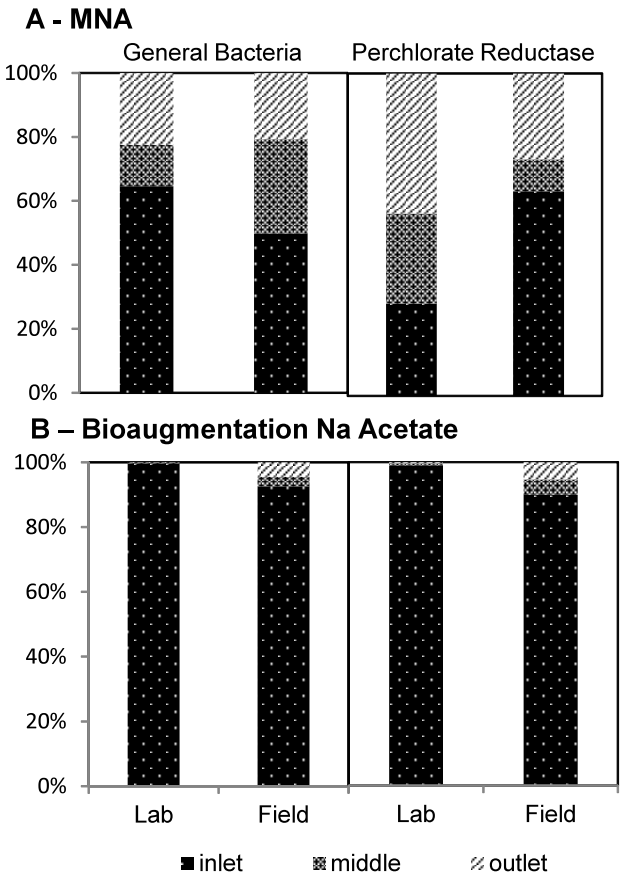

Figure S 5. Relative Bacteria Distribution in Sediment Columns. Results of quantitative PCR targeting the 16S rRNA gene of general bacteria and perchlorate reductase (*pcrA*). Shown are relative results for all column sections.

Limit of detection for lowest detectable degradation rate

Relative standard deviation was determined from 14 samples, each analyzed in triplicate with perchlorate IC 2000 (3 injections from same sample)

	triplicate analyses			MEAN	STDEV	rel. STDEV
1	523.16	513.23	504.69	513.69	9.24	0.02
2	595.65	584.52	598.34	592.84	7.32	0.01
3	623.85	627.03	624.16	625.01	1.74	0.003
4	553.31	548.00	548.36	549.89	2.96	0.005
5	537.64	533.49	532.91	534.68	2.57	0.005
6	545.01	549.23	551.25	548.50	3.18	0.006
7	1509.2	1531.4	1547.2	1529.2	19.09	0.01
8	1430.1	1394.6	1378.8	1401.1	26.27	0.02
9	1555.2	1564.7	1569.4	1563.1	7.22	0.005
10	1685.6	1687.8	1694.5	1689.3	4.61	0.003
11	1512.8	1493.5	1503.5	1503.2	9.66	0.006
12	1468.6	1381.7	1336.5	1395.6	67.13	0.05
13	1348.3	1338.0	1334.2	1340.2	7.30	0.005
14	1418.2	1441.0	1455.4	1438.2	18.76	0.01
				MEAN STDEV		0.011

The average standard deviation was 1.1% (average standard error = 0.7%)

Limit of detection is defined as 3X Stdev. = 3.4%

If we assume 600ppb of influent perchlorate (influent conc. in lab column experiment), 579.6ppb effluent perchlorate (600 – 3.4%) and 10 hrs residence time:

K=[ln(600)-ln(579.6)]/10 = **0.0034 hr-1 = minimum degradation rate that can be detected in MNA column experiments**

APPENDIX E

Peer-reviewed publication not included in dissertation:

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Pharmaceuticals and personal care products in archived U.S. biosolids from the 2001 EPA national sewage sludge survey

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ABSTRACT

In response to the U.S. National Academies' call for a better assessment of chemical pollutants contained in the approximately 7 million dry tons of digested municipal sludge produced annually in the United States, the mean concentration of 72 pharmaceuticals and personal care products (PPCP) were determined in 110 biosolids samples collected by the U.S. Environmental Protection Agency (EPA) in its 2001 National Sewage Sludge Survey. Composite samples of archived biosolids, collected at 94 U.S. wastewater treatment plants from 32 states and the District of Columbia, were analyzed by liquid chromatography tandem mass spectrometry using EPA Method 1694. Thirty-eight (54%) of the 72 analytes were detected in at least one composite sample at concentrations ranging from 0.002 to 48 mg kg^{-1} dry weight. Triclocarban and triclosan were the most abundant analytes with mean concentrations of 36 ± 8 and 12.6 ± 3.8 mg kg^{-1} ($n = 5$), respectively, accounting for 65% of the total PPCP mass found. The loading to U.S. soils from nationwide biosolids recycling was estimated at 210–250 metric tons per year for the sum of the 72 PPCPs investigated. The results of this nationwide reconnaissance of PPCPs in archived U.S. biosolids mirror in contaminant occurrences, frequencies and concentrations, those reported by the U.S. EPA for samples collected in 2006/2007. This demonstrates that PPCP releases in U.S. biosolids have been ongoing for many years and the most abundant PPCPs appear to show limited fluctuations in mass over time when assessed on a nationwide basis. The here demonstrated use of five mega composite samples holds promise for conducting cost-effective, routine monitoring on a regional and national basis.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are common contaminants of the environment, and have been detected in surface water (Kolpin et al., 2002; Cahill et al., 2004; Moldovan, 2006; Roberts and Thomas, 2006; Tamtam et al., 2008), groundwater (Heberer et al., 2000; Lindsey et al., 2001; Fick et al., 2009), and drinking water (Stackelberg et al., 2004; Loraine and Pettigrove, 2006; Loos et al., 2007; Focazio et al., 2008), as well as in agricultural soils subject to land application of digested municipal sludge (Kinney et al., 2008; Kupper et al., 2004; Wu et al., 2009), also known as biosolids. Wastewater treatment plants were identified as one possible source for surface water contamination. Over-the-counter

Abbreviations: DL, Detection limit; EPA, Environmental protection agency; NEBRA, North east biosolids and residuals association; NSSS, National sewage sludge survey; PPCP, Pharmaceuticals and personal care products; RPD, Relative percent difference; TNSSS, Targeted national sewage sludge survey.

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and prescription drugs enter the wastewater via excretion of urine and feces containing parental drugs and their conjugates as well as other metabolites, or from disposal of unwanted or expired medications (Halling-Sorensen et al., 1998; Fent et al., 2006). Similarly, chemical constituents of personal care products may be directly disposed of into domestic wastewater. Removal of PPCPs during municipal wastewater treatment is rarely complete, thereby creating a pathway for entry of these compounds into aquatic environments via wastewater reclamation (Halling-Sorensen et al., 1998; Ternes, 1998; Daughton and Ternes, 1999; Hirsch et al., 1999) and into terrestrial environments via land application of biosolids (Ternes et al., 2004a).

Of the more than 7 million tons of sewage sludge produced in the United States in 2004, about 50% was applied to land as fertilizer or soil amendment, and 45% was disposed of in landfills or as landfill cover (NEBRA, 2007). Terrestrial environments can offer effective biological, physical, and chemical attenuation mechanisms for manmade pollutants. However, they also can act as a source term for chemical migration into surface and groundwater from biosolids runoff and leachate.

Pharmaceutical compounds are designed to be biologically active and therefore may have effects on non-target organisms even at trace concentrations extant in terrestrial and aquatic environments. While acute toxic effects of pharmaceuticals on non-target organisms have been investigated for some compounds, chronic toxicity and potential subtle environmental effects are only scarcely known (Fent et al., 2006). Also insufficiently investigated is the effect of mixtures of pharmaceuticals on aquatic organisms, although biochemical interactions of drugs in humans are well known. Of additional concern is the possible uptake of contaminants into food crops grown on agricultural fields that were fertilized with biosolids (Kumar et al., 2005; Dolliver et al., 2007). Currently, no regulation exists in the United States for PPCPs contained in biosolids, and a need for more information on the occurrence of and risk from these compounds has been noted by the National Research Council of the National Academies of the United States (National Research Council, 2002). In the past, analytical methods were limited, especially for trace analyses of complex environmental samples. In 2007, the release of U.S. EPA method 1694 (USEPA, 2007a) for the analysis of PPCPs in various matrices afforded the opportunity to analyze biosolids samples using a standardized protocol.

The U.S. EPA has performed national sewage sludge surveys (NSSS) in 1989, 2001, and 2007. The survey conducted in 2001 served to evaluate the potential need for regulations of trace levels of dioxins and polychlorinated biphenyls (USEPA, 2007b). After the 2001 survey was completed, unused samples were released to a nationwide repository of biosolids samples now maintained at the Biodesign Institute at Arizona State University.

