4.1 Final publishable summary report

4.1.1 Executive summary

Project overview

The project isoSoil was funded by the EU (FP7:Cooperation, Theme 6: Environment) to support the implementation of the Soil Thematic Strategy by contributing to the development of more precise and reliable site characterisation and monitoring design of contaminated land, thus aiding the improvement in the effectiveness of remediation and mitigation of such sites. isoSoil was active during 36 months in the period 2009 to 2012, had a total budget of ~2100 k€, and involved 11 partners in a pan-European consortium representing six countries. The consortium included partners from both academia and the commercial sector to develop the concept of compound-specific isotope analysis for source apportionment and degradation monitoring of contaminants.

Project objectives

There is an estimated 3.5 million contaminated soil sites within the EU. One urgent identified need to achieve better soil health is more efficient and accurate methods for monitoring and characterization of contaminants in soils. The isoSoil concept is to firmly establish the molecular-isotopic composition as revealed by compound-specific isotope analysis (CSIA) as a novel, user friendly and powerful tool for both degradation monitoring and source apportionment of organic contaminants in soils. CSIA benefits from its independence of non-isotope fractionating processes such as dilution and re-distribution that affect the concentration of contaminants, and therefore provides added value to the conventional concentration/mass balance based methods for site investigations. The objective of isoSoil was to develop and implement methods for statistical optimization of sampling, extraction and isotope analysis of target compounds, and interpretation of the isotope data using purpose-made software.

Project results

Several investigations have been executed using the novel multi-dimensional CSIA concept for a range of compounds, measuring the stable isotopes of carbon, nitrogen and chlorine in the contaminants. Furthermore, an extensive data set has been generated for the combustion derived polycyclic aromatic hydrocarbons of Czech top soils by analysis of stable hydrogen and carbon isotopes, combined with the radioactive radiocarbon isotope, ^{14}C. Microbial and abiotic laboratory experiments have contributed to the baseline data that is needed to apply CSIA in field studies. Several analytical methods have been developed to facilitate such investigations, e.g. novel online analysis of chlorine isotopes and hydrogen isotopes. Finally, the consortium has produced a software package for planning and interpretation of CSIA studies, and studies of the underpinning theory. All foreground has been, and will be, widely disseminated to potential end users.

4.1.2 Summary description of project context and objectives

There is an estimated 3.5 million contaminated soil sites within the EU. One urgent identified need to achieve better soil health is more efficient and accurate methods for monitoring and characterization of contaminants in soils. The isoSoil concept is to firmly establish the molecular-isotopic composition as revealed by compound-specific isotope analysis (CSIA) as a novel, user friendly and powerful tool for both degradation monitoring and source apportionment of organic contaminants in soils.

CSIA makes use of the difference in reaction rates for different isotopes of the chemical element in the reactive position of a compound that undergoes transformation. The breaking of a chemical bond during degradation gives rise to a kinetic isotope effect (KIE) for the elements involved in the bond breaking. Commonly, molecules containing light isotopes (e.g., ^{12}C) react faster than molecules containing heavy isotopes (e.g., ^{13}C), giving rise to measurable isotope enrichment.

This means that during the course of contaminant degradation, the remaining primary contaminant molecules become enriched in the heavier isotope whereas the products (secondary pollutants) may become depleted in heavier isotopes. One particular strength of the CSIA assessment tool is that it provides quantitative diagnostics of key site characteristics while being independent of contaminant concentration, which may fluctuate due to e.g. dispersion, dilution, and volatilization.

Source apportionment is another application where CSIA excels. Different starting material and production mechanisms give rise to source-diagnostic isotopic composition of the contaminants. For instance, the relative contribution to the environmental load of PAHs may be deciphered with natural abundance radiocarbon measurements ($^{14}C/^{12}C$) as fossil fuel combustion and petroleum spills are ^{14}C depleted ("radiocarbon dead") whereas biofuel combustion has a modern ^{14}C signal ("radiocarbon alive"). Similarly, different petroleum feedstock in industrial synthesis may give rise to source-unique CSIA patterns. To illustrate, natural gas and petroleum distillates have very different ($^{2}H/^{1}H$) composition. While the primary sources at a heavily contaminated brownfield may be clear, the more diffuse regional contamination of e.g. agricultural soils through atmospheric deposition and groundwater infiltration may benefit enormously from CSIA-based source apportionment to guide society regarding which sources to target for effective mitigation.

Developments of the analytical methods, the baseline data and the theoretical underpinning will help to make CSIA a viable alternative for e.g. environmental consultants investigating a plot of contaminated land, and a widely applied tool to solve scientific problems. isoSoil set out the following objectives to accomplish this:

- *Building a pan-European consortium from both academia and enterprises*

Dissemination of knowledge outside the consortium was achieved by presentations and house calls by the partners of isoSoil. Both academia, from many different disciplines, and enterprises from both the analytical and the environmental services sector participated in the project, thus

reaching all types of relevant audiences. Increased awareness of the existence and capabilities of CSIA is important to gain acceptance and users.

- *Launching a field-sampling program*

Techniques and concepts have been tested and proven on real field samples from sites where biotransformation processes of the contaminants are active. Sites were chosen to reflect a spectrum of relevant target compounds, e.g. the ubiquitous groundwater pollutants tri- and perchloro ethene, the waste products from Lindane (isomers of hexachlorocyclohexane), nitrogen-containing residues from the manufacture of explosives (nitrotoluenes), and combustion-derived polycyclic aromatic hydrocarbons (PAHs) from a regional sampling campaign in the Czech Republic.