This investigation evaluated occurrences and concentrations of PPCPs in biosolids from the year 2001 to enable risk assessments and to establish a national baseline for evaluating temporal trends of PPCPs in U.S. biosolids. The analysis of composite samples by EPA Method 1694 was employed to determine average concentrations of 72 PPCPs in archived biosolids collected by the EPA, as a representative sample of the more than 16,000 treatment plants located in the contiguous United States.

2. Materials and methods

2.1. Sampling procedure

Biosolids samples with solid contents between 1% and 30% were obtained from 94 wastewater treatment plants in 32 states and the District of Columbia for the 2001 National Sewage Sludge Survey (USEPA, 2007b). They were selected by the U.S. EPA to obtain a representative estimate of the occurrence of chemical contaminants in sewage sludge that is disposed of primarily by land application. Information on the exact sampling locations is available in Table S1 of the supplementary material (SM). This survey aimed to estimate levels of dioxins, dibenzofurans, and coplanar polychlorinated biphenyls in biosolids. The sampling was conducted by the U.S. EPA between February and March 2001, and samples were collected according to sampling procedures developed by the U.S. EPA (USEPA, 2001). Samples were only taken from fully processed sewage sludges intended for disposal. Eighty-nine of the 94 WWTPs had one single system for sludge treatment, therefore one sample was collected. Five facilities had two systems for treating their sludges, therefore two samples were taken from each of these plants. In addition, duplicate samples were collected from 15% of facilities (14 samples) for precision analysis. This amounted to 113 samples overall. After completion of the 2001 NSSS, the samples were acquired by the Halden laboratory for further studies. For the 7-year period between acquisition and analysis in the spring of 2008, samples were stored at −20° C.

2.2. Composite sample preparation

From the 113 biosolids samples acquired from the EPA, three were excluded from analysis because the sample containers were broken or compromised; the remaining 110 samples were randomly grouped into five groups. Composite samples were prepared by weighing out approximately 1 g of dry weight from each sample and pooling it to obtain 5 composites each containing solids from between 21 and 24 individual samples. A duplicate of composite sample #3 was prepared to serve as a blind duplicate.

2.3. Sample analysis

The samples were analyzed by AXYS Analytical Services (2045 Mills Road West, Sydney, British Columbia, Canada V8L 3S8) according to EPA method 1694 (USEPA, 2007a). For the purpose of compound detection, the 72 analytes were divided into four groups. All analytes were separated by liquid chromatography and detected by tandem mass spectrometry. For compounds with a respective labeled analog, the concentration was determined using the isotope dilution technique. The corresponding concentrations are deemed to be of high quality. For compounds where a labeled analog was not available, the concentration was determined using an external calibration. Quantitative data for analytes not determined by the isotope dilution method are judged to be less robust. More detailed information on the analysis method and accuracy and precision criteria for acceptance of analytical data is available in

supplementary material (SM). Further information on study limitations can be found in the discussion section.

2.4. Quality assurance

To ensure system and laboratory performance, several tests were performed before sample analysis. Calibration accuracy was verified using a calibration standard solution with labeled and native analytes. Retention times of native and labeled compounds had to be within ±15 seconds of the respective retention time established during the previous calibration. In addition, ongoing precision and recovery were ensured. Lab blanks were analyzed before each sample analysis. A duplicate sample analysis was performed by the lab for each batch between 7 and 20 samples. In addition to these standard procedures, a blind duplicate was included in the sample set to evaluate analysis precision. Precision is expressed as relative percent difference (RPD) between each pair of measured concentrations. It was calculated using the following equation,

$$\mathrm{RPD}[\%] = \frac{|C_{\mathrm{sample}} - C_{\mathrm{duplicate}}| * 100}{\frac{C_{\mathrm{sample}} + C_{\mathrm{duplicate}}}{2}} \quad (1)$$

where C_{sample} and $C_{\mathrm{duplicate}}$ are, respectively, the concentration detected in the original sample and in its duplicate sample, and RPD is the relative percent difference.

2.5. Modeling of soil and porewater concentration

For assessment of their potential environmental impact, the concentrations of PPCPs after mixing with agricultural soil were calculated. The mixing ratio with soil was assumed based on the EPA-recommended rate of biosolids application of up to 4.5 dry kg per m^2 and by further assuming incorporation into soil to a depth of 10 cm (USEPA, 1994). The soil bulk density was assumed to equal 1.3 g cm^{-3} and the biosolids bulk density 1.6 g cm^{-3}. Though soil moisture content is known to vary widely depending on soil properties, for the purpose of this calculation, a soil moisture content of 22% (v/v) was used, as reported by others for agricultural soil (De Lannoy et al., 2006). A typical soil organic carbon fraction was assumed (Causarano et al., 2008), as was a biosolids organic carbon fraction of 0.4 (USEPA, 2007b). The concentration in soil and porewater at equilibrium was calculated based on the organic carbon fraction of the soil-biosolids mixture and the compound specific organic-carbon distribution coefficient (K_{OC}) of each analyte (Chalew and Halden, 2008). Calculations took into consideration the soil moisture content, as well as the volume of biosolids added to the soil, and were conducted for an environmentally relevant pH range of 7–9. The concentrations of PPCPs in porewater of soil/biosolid mixtures were calculated using the equations below,

$$C_{\mathrm{porewater}} = \frac{\frac{m_{\mathrm{biosolids}}}{m_{\mathrm{soil+biosolids}}} C_{\mathrm{biosolids}}}{\frac{f_{\mathrm{porewater}} * \rho_{\mathrm{porewater}}}{m_{\mathrm{soil+biosolids}}} + K_{\mathrm{OC}} * f_{\mathrm{OC}}} \quad (2)$$

$$C_{\mathrm{soil}} = \frac{m_{\mathrm{biosolids}}}{m_{\mathrm{soil+biosolids}}} C_{\mathrm{biosolids}} - \frac{f_{\mathrm{porewater}} * \rho_{\mathrm{porewater}}}{m_{\mathrm{soil+biosolids}}} C_{\mathrm{porewater}} \quad (3)$$

where m is the dry mass in kg m^{-3} of the solid matrix, C the concentration in μg kg^{-1}, ρ the density in kg m^{-3}, and $f_{\mathrm{porewater}}$ and f_{OC} the dimensionless fractions of, respectively, porewater and organic carbon in the soil/biosolids mixture.

2.6. Drug usage and ecotoxicity data

Information of prescription drug consumption was obtained from Internet sources (www.drugtopics.com). Exotoxicity data were taken from EPA's Ecotox database (www.epa.gov/ecotox).

2.7. Modeling of annual loading to agricultural soil

The annual loading of PPCPs contained in biosolids was based on a production of 5.6–7 million dry tons of sewage sludge in the U.S., of which 50–60% is applied to land (National Research Council, 2002; Jones-Lepp and Stevens, 2007; NEBRA, 2007).

3. Results

3.1. Data quality assurance

Lab blanks showed no detections above the detection limit for any of the analytes except for ciprofloxacin [61 μg kg^{-1} dry weight (dw); detection limit (DL) 32 μg kg^{-1} dw] and erythromycin-H_2O (2.6 μg kg^{-1} dw; DL 1.5 μg kg^{-1} dw)]. However, concentrations detected in biosolids were 100- and 40-times greater than the background level detected in lab blanks. Therefore, measured concentrations for both analytes were accepted.

Recovery for all analytes typically was good with an average of 112% but some notable outliers were observed leading to a range of 12–493% (See Table 1 and Table S2, SM). A total of 10 analytes exceeded the methods' lower and upper control limits. For 5 analytes (anhydrotetracycline, azithromycin, cimetidine, 4-epianhydrochlortetracycline, and 4-epianhydrotetracycline) recovery rates were below the method's lower control limit of 40%. The concentrations reported for these analytes in biosolids may represent underestimates. The recovery rates of 5 analytes (clinafloxacin, enrofloxacin, lomefloxacin, ofloxacin, and sarafloxacin) were above the method's upper control limits. The respective concentrations reported for these analytes may represent overestimations. All of the above mentioned analytes were quantified using labeled surrogate standards. In the case of clinafloxacin, enrofloxacin, lomefloxacin, ofloxacin, and sarafloxacin, $^{13}C_3$-^{15}N-ciprofloxacin was used as an isotope-labeled surrogate standard. The recoveries were determined from spiked quality control samples, where recovery of $^{13}C_3$-^{15}N-ciprofloxacin was below the method's lower control limit. This led to extremely high recoveries of the above mentioned analytes. In the biosolids samples, recovery of $^{13}C_3$-^{15}N-ciprofloxacin was always within the method's control limits. However, the lack of adequate recovery of $^{13}C_3$-^{15}N-ciprofloxacin in quality control samples suggests that concentrations reported in biosolids for clinafloxacin, enrofloxacin, lomefloxacin, ofloxacin, and sarafloxacin should be interpreted with caution.