- *Conducting laboratory experiments to constrain isotope enrichment factors*

Microbial experiments were performed to constrain the degradation-relatable isotope shifts for all target compounds except the PAHs, which are highly recalcitrant. These experiments were based on the actual microbial communities from the field sites to reflect the environmental processes with as high fidelity as possible. Abiotic experiments were also conducted to better understand the intrinsic kinetic isotope effects that underpin the full chain of chemical reactions occurring during microbial and abiotic degradation.

- *Developing methods for isotope analysis*

CSIA has always been at the front line of developments in analytical chemistry, and is still suffering from a high complexity that seldom lends itself to routine analysis. isoSoil has contributed a few solutions to the body of analytical challenges. A technique has been developed to enable accurate online measurements of of $\delta^{2}H$ in organochlorine compounds, that can be employed by any laboratory working with GC-IRMS techniques. Another product of isoSoil is a new method for online determination of $\delta^{37}Cl$ in organochlorines using conventional benchtop GC-quadrupole-MS, which is available in almost all laboratories working with organic analysis. Thus, a potential several fold increase has been achieved w.r.t. analytical capabilities for $\delta^{2}H$ and $\delta^{37}Cl$ in contaminants.

- *Building advanced and easy-to-use software for CSIA use*

Thanks to software companies in the consortium, isoSoil has produced software modules for several purposes. A web interface ties together modules for statistical optimization of sampling, calculations using user-defined reaction schemes and isotope enrichment factors, a large database for kinetic isotope effects (KIEs), and on-the-fly computation of KIEs. The software is available for free trials after contact with the isoSoil consortium.

- *Disseminating the isoSoil foreground*

The outcomes of isoSoil have been disseminated to parties within both academia and the commercial sector through general presentations, specific house calls and publications. We continue to publish isoSoil results during 2012/2013, and to inform about the isoSoil software.

4.1.3 Main S&T results and foreground

isoSoil has been executed according to the Description of Work, forming Annex I of the grant contract. What follows is an account for the different areas of activity. Descriptions according to work package sub-divisions can be found in the periodic reports.

4.1.3.1 Obtaining samples for laboratory experiments and field studies

isoSoil started by launching several sampling campaigns with the aim to provide samples for both laboratory experiments and field studies. Four study sites were selected:

(i) nitrotoluenes in soil at a Swiss site
(ii) hexachlorocyclohexanes at the Spolana site, Czech Republic
(iii) chlorinated solvents (tri- and tetra-chloroethene) at the SAP site, Czech Republic
(iv) polycyclic aromatic hydrocarbons (PAHs) from forest soils, Czech Republic

Sampling was performed for spatial coverage of biotransformation processes pertaining to the target compounds, but was augmented to also yield soil samples for preparation of cultures for microbial experiments. Soil sampling for (i) and (ii) was done simply by manual collection of soil. Groundwater sampling for (iii) relied on a subsample of the existing grid of observation wells, whereas soil for microbial cultures was obtained using 3 core samples from different part of the investigated area and one background sample away from the contaminated area. In total, some 50 samples were retrieved, forming the basis for the field studies within isoSoil.

4.1.3.2 Microbial experiments to obtain the isotope fractionation during degradation

In order to determine the isotope fractionation factor associated with the microbial degradation of the target compounds, a series of reference biodegradation experiments were performed and samples corresponding to different extents of degradation were collected. The results hereof will be published during 2012/2013 and some results are still pending. So far, a chlorine isotope enrichment factor of approximately -2.0 ‰ has been found for the PCE degradation experiments. The corresponding experiments for dioxygenation of 2,4- and 2,6-DNT gave enrichment factors of -8.2 ± 0.3 and -8.8 ± 0.7‰ for $\delta^{13}C$ for 2,4- and 2,6-DNT respectively, and -1.3 ± 1.4‰ for $\delta^{15}N$ for 2,4-DNT.

The experiments leading to these enrichment factors were conducted as follows. For the implementation of the DNT experiments a fresh culture of mixed DNT-degrading bacteria was prepared using surface soil from Spot 3 site (Switzerland). The concentrations of 2,4-DNT and of 2,6-DNT were regularly monitored over the course of degradation and the cultures were sacrificed at specific extents of degradation by the addition of concentrated HCl. After the end of the experiments, the sacrificed cultures (by HCl addition) were sealed in amber glass vials and shipped to partners (ETH-Z) for the implementation of compound specific isotopic analysis.

For the enrichment of PCE/TCE-degrading bacteria, four soil samples were collected by our partners (Earth Tech CZ s.r.o.; ETCZ) from a carcass disposal plant at north Bohemia, Czech Republic. The enrichment of PCE/TCE-degrading bacteria under anaerobic conditions was a very challenging task. To circumvent this difficulty, different types of culture media and conditions were applied to the four soil samples in order to achieve bacterial growth and biodegradation of chlorinated ethylenes. Based on the results, the Cole's medium was found to be the most appropriate for the biodegradation of chlorinated ethylenes under anaerobic conditions. Two series of biodegradation experiments were performed for PCE and one series for TCE using fresh enrichment cultures of mixed bacteria from soil sample Z-53. After the end of the experiments, the sacrificed cultures (by HCl addition) were sealed and shipped to partners (SU) for the implementation of compound specific isotopic analysis. Following the results from the analytical methods applied for ethylene measurements the method of in-vial microscale liquid-liquid extraction for the analysis of hexachlorocyclohexanes was also examined.