The duplicate analysis revealed 18% relative percent difference (RPD) for all analytes. The blind duplicate analysis of a subset of 10 analytes revealed 28% RPD. Both RPD values

Table 1 – Analytical results and summary statistics for pharmaceuticals and personal care products detected in U.S. biosolids collected in 2001 and reported on a dry weight basis.

Substance name	CAS RN	Detection limit [μg kg^{-1}]	Standard deviation of detection limit [μg kg^{-1}] $n=5$	Frequency measured [%]	Maximum concentration [μg kg^{-1}]	Mean detected concentration [μg kg^{-1}]	Standard deviation of mean concentration [μg kg^{-1}] $n=5$	Recovery [%]	Isotope dilution quantification	Use	Lowest effect concentration for aquatic biota [μg L^{-1}]	Projected annual land application [kg yr^{-1}][f]
Anhydrotetracycline[g]	4496-85-9	74.1	7.4	100	880	392	249	51		Antibiotic		1300–1600
Azithromycin [g]	83905-01-5	5.6	1.6	100	1220	838	224	12		Antibiotic		2800–3500
Caffeine	58-08-2	59.0	16.4	100	643	248	200	78	✓	Stimulant	0.05 [a] (Bantle et al., 1994)	830–1000
Carbamazepine	298-46-4	5.6	1.6	100	238	163	56.4	139		Anticonvulsant	100 [b] (Jos et al., 2003)	550–680
Chlortetracycline	57-62-5	22.9	6.2	60	43.5	23.4	16.9	159		Antibiotic	36 [c] (Brain et al., 2004)	80–100
Cimetidine [g]	51481-61-9	6.4	2.0	100	893	504	208	41		Antacid		1700–2100
Ciprofloxacin	85721-33-1	20.9	6.4	100	10800	6858	2348	98	✓	Antibiotic	5[d] (Halling-Sorensen, 2001)	23,000–28,000
Clarithromycin	81103-11-9	5.6	1.6	100	94.6	66.2	25.5	114		Antibiotic		220–270
Codeine	76-57-3	11.2	3.2	20	29.7	n.d.		114		Analgesic		
Cotinine	486-56-6	9.9	3.3	100	38.6	28.1	8.3	103	✓	Nicotine metabolite		100–120
Diltiazem	42399-41-7	1.3	0.1	100	109	45.2	34.2	110		Antianginal		150–190
Diphenhydramine	58-73-1	2.3	0.6	100	1740	1166	516	101		Antihistamine		3900–4800
Doxycycline	564-25-0	23.9	6.3	100	1780	966	436	85		Antibiotic		3200–4000
Enrofloxacin[h]	93106-60-6	19.5	6.5	60	28.6	n.d.		190		Antibiotic	49 [d] (Robinson et al., 2005)	
4-Epianhydro-tetracycline [g]	4465-65-0	77.1	20.2	100	399	261	71.7	39		Antibiotic		900–1100
4-Epichlor-tetracycline	14297-93-9	56.3	16.0	40	93.0	n.d.		161		Antibiotic		
4-Epitetracycline	23313-80-6	74.4	18.8	100	3040	2376	517	82		Antibiotic		8000–9800
Erythromycin-H_2O	114-07-8	1.6	0.2	100	183	81.5	52.3	97	✓	Antibiotic	22 700 [c] (Williams et al., 1992)	270–340
Fluoxetine	54910-89-3	8.2	1.0	100	258	171	46.6	89	✓	Antidepressant	36[h, c] (Flaherty and Dodson, 2005)	580–710
Gemfibrozil	25812-30-0	12.2	12.3	100	159	152	13.2	107	✓	Antihyper-lipidemic	30,040 [c] (Zurita et al., 2007)	510–630
Ibuprofen	15687-27-1	122	123	80	359	246	121	109	✓	Anti-inflammatory		830–1000
Isochlortetracycline	514-53-4	22.5	6.4	60	36.0	n.d.		70		Antibiotic		
Lomefloxacin[h]	98079-51-7	13.2	2.1	40	16.1	n.d.		388		Antibiotic	106 [e] (Robinson et al., 2005)	
Metformin	657-24-9	119	32.5	80	456	305	152	116	✓	Antidiabetic		1000–1300
Miconazole	22916-47-8	5.8	1.3	100	1100	777	266	86		Antifungal		2600–3200
Minocycline	10118-90-8	1880	524	80	2630	1884	939	54		Antibiotic		6300–7800

(continued on next page)

Table 1 (continued)

Substance name	CAS RN	Detection limit [μg kg^{-1}]	Standard deviation of detection limit [μg kg^{-1}] $n=5$	Frequency measured [%]	Maximum concentration [μg kg^{-1}]	Mean detected concentration [μg kg^{-1}]	Standard deviation of mean concentration [μg kg^{-1}] $n=5$	Recovery [%]	Isotope dilution quantification	Use	Lowest effect concentration for aquatic biota [μg L^{-1}]	Projected annual land application [kg yr^{-1}][f]
Naproxen	22204-53-1	24.3	24.6	100	273	119	79	106	✓	Anti-inflammatory		400–500
Norfloxacin	70458-96-7	63.5	6.3	100	418	289	74.0	136		Antibiotic	18 000 [b](Yang et al., 2008)	970–1200
Ofloxacin[h]	82419-36-1	7.7	4.2	100	8140	5446	1941	493		Antibiotic	21 [d](Robinson et al., 2005)	18000–23,000
Oxytetracyclin	79-57-2	22.8	5.8	100	114	87.5	22.2	90		Antibiotic	50 [d](Hanson et al., 2006)	300–360
Ranitidine	66357-35-5	6.5	1.7	100	30.1	21.0	8.0	51		Antacid		70–90
Sulfamethoxazole	723-46-6	2.9	0.7	20	3.3	n.d.		98	✓	Antibiotic	9 [e](Brain et al., 2008)	
Sulfanilamide	63-74-1	56.2	16.0	40	87.3	n.d.		101		Antibiotic		
Tetracycline	60-54-8	57.5	15.5	100	2790	1914	691	124		Antibiotic	47 [e](Brain et al., 2004)	6400–7900
Thiabendazole	148-79-8	5.9	1.2	100	370	110	131	103	✓	Fungicide	310 [c](EPA, 2000)	370–460
Triclocarban	101-20-2	183	67.0	100	48100	36060	8049	96	✓	Disinfectant	0.101 [c] (EPA/OTS, 1992)	120,000–150,000
Triclosan	3380-34-5	487	493	100	19700	12640	3816	105	✓	Disinfectant	0.12 [b](Wilson et al., 2003)	42,000–52,000
Trimethoprim	738-70-5	10.6	3.8	60	60.5	26.0	21.5	97	✓	Antibiotic	16 000 [b](Luetzhoft et al., 1999)	90–110

a Amphibium.
b Green algae.
c Crustacean.
d Cyanobacteria.
e Macrophyte.
f Projected deposition rates of PPCPs on land based on 5.6–6.9 million dry tons of annual sewage sludge production and a land application rate of 60% (National Research Council, 2002; USEPA, 2003; USGS, 2006; Jones-Lepp and Stevens, 2007).
g Concentration may be underestimated due to low recovery in quality control samples.
h Concentration may be overestimated due to high recovery in quality control samples.

were in control with respect to the target RPD of 30% or less. The RPD value improved to 11% for 9 analytes, when excluding results for metformin, whose lack of detection in the blind duplicate unfavorably increased the summary statistic for measurement precision. A non-blinded duplicate analysis, performed by the contract laboratory for these 10 analytes, showed an RPD value of 11%.

3.2. *Study representativeness and sample integrity*

The prolonged storage of samples between sampling event and analysis may have allowed for the chemical degradation of labile analytes to occur. Therefore, any results of this study are conservative with respect to the detection frequency and concentration of compounds found. In other words, due to pooling of a large number of samples, analytes occurring infrequently and at low concentrations may have been diluted out to below the detection limit. A comparison of the presented data with the EPA data (USEPA, 2009), reveals that the mean concentrations of all analytes show no statistically significant difference within the 95th percentile confidence interval. Therefore, the prolonged storage did not impair the detection of multiple analytes at elevated concentrations in archived samples.

3.3. *Occurrence of PPCPs in biosolids*

All composites tested positive for at least 26 analytes. Of the 72 PPCPs targeted, 38 (54%) were detected at concentrations ranging from the low parts-per-billion (ppb) to the parts-per-million (ppm) range. These 38 PPCPs include 8 that have not previously been reported in biosolids in the peer-reviewed literature but that also were observed in the U.S. EPA's TNSSS published online (USEPA, 2009). The remaining 34 PPCPs were not detected in any of the composite biosolids samples. The mean total concentration of all targeted PPCPs combined in the five composite samples was 74.4 mg kg^{-1} dw of sewage sludge ± 21.4 mg kg^{-1} standard deviation ($n = 5$).

The two most abundant contaminants were the disinfectants triclocarban (48% of total detected PPCP mass) and triclosan (17%) (Fig. 1). Their mean concentrations were 36 ± 8 and 12.6 ± 3.8 mg kg^{-1} dw ($n = 5$), respectively (Table 1). The second most abundant class of PPCPs found was antibiotics. In order of decreasing concentration, ciprofloxacin, ofloxacin, 4-epitetracycline, tetracycline, minocycline, doxycycline and azithromycin were found at concentrations between 6.8 ± 2.3 and 0.8 ± 0.2 mg kg^{-1} dw ($n = 5$) (Table 1). The combined mass of all antibiotics constituted about 29% of the total mass of PPCPs per sample.

In addition to the 3 tetracyclines reported above, three tetracycline antibiotics were found for which peer-reviewed occurrence data in sewage sludge thus far were lacking. Anhydrotetracycline, 4-epianhydrotetracycline, and chlortetracycline (in order of decreasing concentration) together accounted for 6.9 ± 2.5 mg kg^{-1} dw ($n = 5$) (Table 1). Also not previously reported in biosolids were two prescription drugs, metformin and ranitidine. They together accounted for 0.4% of the total mass of PPCPs found in sewage sludge composites.

4. Discussion

4.1. *Study limitations*

For this study, a relatively large number of individual samples were combined to form 5 composite pools or mega composites. This approach served to reduce the number of samples to be analyzed in order to obtain a defensible estimate of mean

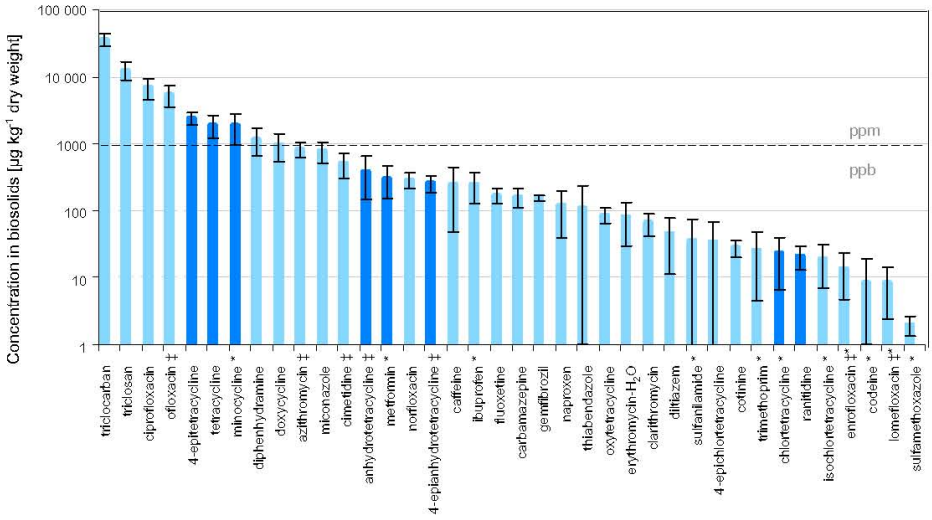

Fig. 1 – Rank order of mean concentrations for 38 PPCPs detected in composites of a total of 110 U S biosolids samples from 94 treatment plants in 32 states and the District of Columbia. Newly detected compounds are shown in darker hue. Error bars depict ± one standard deviation ($n = 5$). Some concentrations represent estimates only (‡) and some analytes were detected inconsistently (*).

analyte concentrations across the various treatment facilities represented. While being efficient and economical for the intended purpose, this approach was not well suited to capture the full spectrum of concentrations of individual PPCPs as a function of plant type, treatment processes employed, populations served, as well as of geographical locations and climate zones represented. As a comparison with the EPA TNSSS data reveals (USEPA, 2009), the detection frequency of less abundant analytes was significantly reduced in composite samples compared to individual sample analysis. Therefore, analytes that were not detected will represent a conservative estimate, and may still occur at detectable concentrations in individual samples from specific plants. While the mega composite approach cannot serve to determine variability between the large numbers of WWTPs studied, it was found to be suitable for identifying major contaminants of concern as well as their average concentration in a large sample set.

4.2. *Sanitizing agents*

Triclocarban, which in previous U.S. studies had been found in concentrations ranging from 5.97 to 51 mg kg^{-1} dry weight (dw) (Heidler et al., 2006; Chu and Metcalfe, 2007; Sapkota et al., 2007), was detected in every composite sample assayed. Also found in significant amounts was triclosan, which has been observed in a number of U.S. studies of sewage sludge with reported concentrations ranging from 0.53 to 30 mg kg^{-1} dw (Chu and Metcalfe, 2007; Heidler and Halden, 2007; Kinney et al., 2008; McAvoy et al., 2002; USEPA, 2003). The EPA TNSSS (USEPA, 2009) found triclocarban and triclosan at concentrations of up to 441 and 133 mg kg^{-1} dw, respectively, with mean concentrations at 38.7 ± 59.7 and 12 ± 18 mg kg^{-1} dw ($n = 74$), respectively. The relatively high mean concentrations of triclocarban and triclosan reported here and by the U.S. EPA (USEPA, 2009) in U.S. biosolids are in line with the intense usage of these antimicrobials and their high octanol/water partitioning coefficient (log K_{OW}) of 4.9 and 4.8, respectively (both at neutral pH), which indicates significant potential of both compounds for sorption to biosolids (Halden and Paull, 2005). In addition, triclosan was found to be persistent in biosolids after aeration (Ying et al., 2007). Both triclosan and triclocarban concentrations found in the present study fall within the range of concentrations previously reported. Information on previously reported concentrations of PPCPs in biosolids is available in Table S2, SM.

4.3. *Antibiotics*

The most abundant antibiotic was ciprofloxacin (6.8 ± 2.3 mg kg^{-1} dw; $n = 5$), which is among the 30 most prescribed drugs in the United States, according to Internet sources. Ciprofloxacin, a metabolite of enrofloxacin, is polar and therefore prone to electrostatic interactions with the negatively charged surfaces of microbes that are found in high concentrations especially in secondary sludge (Ternes et al., 2004b). Ciprofloxacin has been detected in sewage sludge by several studies conducted in Sweden and Switzerland. Detected concentrations ranged from 6×10^{-5}–11 mg kg^{-1} dw (Golet et al., 2002; Lindberg et al., 2005, 2006, 2007). Therefore, the mean concentration found in the present study (6.8 ± 2.3 mg kg^{-1} dw; $n = 5$) falls in the mid range of concentrations reported from outside of the U.S. The EPA TNSSS also identified ciprofloxacin as an abundant microcontaminant in sewage sludge, which was detected in every sample analyzed, yielding a mean concentration of 8.7 ± 8.5 mg kg^{-1} dw.

At a mean concentration of 5.4 ± 1.9 mg kg^{-1} dw, ofloxacin was the fourth most abundant contaminant found in biosolids. It is fairly hydrophilic (log K_{OW} of −0.2) and does not appear on the list of the top 200 prescription drugs. Its detection at elevated levels in every composite sample came as a surprise. However, ofloxacin had been found in concentrations of up to 2 mg kg^{-1} dw in biosolids from Sweden (Lindberg et al., 2005). It can be speculated that the carboxyl group contained in ofloxacin may form an ion complex with exchangeable cations associated with negatively charged surfaces.

Among the 10 most abundant PPCPs of the present study were three tetracycline antibiotics, 4-epitetracycline, tetracycline and minocycline, which had not been reported in biosolids before in the peer-reviewed literature. In addition, doxycycline was found as the ninth most abundant PPCP at a mean concentration of 1 ± 0.4 mg kg^{-1} dw. It had not been detected in biosolids from the U.S. before and only once in a Swedish study that found concentrations similar to those reported here (Lindberg et al., 2005). All tetracycline antibiotics are fairly hydrophilic (log K_{OW} of −1.33 for tetracycline/4-epitetracycline, −0.42 for minocycline, and −1.36 for doxycycline). Despite their hydrophilic character, the mean concentrations reported here are around 1–2 mg kg^{-1} dw. Tetracycline antibiotics are known to precipitate with ions of magnesium, calcium and ferric iron, and therefore accumulate in the solid fraction during wastewater treatment. Tetracycline, doxycycline and minocycline also rank among the 200 prescription drugs most widely used in the U.S. Other tetracycline antibiotics analyzed in this study were only found at mean concentrations below 0.4 mg kg^{-1} dw, but the concentrations of anhydrotetracycline, 4-epianhydrotetracycline and 4-epianhydrochlortetracycline were possibly underestimated due to low recoveries. The sum of tetracycline antibiotics found in sewage sludge constitute about 8 ± 1.3 mg kg^{-1} dw, which is similar to the findings of the EPA TNSSS that found about 5 mg kg^{-1} dw (USEPA, 2009).