For the enrichment of α-HCH/β-HCH-degrading bacteria, five soil samples were collected by our partners (Masaryk University) from the ground left after the destruction of contaminated building and BCD remediation of surface soils at Spolana Neratovice, Czech Republic. For the enrichment of α-HCH/β-HCH-degrading bacteria under anaerobic conditions we followed the same protocol as for the PCE/TCE-degradation experiments. The anaerobic experiments were performed using the reduced anaerobic medium described by Cole et al. (Cole et al. 1994. Appl. Environ. Microbiol. 60, 3536-3542). One series of biodegradation experiments was performed for α-HCH and one for β-HCH using a fresh enrichment culture of mixed bacteria from soil sample 2B. After the end of the experiments, the sacrificed cultures (by HCl addition) were sealed and shipped to partners (UB) for the implementation of compound specific isotopic analysis.

4.1.3.3 Characterization of the microbial communities used in microbial experiments

Sequence analysis of polymerase chain reaction (PCR)-amplified and cloned 16S ribosomal RNA genes (16S rDNA) is a widely used approach to assess microbial diversity and community composition in environmental samples. In order to investigate microbial community composition changes during degradation experiments, a total of three samples were chosen for 16S rRNA gene clone library analysis. These samples corresponded to the beginning (~100% remaining fraction of the compound), the middle (~50% remaining fraction of the compound) and the end (~5% remaining fraction of the compound) of the degradation experiments.

For the 2,4-DNT biodegradation experiments the samples S2 (79% remaining fraction), S4 (40%) and S7 (5.2%) were chosen for 16S rRNA gene clone library analysis. In sample S2 a total of three phyla were identified the Bacteroidetes, Betaproteobacteria and Gammaproteobacteria. In sample S4 the diversity was much higher but mostly dominated by Pseudomonas species. In sample S7 the diversity was also high and also dominated by Pseudomonas species together with Stenotrophomonas species. For the 2,6-DNT biodegradation experiments the samples S9 (59% remaining fraction), S8 (49%), and S4 (5.1%) were chosen for 16S rRNA gene clone library analysis. The sample S9 was dominated by Pseudomonas species. In sample S8 the diversity was also very high but mostly dominated by Pseudomonas and

Achromobacter species. In sample S4 the diversity was also high but mostly dominated by Pseudomonas species whereas Stenotrophomonas species in this fraction were not detected.

For the PCE biodegradation experiments the samples 1 (100% remaining fraction), 10 (53% remaining fraction) and 9 (7.6%) were chosen for 16S rRNA gene clone library analysis. The sample PCE1 was dominated by a phylotype closely related to Clostridium sp. strain DR7 accounted for up to 76% of the total analyzed clones. In sample PCE10 the diversity was also high and dominated also by a phylotype closely related to the strain detected in the previous fraction (accounted for up to 63% of the total analyzed clones). The sample PCE9 was dominated by two phylotypes closely related to Desulfitobacterium aromaticivorans UKTL and Clostridium sp. strain DR7 which both of them dominated the previous fraction.

For the α–HCH biodegradation experiments the samples S2 (104.4% remaining fraction), S7 (56.8%) and S4 (6.8%) were chosen for 16S rRNA gene clone library analysis. The sample S2 was dominated by two phylotypes closely related to Clostridium sp. and to Dendrosporobacter quercicolous strain DSM1736 accounted for up to 15% and 44% of the total analyzed clones, respectively. In sample S7 the diversity was much higher compared to the previous fraction. In sample S4 the diversity was much higher compared to the other two cultures. For the β–HCH biodegradation experiments the samples 1 (92.7% remaining fraction), 6 (44%) and 12 (6.2%) were chosen for 16S rRNA gene clone library analysis. The diversity of the first two samples was extremely high compared to all other experiments performed within WP2. This culture was highly diverse mostly dominated by Pseudomonas species. These results indicated that the degradation of βHCH is mediated by a microbial consortium with representatives by the taxonomic phyla of Firmicutes and Gammaproteobacteria.

Overall, the implementation of reference biodegradation experiments were successful for all the range of pollutants under investigation (i.e. PCE, TCE, 2,4-DNT, 2,6-DNT, αHCH and βHCH). Microbial community composition data during degradation experiments of the target compounds (beginning, middle and end of degradation) has been generated and demonstrated that diverse soil communities mostly closely related to Pseudomonas species mediated the biodegradation processes of the target compounds. Samples from these reference biodegradation experiments were shipped to isoSoil partners to investigate isotopic fractionation associated with microbial degradation.

4.1.3.3 Laboratory experiments to obtain the enrichment factors of abiotic reactions

The microbial experiments provide information about the isotope fractionation that is observed in the field during biotransformation of the target compounds. Further insights to the underpinning mechanisms can be found through abiotic experiments, i.e. by mimicking individual reaction steps that are part of the overall microbially mediated reaction. The long-term scientific goal is to better understand and predict the isotope fractionation in the context of reactions occurring in the environment.

Table 1. Overview of contaminants and reactions that will be analyzed for isotope fractionation in WP3.

Contaminants	Process	Transformation Pathway	Stable isotope system

	no.	considered	investigated
PCE and TCE (per- and tri-chloroethene)	1	hydrogenolyis	C, Cl
α-/β-HCH (hexachlorocyklohexane)	2	reductive β-elimination	C, H, Cl
α- β-HCH	3	dehydrochlorination	C, H, Cl
α-/β-HCH	4	volatilization	C, Cl
2,4-/2,6-DNT (dinitrotoluene)	5	dioxygenation	C, N
2,4-/2,6-DNT	6	nitro group reduction	N

One workpackage was dedicated to providing isotope fractionation factors from abiotic reference experiments and from literature data to support the interpretation of observations made at the isoSoil field sites. The processes and reactions mimicked in abiotic reference experiments are different for the three classes of contaminants examined for degradation kinetics in isoSoil and are listed below. In Table 1, the most likely transformation and loss processes are listed, for which systematic data on isotope effects are essential in isoSoil.