Azithromycin was found at 0.8 ± 0.2 mg kg^{-1} dw. It ranked as the 6th most frequently prescribed drug in 2007 and is also fairly hydrophobic. Due to both these properties and the fact that biodegradation of azithromycin was found to be insignificant (Ericson, 2007), one would expect high concentrations in biosolids. Yet, there are 7 drugs (not counting triclocarban and triclosan) that were found at higher concentrations. However, a low recovery of azithromycin of only 12% may indicate that actual azithromycin concentrations in biosolids are much higher than the detected concentration. Azithromycin has been detected in previous studies at concentrations of up to 6.5 mg kg^{-1} dw in the U.S. (Jones-Lepp and Stevens, 2007) and at up to 0.16 mg kg^{-1} dw in sludge from Germany and Switzerland (Gobel et al., 2005a, 2005b).

4.4. *Bioavailability and soil/porewater equilibria*

To explore the importance of bioavailability of PPCPs sequestered in biosolids, the concentrations of individual PPCPs that are anticipated to occur in the solid and liquid

phases upon mixing of land applied biosolids into soil were calculated (Fig. 2). In fully equilibrated soil-biosolids mixtures (assuming an EPA-recommended mixing ratio of approximate 25:1), concentrations of PPCPs on soil particles are expected to fall into the ppb range for most analytes, except for triclocarban, which was projected to be present at around 1 $ppm_{(w/w)}$ dw. Since most of the PPCPs found in biosolids are fairly hydrophobic, their calculated concentrations in porewater at equilibrium typically were quite low (Fig. 2 B) (Kinney et al., 2008). A comparison of these estimated dissolved PPCP levels in porewater with the lowest effect concentrations for aquatic organisms (red circles in Fig. 2 B) suggests that the leaching of dissolved PPCPs into surface waters probably does not present an important mechanism for exposure of aquatic biota for the majority of analytes detected. Concentrations calculated for soil porewater typically were several orders of magnitude below the lowest effect concentration reported for aquatic organisms. Notable exceptions were 6 analytes (Fig. 2) that are expected to yield potentially problematic concentrations in porewater after land application and partitioning of biosolids-derived PPCPs. These include the antibiotics ciprofloxacin, ofloxacin and tetracycline, as well as the stimulant caffeine, and the two sanitizing agents triclosan and triclocarban (Fig. 2).

4.5. *Risk assessment data gaps*

These results suggest that aside from the 7 notable exceptions discussed above, the majority of the PPCPs detected in biosolids in this study likely exert no acute effects on aquatic organisms, assuming that biosolids are applied as regulated by the EPA (USEPA, 1994) and that the migration of solids from agricultural land to surface water via soil erosion and runoff can be completely prevented. While the former assumption is plausible, the latter may not always apply, as soil erosion is a common phenomenon.

However, chronic toxicity as well as effects from mixtures of PPCPs on non-target organisms cannot be assessed due to lack of appropriate toxicity data. It has been speculated that the presence of sub-therapeutic concentrations of antibiotics may adversely affect soil microbial community structures as well as induce spreading of resistance among bacterial pathogens. In addition to influencing microbial populations, it has been shown that some

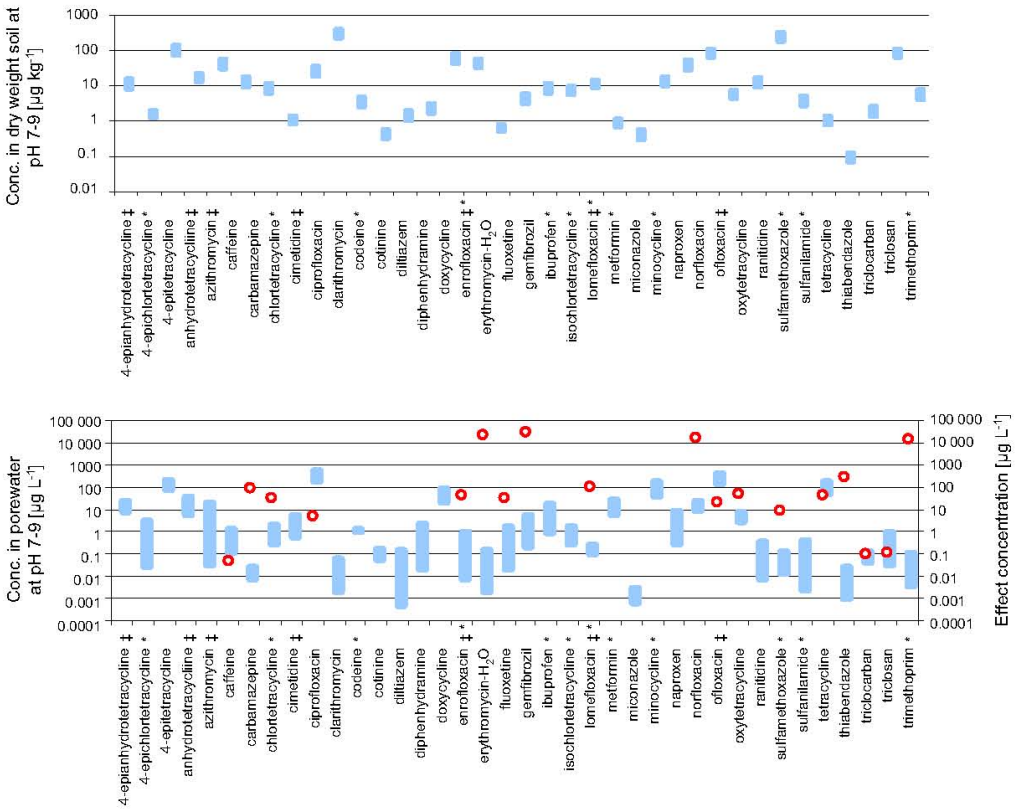

Fig. 2 – Predicted equilibrium concentrations of PPCPs associated with particulates of soil-biosolids mixtures (top) and dissolved in porewater (bottom) after land application of biosolids on agricultural soil. Data depict the environmentally relevant range between pH 7 and 9. Circles represent the lowest ecological effect concentrations contained in the EPA Ecotox database. Some concentrations were calculated based on estimates only (‡) and some analytes were detected inconsistently (*).

antibiotics, specifically drugs belonging to the tetracyclines, fluoroquinolones and sulfonamides, may be taken up by crop plants (Migliore et al., 2003; Kumar et al., 2005; Dolliver et al., 2007). This presents a potential exposure pathway to humans through ingestion of contaminated food and may result in the promotion of resistant bacteria in humans (Shoemaker et al., 2001).

Furthermore, information is lacking to determine risks to the health of agricultural soils and soil-dwelling organisms. Few studies have examined the half-lives in soils of PPCPs sequestered in biosolids. The effect of ppm levels of sanitizing agents on soil microbial communities has rarely been investigated to date (Liu et al., 2009). Similarly, the half-life of PPCPs in biosolids-amended soils will require additional research to inform risk assessment analyses. Passage through municipal digesters and chemical aging may reduce the bioavailability of PPCPs and with it the risk of chemical uptake of and exposure to soil-dwelling organisms. However, a reduced bioavailability also may imply a prolonged half-life of these compounds in the environment, with possible delayed release in sensitive compartments. Furthermore, studies are lacking on the potential of biosolids-derived antimicrobials and antibiotics to exert selective pressure for the enrichment of drug-resistant microorganisms, a scenario demonstrated in vitro (Braoudaki and Hilton, 2004). Also unavailable are threshold concentrations for toxic effects in terrestrial organisms of many PPCPs, including some that have been detected in biosolids at ppm levels. The EPA's Ecotox database (www.epa.gov/ecotox) provides only some toxicity values in aquatic organisms for the PPCPs investigated here. Bioaccumulation and biomagnification are other aspects that will need to be considered for risk assessment purposes. Although PPCPs are not typically thought of as representing persistent hydrophobic pollutants, some may be subject to bioaccumulation and possibly biomagnification thereafter in both terrestrial and aquatic environments. Bioaccumulation was demonstrated for triclosan and triclocarban in lab and field studies that examined uptake of these compounds from soil, sediment and water (Coogan and La Point, 2008; Higgins et al., 2009; Kinney et al., 2008).

5. Conclusions

Overall, this study reemphasizes the significance of biosolids recycling as a mechanism for the release of PPCPs into the environment. Based on the mean concentrations of all analytes detected, it is estimated that the total loading to U.S. soils from nationwide biosolids recycling is on the order of 210–250 metric tons per year for the 72 PPCPs investigated here.