For processes no. 1-5, experimental systems for the investigation of isotope fractionation have been developed (see Table 2), while process no. 6 has been dealt with based on literature data.

Table 2. Overview of proposed experimental systems and mimicked reactions/processes.

Process no.	Transformation pathway considered	Experimental system
1	hydrogenolyis	Suspensions containing Zn(0)- or Fe(0)-particles, side reaction to be monitored: reductive β-elimination
2	reductive β-elimination	Suspensions containing Zn(0), Fe(0) or homogeneous reduction by Cr(II)
3	dehydrochlorination	Base-catalyzed elimination in aqueous solution.
4	volatilization	(1) Comparison of isotope signatures form HCHs in artificially contaminated soils vs. pure HCH signatures. (2) Comparison of isotope signatures form HCHs soils vs. HCHs from passive samplers at Spolana site.
5	dioxygenation	Oxidation of substituted nitroaromatic compounds with MnO_4^- in homogeneous solution

Process no. 5: Permanganate catalyzed oxidations of dinitrotoluenes

Laboratory batch model systems for abiotc oxidations of 2,4- and 2,6-DNT have been completed and showed significant C and H isotope fractionation while isotope effects for N were only minor. A typical results is shown in Figure 1 for 2,4-DNT concentration-time courses and $\delta^{13}C$ isotope signature trends.

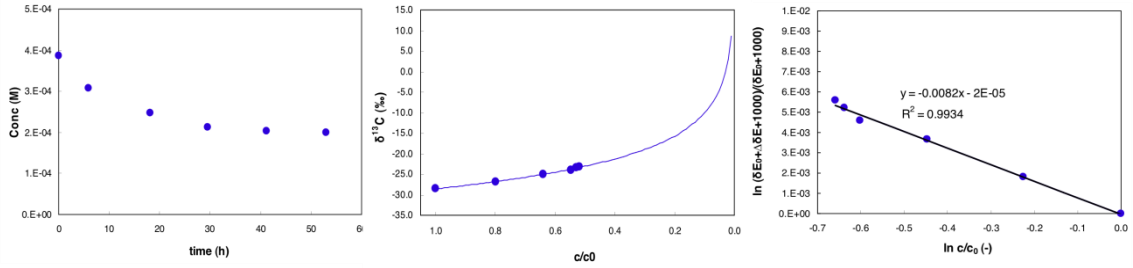

Figure 1. MnO4-catalyzed oxidation of 2,4-DNT and correspondig C isotope fractionation

The results from the permanganate catalyzed oxidations of dinitrotoluenes are presented together with the numbers for reductive transformations compiled from the literature, see Table 3.

Table 3. Isotope enrichment factors from abiotic experiments with nitrotoluenes.

Process	Compound	^{13}C [‰]	^{15}N [‰]	^{2}H [‰]
Dioxygenation	2,4-DNT	–8.2 ± 0.3	not significant	–55 ± 14
Dioxygenation	2,6-DNT	–8.8 ± 0.7	not significant	–111 ± 10
Reduction	2,4-/2,6-DNT	not applicable	–30-40‰	not applicable

Process no. 1-4: Samples awaiting isotope analysis

Experiments with PCE/TCE and HCHs are pending and will be performed by partner SU after the end of isoSoil. Furthermore, laboratory and field experiments addressing the shifts in the isotopic ratios of HCHs caused by volatilization from contaminated soils were completed. The samples have been sent to partner 1-SU and 9-UB, for δ^{37}Cl and δ^{13}C isotopic analysis respectively.

4.1.3.5 Preparing samples for isotope analysis

Preparation of samples for isotope analysis, i.e. extraction, pre-cleaning and quantification, was performed as a separate work package. Compounds of interest were volatile compounds such as tetrachloroethylene (PCE) and trichloroethylene (TCE) in groundwater, and a suite of semi/non volatile compounds in soil: nitrotoluenes, two isomers of hexachlorocyclohexane (HCH), and polycyclic aromatic hydrocarbons (PAHs).

Nitrotoluenes

All soil samples collected from the Swiss site by Partner 5 were dried, mixed and ground to pass a 5 mm sieve. Aliquots of 10 g were extracted in an ultrasonic bath for 18 hours in dichloromethane and analyzed by GC-MS (US EPA method 8330, see Table 4). Pre-tests with spiked soil samples showed recoveries of up to 100 % for 2,4-DNT, 2,6-DNT and TNT.

Table 4. Comparison of δ15N and δ13C isotope signatures of 2,4-DNT, 2,6-DNT and TNT from spiked soil samples with EA-IRMS values (i.e. the δ values of pure standard compounds)

Substance	δ^{13}C (‰)		$\Delta\delta^{13}$C(EA-IRMS - after procedure)	δ^{15}N (‰)		$\Delta\delta^{15}$N (EA-IRMS - after procedure)
	EA-IRMS	after procedure		EA-IRMS	after procedure	

2,6-DNT	-28.4 ± 0.3	-28.3 ± 0.2	**-0.1**	1.2 ± 0.1	1.5 ± 1.26	**-0.3**
2,4-DNT	-28.8 ± 0.3	-29.0 ± 0.3	**0.2**	-4.3 ± 0.2	-3.9 ± 1.14	**-0.4**
TNT	-26.1 ± 0.3	-25.8 ± 0.1	**-0.3**	16.1 ± 0.3	16.7 ± 0.6	**-0.6**

For GC/IRMS analysis, aliquots of soil samples were extracted by an accelerated solvent extraction system (Dionex ASE 350) with elevated pressure and temperature and acetone as solvent followed by a solid phase extraction with dichloromethane as elution solvent and if necessary with a subsequent evaporation step. This method was tested to ensure that the isotope composition of the sample remained intact throughout the entire analytical sequence (Table 4).

Hexachlorocyclohexanes

Aliquots of 5 grams of dry soil were extracted from each soil sample using dichloromethane. The extracts were pre-cleaned using sulfuric-acid modified silica gel columns and analyzed on GC-MS for the content of the isomers of hexachlorocyclohexane. Based on the concentrations found in the individual soil samples in the previous step, the soil sample size for CSIA was adjusted to provide a sufficient quantity of α-HCH and β-HCH for the compound-specific isotope analysis. It varied between 10 g and 1.5 kg of soil among the soil samples. Respective amounts of soil were extracted and extracts were pre-cleaned using large volume sulfuric-acid modified silica gel columns. Samples were concentrated to the final volume of 1 ml and aliquots were analyzed to determine the final extracted amounts of α-HCH and β-HCH.

Polyaromatic hydrocarbons

An aliquot of 5 grams of soil was extracted from each soil sample using dichloromethane. The extracts were pre-cleaned using silica gel columns and analyzed on GC-MS for the content of polyaromatic hydrocarbons, but also polychlorinated biphenyls and organochlorine pesticides.
For the purpose of contaminant-specific isotope analysis, only the samples from the top (organic) horizons were selected and extracted since those were believed to yield the highest concentrations of target compounds. Based on the concentrations found in the individual soil samples in the previous step, the soil sample size needed to provide a sufficient quantity of selected PAHs (fluoranthene, pyrene, (benzo(a)pyrene). The required sample size varied between 0.5 and 1.5 kg of soil among the top soil samples. Extracted samples were pre-cleaned using large-volume silica-gel columns. As the mountain forest soils contain large amounts of organic material, additional clean-up step was needed. A gel-permeation chromatography was applied to remove high molecular weight compounds from the samples. Samples were concentrated to the final volume of 1 ml and aliquots were analyzed to determine the final extracted amount of each compound.

Samples were additionally processed to obtain pure isolates of the target PAHs. This was done using preparative capillary gas chromatography. Each sample was repeatedly injected to the system where the target compounds and matrix components are resolved in the GC column. The desired compounds were "harvested" as they eluted from the column, using an automated Gerstel preparative fraction collector. The accumulated mass of pure compound, in excess of 25 µg carbon, was split in two fractions and sent for analysis of $\delta^{13}C$ and $\delta^{2}H$ within the isoSoil consortium. The main fraction of the isolate was however sent for radiocarbon analysis at

National Ocean Sciences Accelerator Mass Spectrometry Facility (NOSAMS; Woods Hole, Massachusetts, U.S.).

PCE and TCE

The samples containing chlorinated ethenes (tetrachloroethylene, PCE; trichloroethylene, TCE; and cis-dichloroethylene, cis-DCE) were collected from SAP Mimoň, Czech Republic. Field measurements and laboratory analyses of groundwater samples were performed by Partner 7 using portable equipment, whereas laboratory analyses were executed in the accredited laboratories ALS CZ according to the certified methodology. All groundwater samples were sent to Stockholm University for further extraction and analysis, using the data from Partner 7 as guidance.
Once received at SU, the groundwater samples were spiked with about 3 mL HCl (conc., 37%) to reach pH 1, in order to avoid any further biodegradation, and stored in a fridge at 4 C. Aliquots of the samples were extracted with cyclopentane and the concentration in each extract was measured by GCqMS. Based on the concentration data, the volumes of sample and solvent needed for the extractions prior to CSIA were evaluated in order to provide sufficient material for the chlorine-specific isotope analysis. An aliquot of each sample was sent to partner 9, where the target compounds were extracted for $\delta^{13}C$ measurements using identical procedures as in the laboratory of partner 1.

The same approach was taken for all samples resulting from the microbial experiments, since samples were delivered as aqueous solutions (growth media). Degradation experiments with microbial cultures cultivated were terminated by addition of 500 µl of HCl at various stages of degradation. All samples were then extracted by dichloromethane using liquid-liquid extraction technique. The extracts were pre-cleaned on sulfuric-acid modified (30 % w/w) silica column eluted by 40 ml of dichloromethane / hexane mixture (1:1). A gel permeation chromatography was employed to remove high molecular weight interfering compounds. An eluate was concentrated and target compounds quantified on a GC/MS instrument.

4.1.3.6 Developing methods for isotope analysis

Several methods have been developed to measure stable isotope in the selected target compounds. Furthermore, significant efforts have been spent in the investigations of a purge-and-trap system for off-line preparation of chlorinated solvents for $\delta^{13}C$ and $\delta^{37}Cl$ analysis.