It is concluded that, despite large variations found by the U.S. EPA between different treatment plants, mean concentrations of PPCPs in U.S. biosolids on a nationwide basis have remained fairly constant between 2001 and 2007 and possibly longer. Good agreement between this and the U.S. EPA study further suggests that the here demonstrated use of mega composite samples represents a cost-effective approach for collecting regional and nationwide information on average concentrations of contaminants in biosolids.

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Appendix. Supplementary material

Additional details regarding experimental procedures and results can be found in the Supplementary Material accompanying this article. This information is available free of charge via the Internet at www.iwaponline.com.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.watres.2009.12.032.

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APPENDIX F

Peer-reviewed publication not included in dissertation:

Kristin McClellan, Rolf U. Halden: "Pharmaceuticals and Personal Care Products in U.S. Biosolids", in ACS Symposium Series, Vol. 1048 "*Contaminants of Emerging Concern: Pharmaceuticals, Personal Care Products and Organohalogens in Municipal Waters and Biosolids"*, Chapter 8, pp 199–211.

Chapter 8

Pharmaceuticals and Personal Care Products in U.S. Biosolids

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Since the 1988 U.S. ban on ocean dumping of sewage sludge, the majority of these materials are disposed of on land as biosolids. To provide fundamental data for risk assessment concerning the environment, crop plants, and humans, several studies have been conducted that aimed at quantifying the load of pharmaceuticals and personal care products (PPCPs) as well as other emerging pollutants of concern in biosolids. So far, two large studies exist that analyzed biosolids samples representing the whole U.S. Both sample sets were collected by the U.S. Environmental Protection Agency (EPA) and analyzed by the same contract laboratory that developed the EPA method 1694 for analysis of PPCPs in biosolids and other matrices. The samples were analyzed using liquid chromatography tandem mass spectrometry and quantified using isotope dilution as well as conventional use of internal and external standards. The present meta analysis scrutinizes the findings and approaches of the two studies and puts them in context to potential environmental risks that should be considered.

Both the EPA's Targeted National Sewage Sludge Survey (TNSSS) and the analysis of a comparable sample set collected in 2001 revealed concentrations of several antimicrobials and antibiotics in the mg kg^{-1} dry weight range. Prevalent contaminants were triclocarban, triclosan, ciprofloxacin and ofloxacin, followed by a number of tetracycline antibiotics.

A comparison of the two datasets and study designs showed that combining a large number of individual samples to mega-composite samples is a suitable approach for identifying

prevalent contaminants and for obtaining representative mean concentrations. Whereas the use of mega composite samples can result in significant time and cost savings, this study design strategy tends to yield lower numbers of total analytes detected, lower detection frequencies for individual analytes and it limits the detection of spatial (geographical) patterns in analyte occurrence.

The findings of both nationwide studies provide a critical data basis for future risk assessment concerning the safety of biosolids application on agricultural and recreational land. Risks of primary concern identified in this work are the promotion of antibiotic resistance in the environment, adverse effects on soil microbial communities and plants, as well as the possibility of direct exposure of consumers to antibiotic residues contained in food crops grown on biosolids amended fields.

Introduction

Pharmaceuticals and personal care products (PPCPs) enter the wastewater stream through multiple pathways, such as ingestion and excretion of pharmaceuticals and their metabolites with urine and feces, direct disposal of unused medications, and washing off after dermal application. In many cases removal during wastewater treatment is incomplete, and trace amounts of these biologically active compounds are discharged to aquatic environments (*1*, *2*). Even if significant removal is achieved during the treatment process, this does not always mean that the compound has been degraded during treatment (*3*, *4*). Specifically, hydrophobic PPCPs have the potential to sorb to primary and secondary sludge and, therefore, can evade biological degradation due to their reduced bioavailability (*5*). The majority of sludge from wastewater treatment plants is further treated for land application and the resulting biosolids are used as soil amendment on agricultural land, in forests and at reclamation sites (*6*). The U.S. Environmental Protection Agency (EPA) estimates that approximately 0.1% of agricultural land is amended with biosolids annually. The remainder of sewage sludge mass produced is either disposed of in landfills or burned in municipal incinerators.

In response to the U.S. National Research Council's call for better characterization of residual contaminantion of biosolids, the EPA conducted the Targeted National Sewage Sludge Survey (TNSSS) in 2006/2007 to obtain national estimates of certain pollutants in biosolids. In addition to various organic and inorganic compounds, a suite of 72 PPCPs was analyzed for their occurrence in biosolids originating from across the U.S. In 2001, the U.S. EPA had conducted a similar survey focusing on dioxins and dibenzofurans in biosolids destined for land application. Later in 2008, when analytical methods for the analysis of PPCPs in biosolids became available (*7*), archived samples from the 2001 EPA biosolids survey were examined for PPCP contaminant levels (*8*). Thus,

today two recent U.S. nationwide datasets on PPCPs exist, one representing contaminant levels in biosolids originally collected in 2001 for analysis of dioxins and a second one, the TNSSS conducted specifically for PPCP determination. The sampling campaigns for both surveys were conducted by the EPA and the samples collected in 2001 and in 2006/2007 were analyzed by the same contract laboratory that developed EPA method 1694 for PPCP analysis in biosolids.

This article aims to compare the findings of the two independent studies in regards to concentrations of PPCPs found as well as the analysis approaches taken and the quality and informational value of the resulting data.

Secondary Data Analysis Strategy

Data Sources

Analytical results from the two large nationwide U.S. biosolids studies were obtained from the TNSSS website (http://www.epa.gov/waterscience/biosolids/tnsss-overview.html) and from the peer-reviewed literature (*8*).

Origin of the Biosolids Analyzed in Prior Studies

In order to properly understand the commonalilties and differences between the two studies compared here, it is important to consider the origin of the samples for which analytical data were obtained. Both the 2001 and the 2006/2007 sample sets were collected by the EPA according to established protocols (*9*).

In the spring of 2001, the EPA collected sewage sludge samples from 94 wastewater treatment plants in 32 states and the District of Columbia. Five of the 94 plants had two treatment systems, therefore, 2 samples were collected from these facilities. In addition 14 duplicate samples were collected for quality control. This resulted in 113 sewage sludge samples that were analyzed for dioxins and dibenzofurans and subsequently given to the National Biosolids Repository, currently maintained at Arizona State University, where they were stored at -20°C. In the spring of 2008, 110 of the samples were combined into five composite mega-samples, consisting of 21 to 24 randomly selected individual samples. These five composite samples were subsequently analyzed.

In 2006/2007, the EPA collected sewage sludge samples from 74 treatment plants in 35 states. In addition, 10 samples were collected for duplicate analysis or because the plant had more than one treatment system. Only treatment plants that processed more than 1 million gallons of wastewater per day (MGD) were considered for sampling. Together, this group of facilities treats approximately 94% of all wastewater in the U.S. All treatment plants sampled employed at least secondary treatment. Sewage sludge produced at these faciclities was disposed of either through land application, incineration or surface disposal.

Overview of Analytical Strategies Utilized by Prior Studies

Both sample sets were analyzed by the same analytical laboratory, Axys Analytical Services (2045 Mills Road West, Sydney, British Columbia, Canada

V8L 3S8), according the EPA method 1694 (*7*). A detailed description of this liquid chromatography tandem mass spectrometry method, a.k.a. EPA method 1694, can be found at www.epa.gov/ost/methods/method/files/1694.pdf.

For the purpose of compound detection, the 72 analytes were divided into four groups in EPA method 1694. All analytes were separated by liquid chromatography and detected by tandem mass spectrometry (MS/MS). For some compounds for which stable isotope-labeled analogs were available, concentrations were determined using the isotope dilution technique (*7*). For compounds where a labeled analog was unavailable, corresponding concentrations were determined using conventional calibration by employing internal and external standards.

Statistical Tools Employed and Literature Information Source

Data from the two large nationwide studies were extracted from the literature and entered into a Microsoft Excel data spreadsheet. Analytical results were compared using standard statistical analysis such as scatter plots and linear regressions using Microsoft Excel. Literature information for the risk assessment section was obtained from the peer-reviewed literature indexed in the Web of Science (ISI Thompson; http://thomsonreuters.com/).