$\delta^{13}C$ and $\delta^{2}H$ in chlorinated ethenes and cyclohexanes

We have successfully established methods for the determination of compound-specific $\delta^{13}C$ values of chlorinated ethylenes (PCE, TCE and cis-1,2-DCE) and the α, β, γ, and δ 1,2,3,4,5,6-hexachlorocyclohexane enantiomers in the low ng range. In addition, we have also established methods for the determination of $\delta^{2}H$ and $\delta^{13}C$ values for individual polycyclic aromatic hydrocarbons.

We have developed an on-line reduction reactor which promotes the production of H_2 and prevents HCl production via trapping of the Cl in HCl as $CrCl_3$, and liberation of the H in HCl as H_2. This results in a very large improvement in the conversion efficiency of organochlorine

compounds, from ca. 15% using a traditional glassy carbon reactor to 80-90% using nickel/chromium alloy or stainless steel based designs. Importantly, replicate analyses produced δ^2H values with standard deviations revealing precisions of better than 5‰. Accuracy was tested via analysis of n-alkanes within run, whose δ^2H values were determined to be within 5‰ of their known isotopic compositions (Figure 2). Furthermore, the δ^2H values determined for organochlorine compounds were statistically identical when analysed under the optimal conditions for both new reactor designs. A chromatogram showing the m/z 2 and 3 traces acquired during the analysis of a standard mixture of α and β-HCH and three n-alkanes, with each component containing equimolar quantities of H.

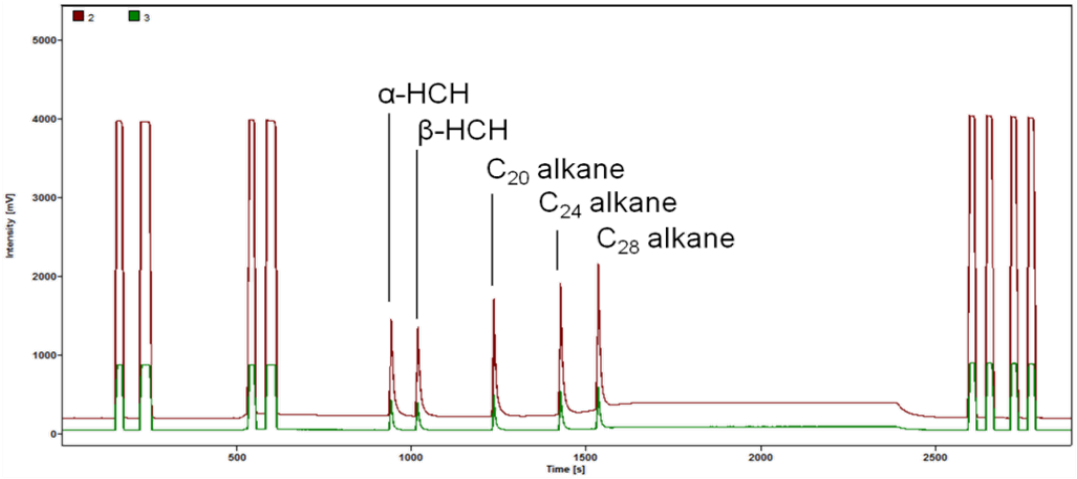

Figure 2. Mass chromatogram showing the m/z 2 and 3 traces during an analytical standard run containing two HCHs and three n-alkanes

The successful development of a reactor capable of the compound-specific δ^2H analysis of highly chlorinated compounds will open the way for contaminant degradation studies based upon ^{1}H/^{2}H isotope effects with the potential to give us new insights into the nature of the degradation processes involved and the extent to which they are occurring. Furthermore, this development could provide an alternative to the 1450°C glassy carbon reactor design commonly used in the deuterium isotope analysis of many organic compounds, meaning that researchers without the dedicated equipment required for these analyses will be able to do so using lower reactor temperatures of around 1000°C, which are achievable in standard GC-C-IRMS instrumentation.

δ^{13}C and δ^{15}N in nitroaromatic compounds

We established a new analytical procedure that enables, for the first time, the analysis of C and N isotopes in nitroaromatic compounds bound in soil. A schematic representation of the different steps leading from the extraction of the analytes from soil organic material to the analysis by GC/IRMS is shown in Figure 3A. Soil material was extracted by accelerated solvent extraction (ASE), filtration, enrichment by solid phase extraction (SPE), elution from the sorbent, and evaporation of the solvent. Despite slight analyte losses during the extraction of nitrotoluenes from soil by accelerated solvent extraction and further processing by solid phase extraction, elution, and solvent evaporation, we confirmed that no isotope signatures of original soil contamination was altered by experimental artefacts. In addition to measurements of soil samples, we established C and N isotope analysis by solid-phase micro-extraction (SPME)

coupled to GC/IRMS for dissolved nitroaromatic compounds (Figure 3), which enabled one to access 1000-fold lower concentration ranges.