Results and Discussion

Statistical Comparison of the 2001 Survey and EPA Targeted National Sewage Sludge Survey 2006/2007

Both surveys analyzed sewage sludge samples for a set of 72 PPCPs. The TNSSS conducted by the EPA relied on the analysis of individual samples. In contrast, the dataset on PPCP concentration present in sludge samples collected in 2001 was obtained by analyzing a small number of mega composite samples (*8*). In mega composites mixed from individual samples collected in 2001, 38 PPCPs were found in at least one of the mega composite samples analyzed, and a total of 26 analytes were detected in all five mega composite samples (*8*). In the TNSSS in which individual samples from discrete plants were analyzed, 69 PPCPs were detected in at least one sewage sludge sample. Three analytes, namely ciprofloxacin, diphenhydramine, and triclocarban, were found in each of the 84 samples examined. The observed differences in detection frequency can be linked to the very different experimental approaches of the two studies, i.e., the use of mega composite samples versus individual samples from discrete treatment plants. Despite these expected differences in detection frequency, a comparison of mean concentrations identified in both surveys shows that both studies produced similar findings (Figure 1), especially for analytes found in high concentrations, i.e., ciprofloxacin, ofloxacin, triclocarban, and triclosan.

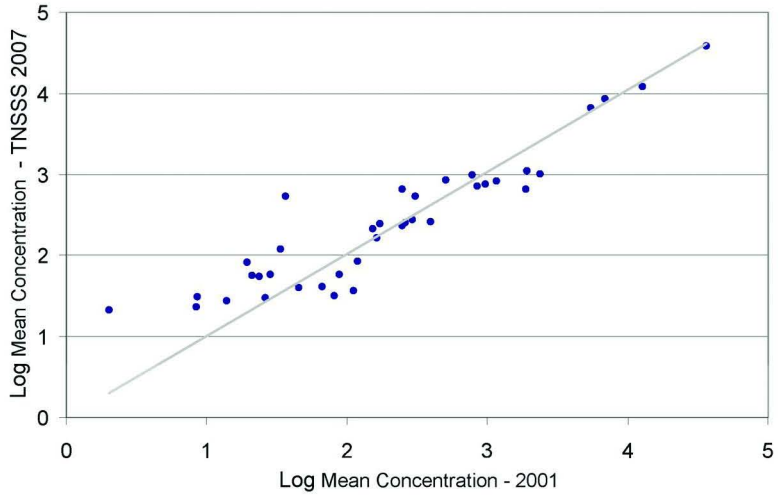

Figure 1. Comparison of mean concentrations for pharmaceuticals and personal care products in sewage sludges collected in 2001 and 2006/2007 by the U.S. Environmental Protection Agency. Excluded are mean concentrations below the detection limit; line denotes linear fit (y = 1.012 x with an R^2 value of 0.778). (see color insert)

A comparison of the range of concentrations detected reveals that the analysis of individual samples resulted in a much larger range of concentrations for all analytes detected (Figure 2). This comes to no surprise as the composite samples represent an average of all individual samples they are comprised of.

In addition, systematic differences in detection frequencies were revealed between the two datasets (Figure 3). These differences in detection frequency also originate from the different sampling approaches (individual vs. composite samples). Incorporation of uncontaminated materials into composite samples can lead to a dilution of concentrations of rare analytes. This may cause concentrations in composite samples to fall below the limit of detection, as evidenced by the lower number of analytes detected in any one of the composite samples when compared to the individual samples of the TNSSS. A similar but reverse effect was observed for analytes featuring a high frequency of occurrence in individual samples. For composite samples, detection frequencies of these types of analytes was 100%, whereas the analysis of individual samples from the TNSSS revealed detection frequencies of less than 100% for most analytes.

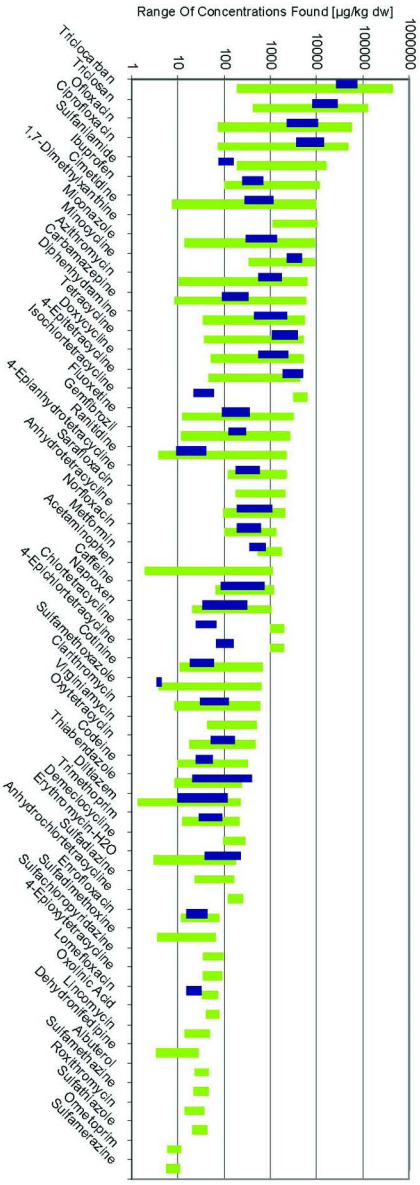

Figure 2. Comparison of the range of concentrations found in mega composite samples collected in 2001 (dark blue) and in EPA's Targeted National Sewage Sludge Survey (light green); excluded are concentrations below detection limit. (see color insert)

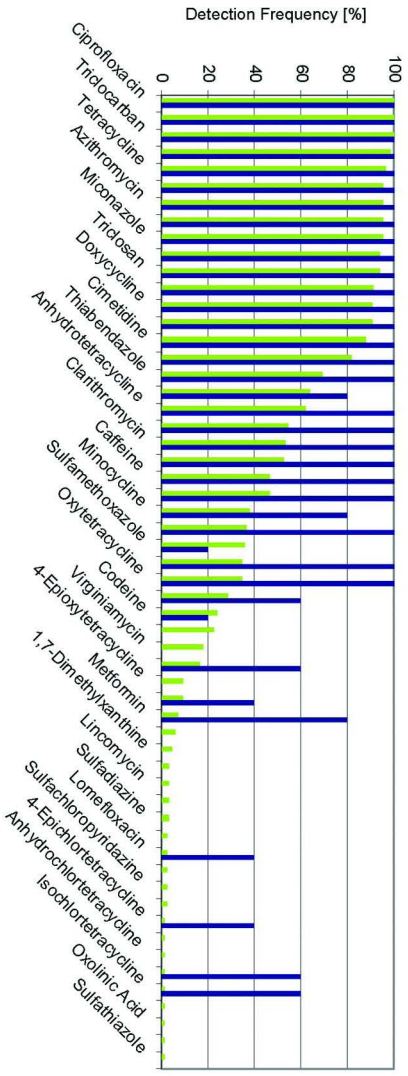

Figure 3. Comparison of detection frequencies of analytes featuring concentrations above the detection limit in mega composite samples collected in 2001 (dark blue; n = 5) and in individual samples analyzed as part of the EPA's TNSSS (light green; n = 84). (see color insert)

Comparison of the 2001 and 2006/2007 Datasets on PPCPs in Biosolids to Literature Values

Both sewage sludge surveys identified two biocides, triclocarban and triclosan, as the primary contaminants among the PPCPs analyzed. Triclocarban was found at mean concentrations of 36.1 ± 8.0 mg kg^{-1} dry weight (dw) (2001) and 39.4 ± 60 mg kg-1 dw (2006/2007), whereas triclosan was measured at 12.6

± 3.8 mg kg^{-1} dw (2001) and 16.1 ± 65 mg kg^{-1} dw (2006/2007). Both analytes have been found previously in sludge. Triclocarban has been found at lower concentrations ranging from 3.05 to 25.9 mg kg^{-1} dw in biosolids (*10–12*). In contrast, triclosan has been found in a wide range of concentrations from 0.09 mg kg^{-1} dw up to 55 mg kg^{-1} dw by different studies (*3*, *5*, *11–15*), which includes the range of values reported above. Furthermore, both surveys identified two fluoroquinolone antibiotics as prevalent contaminants. Ciprofloxacin was found at 6.8 ± 2.3 mg kg^{-1} dw (2001) and 10.5 ± 17.6 mg kg^{-1} dw (2006/2007), while ofloxacin was found at mean concentrations of 5.4 ± 1.9 mg kg^{-1} dw (2001) and 8.6 ± 22 mg kg^{-1} dw (2006/2007). These four contaminants were identified by both surveys as having the highest concentrations among all PPCPs analyzed. Other fluoroquinolone antibiotics identified in both surveys were norfloxacin, with mean concentrations between 0.1 and 1 mg kg^{-1} dw, as well as enrofloxacin and lomefloxacin, both at mean concentrations of around 0.01 mg kg^{-1} dw. In addition, the 2006/2007 TNSSS found sarafloxacin at a mean concentration of 0.3 ± 0.7 mg kg^{-1} dw, while it was not detected in any of the 2001 samples. The group of fluoroquinolones accounted for 12.6 mg kg^{-1} dw (2001) and 16 mg kg^{-1} dw (2006/2007) in sewage sludge samples. Ciprofloxacin has been found in concentrations from 22.6 to 46.4 mg kg^{-1} dw in biosolids from Ohio (*16*), which are even higher than the mean concentrations found in samples representing biosolids nationwide. Other fluoroquinolone antibiotics have so far not been reported in biosolids from the U.S.

Tetracyclines are another group of antibiotics identified by both surveys as PPCPs prevalent in biosolids. Both surveys found tetracycline and 4-epitetracycline at mean concentrations above 1 mg kg^{-1} dw, and doxycycline, minocycline, anhydrotetracycline (in order of decreasing mean concentration) were found at concentrations between 0.1 and 1 mg kg^{-1} dw in both surveys. In addition, the 2006/2007 TNSSS showed mean concentrations of anhydrochlortetracycline and 4-epichlortetracycline in the same range, whereas these substances either were not detected in any of the samples from 2001 (anhydrochlortetracycline) or at concentrations below 0.1 mg kg^{-1} dw (4-epichlortetracycline). Furthermore, isochlortetracycline, oxytetracycline and chlortetracycline were detected in both surveys at mean concentrations between 0.01 and 0.1 mg kg^{-1} dw. Lastly, 4-epioxytetracycline and demeclocycline were found in samples from 2006/2007 in the same concentration range, but not in any of the samples collected in 2001. Together, tetracycline antibiotics accounted for 6 mg kg^{-1} dw (2001) and 3 mg kg^{-1} dw (2006/2007). So far, only one peer-reviewed study (*16*) has reported tetracycline in biosolids from the U.S. Tetracycline was detected in only one biosolids sample from Ohio at a concentration of 15.7 mg kg^{-1} dw, which is about one order of magnitude more than mean concentrations found for biosolids samples from the entire U.S.

Other antibiotics found by both surveys at mean concentrations above 0.1 mg kg^{-1} dw were azithromycin and sulfanilamide. Virginiamycin was only found by the 2006/2007 TNSSS at a mean concentration of 0.14 ± 0.23 mg kg^{-1} dw. Notably, only the EPA's TNSSS found 1,7-dimethylxanthine, a stimulant, at a mean concentration of 1.18 ± 8.7 mg kg^{-1} dw, while this substance was not detected in any of the samples collected in 2001. Concentrations for 1,7-dimethylxanthine

have not been reported elsewhere for U.S. biosolids, however, the compound has been found in surface water before (*17*). Both surveys also found a variety of over-the-counter and prescription drugs at mean concentrations between 0.1 and 1 mg kg^{-1} dw, namely caffeine, carbamazepine, cimetidine, diphenhydramine, fluoxetine, gemfibrozil, ibuprofen, and miconazole. Most of these compounds have been found by other U.S. studies. Some PPCPs have previously been found at higher concentrations, namely caffeine which has been reported in one U.S. study at concentrations of around 5 mg kg^{-1} dw (*16*), carbamazepine, which was found at concentrations of 4.7 to 12.8 mg kg^{-1} dw by the same study and diphenhydramine, which was found at concentrations up to 22 mg kg^{-1} dw (*3*, *15*). Some compounds that were previously found in lower concentrations are cimetidine, which has been found in concentrations of up to 0.07 mg kg^{-1} dw (*3*), fluoxetine of up to 0.06 mg kg^{-1} dw (*3*) and miconazole, which has been found at concentrations of up to 0.46 mg kg^{-1} dw (*3*). Gemfibrozil has been found in a wide range of concentrations from 0.06 to 3.4 mg kg^{-1} dw (*3*, *16*). Only ibuprofen has not been previously reported for U.S. biosolids, but has been detected in biosolids in Spain (*18*).

Potential Effects

Since the majority of sewage sludge is applied on land, these findings warrant further evaluation regarding the fate and effects of these contaminants in the environment. To predict the fate of biosolids contaminants in the environment, multiple physicochemical mechanisms have to be considered. Once applied on land, biosolids are typically mixed into top soil through tilling. Organic contaminants introduced into soils in biosolids can then become dissolved in soil porewater and leach out during rain events. They also may be removed in the sorbed state by soil erosion. The extend of compound migration depends on a compound's solubility in water, its sorption coefficient for soil and its tendency to form insoluble salts through complex formation. Some PPCPs have functional groups which are capable of complex formation with metallic cations, which can reduce their bioavailability.

More likely for hydrophobic chemicals is the process of sorption to soil particles. Once sorbed to soil, it cannot be easily be predicted to what extent chemicals desorb under varying environmental conditions, or to what extent they are still available to biodegradation or biologically active, in the case of pharmaceuticals.

Antibiotics and antimicrobials are designed to affect bacteria and other microorganisms. Therefore, even low concentrations in the environment might affect microbial communities. These effects can occur in the form of direct toxicity to soil bacteria, therefore influencing soil quality of agricultural land after application of biosolids. Evidence for this has been observed in a soil microbial community, where 10 μg kg^{-1} tetracycline still had significant effects on the metabolic quotient 8 weeks after a one-time application (*19*). Besides direct toxicity, the potential of antibiotics to promote resistance among bacteria has to be considered. Although some details of the mechanisms leading to the spread of antibiotic resistance in the environment are still unclear, it is generally accepted that sub-therapeutic concentrations in environments with high bacterial density

(such as soil) constitute conditions for the development and spread of antibiotic resistance. However, it has been suggested that the input of already resistant bacteria into the environment may be more important for resistance occurring in the environment than the presence of antibiotics (*20*). Biosolids may also be a direct source of resistant bacteria. Several studies have shown that resistance against multiple groups of antibiotics have been found in bacteria contained in sewage sludge (*21–23*) and that these are not always being eliminated during conventional anaerobic sludge digestion (*24*).

Presence of pharmaceutical contamination in biosolids also constitutes a risk to crops grown on sludge-amended agricultural land. Plants may take up therapeutic agents from soil which can affect growth and development of the plants in question. Evidence for plant uptake as well as adverse effects has been found for several antibiotics. Uptake of sulfamethazine has been demonstrated for lettuce, potato and corn (*25*). Another study demonstrated uptake of chlortetracycline from a manure-soil mixture by corn, green onions and cabbage (*26*), while tylosin was not absorbed by these plants. A study focusing on veterinary medicines showed uptake of florfenicol, levamisole and trimethoprim by lettuce, while diazinon, enrofloxacin and trimethoprim were taken up by carrot roots (*27*). These studies show that uptake of PPCPs is dependent on the plant in question as well as the compound. Besides active uptake of dissolved PPCPs, hydrophobic compounds also have the potential to bioaccumulate in plant tissue that is in direct contact with contaminated soil. Bioaccumulation by plants has been demonstrated by several studies (*28–31*), which is of particular concern for root vegetables.

Toxic effects on plants also have been demonstrated. In one study 400 ppm tetracycline suppressed free-branching of poinsettia in laboratory studies (*32*). Also, 300 ppm sulfadimethoxine depressed growth of several plants (*29*, *33*). It was further found that 50 ppm of enrofloxacin caused hormesis in several plant species, while 5000 ppm had toxic effects (*34*). However, adverse effects seem to be plant and compound specific. Another study found adverse effects on pinto beans by oxytetracycline and chlortetracycline, while the same antibiotics had no effect on corn, and even stimulated the growth of radish and wheat plants (*35*). In addition to the plant species and the therapeutic agent, the nature and extent to which adverse effects occur also depend on soil properties such as organic carbon, pH, mineral concentration, clay composition and temperature.

While the uptake and effects of antibiotics in crop plants are variable, these findings indicate potential risks of antibiotic contamination of food supplies. Associated health risks for consumers can include allergic or toxic reactions, chronic toxic effects due to low-level exposure, disruption of the human gut microbial flora and spread of resistant bacteria.

Conclusions

Both the EPA's TNSSS and the analysis of samples collected in 2001 revealed concentrations of several antimicrobials and antibiotics in the mg kg^{-1} dw range.

Prevalent contaminants were triclocarban, triclosan, ciprofloxacin and ofloxacin, followed by a number of tetracycline antibiotics.

The statistical comparison of the results of the two studies showed that combining a large number of individual samples to mega composite samples is a suitable approach to identify prevalent contaminants and obtain representative mean concentrations. Shortcomings of this analysis approach potentially include unrepresentatively high frequency of non-detect values for rare contaminants, and reduced informational value concerning geographical distributions of contaminants. However, the use of mega composite samples can result in significant cost savings and it is accomplished much quicker than the analysis of many individual samples.

The two studies compared here provide a fundamental knowledge basis for further risk assessment of the practice of biosolids application on agricultural and recreational land. Areas of concern include the promotion of antibiotic resistance in the environment, adverse effects on soil microbial communities and plants, as well as direct exposure of consumers to drug residues in food crops grown on biosolids amended fields.

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