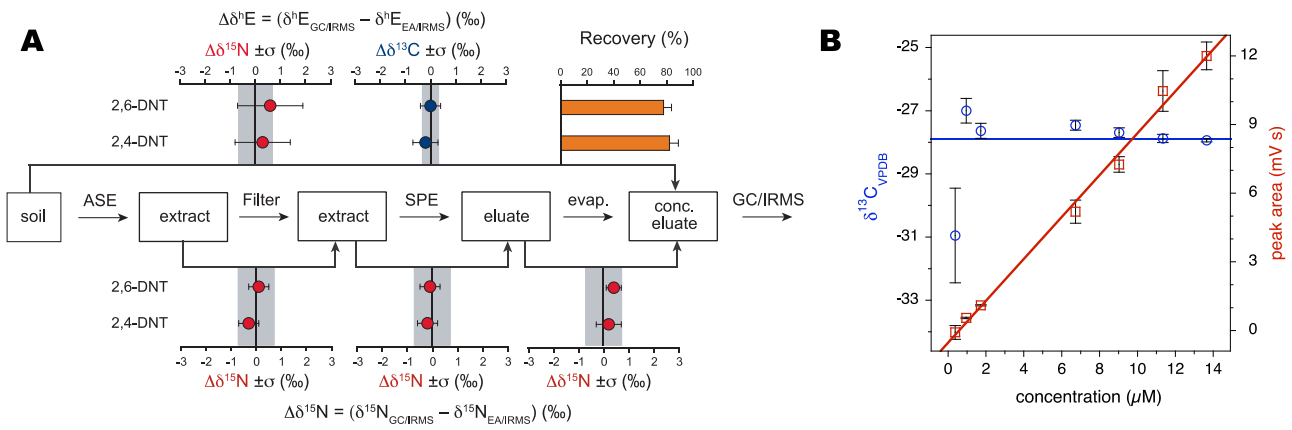

Figure 3. A: Schematic representation of the sample preparation procedure for the analysis of nitrotoluenes from soil material including the evaluation of potentially isotope fractionating procedural steps. (Abbreviations: ASE accelerated solvent extraction; SPE = solid phase extraction; evap = evaporation of solvent.); B: $\delta^{13}C$ measurements of 2,4-DNT by SPME-GC/IRMS.

$\delta^{37}Cl$ of chlorinated organic compounds

Partner 1 investigated whether the chlorine isotope measurements using thermal-ionization mass spectrometry (TIMS) could be made more time efficient. Different approaches were tested to improve the sample preparation and thereby increase the method reliability. It was concluded that the method was not suitable for large-scale investigations, such as that of isoSoil or the needs of an actor on the market for analytical services. Instead, a new method for measurements using highly sensitive on-line gas-chromatography quadrupole mass spectrometry was developed, published and adopted in isoSoil. This method is now published (Aeppli et al., 2010, Analytical Chemistry) and used for all the chlorine isotope measurements except for the determination of standard materials. The precision is ~0.5‰ (1 σ) and the limit of quantification is in the picomol range for PCE and TCE. Samples can be processed completely online, starting with an extract in organic solvent. The method requires authentic standards to yield accurate results, and such standards have been produced using the older in-house method for thermal-ionization mass spectrometry.

4.1.3.7 Extraction of chlorinated solvents from groundwater

The initial plan to use the purge and trap system for the extraction of CEs from groundwater samples has been abandoned. Significant troubles were encountered in efficiently trapping the compounds offline (i.e. without connecting the purge and trap to a GC/MS, which is commonly used by other laboratories). Although different trapping systems, along with different temperature and flow control systems have been tested, all attempts brought unsatisfying results. Therefore the extraction of target compounds from the samples generated in WP2 is ongoing using the liquid-liquid extraction method used for the groundwater samples from SAP site.

4.1.3.8 Field-site studies

In all, partner 9 - UB has performed >300 $\delta^{13}C$ and >240 $\delta^{2}H$ analyses, thus more than fulfilling the commitment in Annex I. Partner Eawag has totalled >280 injections, and SU >1000 injections. These numbers are conservative, since each analysis normally consists of multiple trials before all parameters are optimized for high-quality results. Herewith is a brief presentation of some of the results so far, all in the stage of interpretation prior to publishing.

Czech soil – three-dimensional CSIA for sourcing of PAHs

As part of the isoSoil goal to merge data from several isotope systems, so called multi-dimensional CSIA, we analyzed PAHs from Czech Republic top soil for the stable isotopes of hydrogen and carbon, and for radiocarbon. The results, forming one of the largest ^{14}C-PAH datasets ever produced, constrain quantitatively that 80-90% of PAHs in this central European region stem from fossil fuel combustion and that only 10-20% comes from biomass burning. These numbers can be directly translated into a top-down estimate of the overall fossil vs. biomass combustion in the region. By using the added resolving power of the stable isotopes, it might be possible to differentiate the sources of the fossil/biomass compartments (e.g. fossil sources like diesel, gasoline, coal, natural gas). Such interpretations will be facilitated by our newly developed methods for Monte Carlo simulations of endmember source distributions vs. the distributions found in the results from sample analysis.

Swiss soil – two-dimensional CSIA to investigate the fate of dinitrotoluenes

Analysis of 2,4-DNT in a soil profile at the contaminated site revealed a characteristic trend in $\delta^{15}N$ vs. $\delta^{13}C$-values with isotope fractionation of 10 and 5‰, for N and C, respectively (Figure 4B). The observed trend is indicative for biodegradation via initial dioxygenation because bonds to C and N are weakened during dioxygen attack at the aromatic ring (Figure 4A). From the historic analysis of explosives production at the contaminated site, a tentative degradation rate could be estimated that corresponded to a half-life of approx. 60 to 80 years. C isotope fractionation of 2,6-DNT, the other contaminant selected in the project, also suggests some form of oxidative transformation. This pathway, however, is not yet fully explored for mechanistic interpretations.

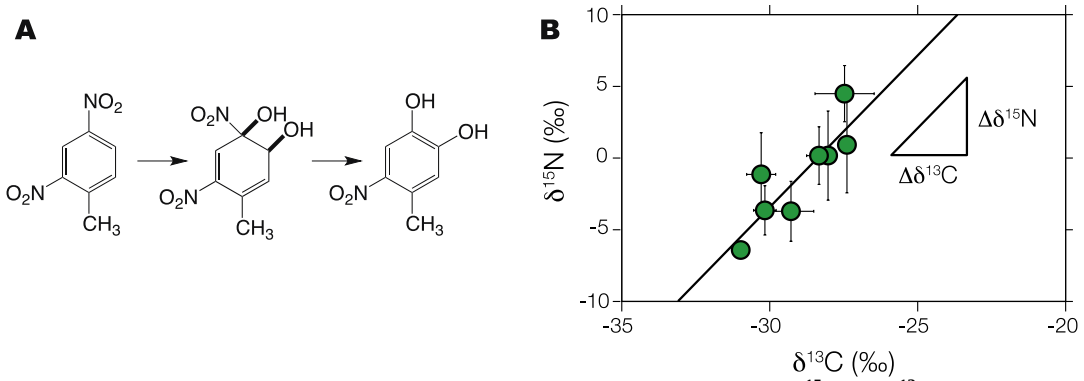

Figure 4. A: Initial reaction steps of 2,4-DNT dioxygenation mechanism; B: $\delta^{15}N$ vs. $\delta^{13}C$ trends of 2,4-DNT during oxidative biodegradation.

A complete characterization of explosives biodegradation by CSIA bears potential to be applied at contaminated sites. Partner 13 – Eawag intends to inform environmental protection agencies and related authorities as well as environmental consultants via established publications of their institution (Eawag news) and offer measurements once the methods and their interpretation are fully established.

Spolana site – two-dimensional CSIA to investigate degradation of hexachlorocyclohexanes in soil

Compound-specific $\delta^{13}C$ value determinations of hexachlorocyclohexanes in soils from the Spolana site revealed significant degradation for α-,β-,δ- and γ-HCHs throughout the site, with the highest proportion of HCHs degraded at the likely source of contamination (Figure 5).

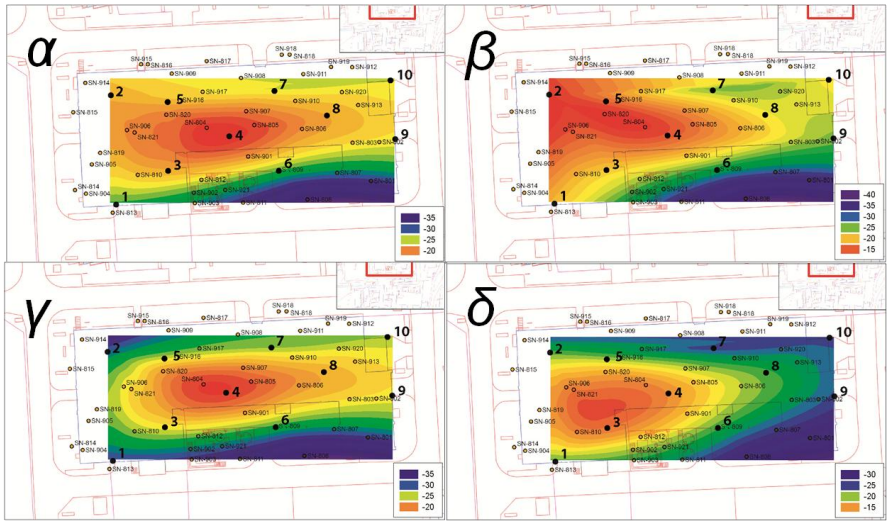

Figure 5. Spatially resolved δ13C values determined for Spolana HCHs.

SAP site – two-dimensional CSIA to investigate degradation of chlorinated solvents in groundwater

$\delta^{13}C$ and $\delta^{37}Cl$ values for trichloroethylene (TCE) and perchloroethylene (PCE) in groundwater samples from the SAP site have been successfully determined, and are shown along with CE concentration data in Figure 6 and Figure 7.

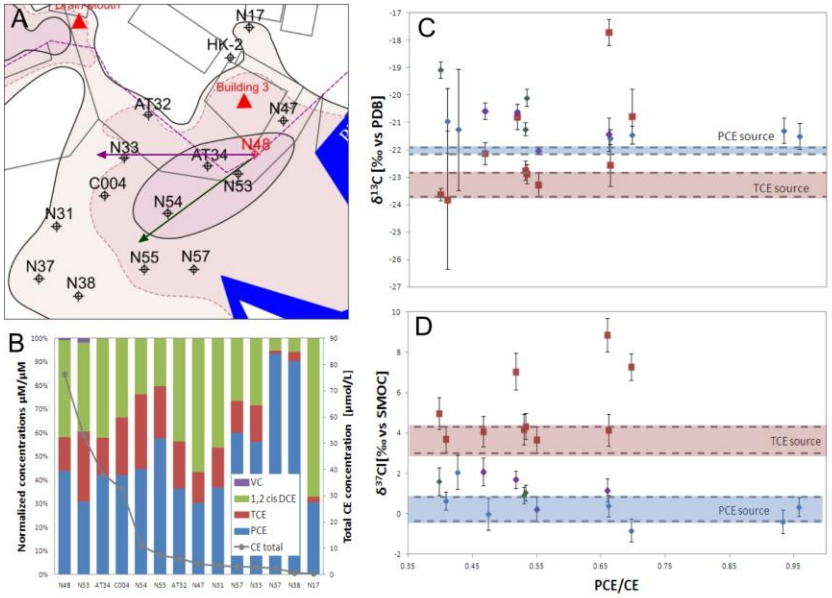

Figure 6. A: The investigated area; B: Concentrations of CE at the monitored wells. The gray dots represent the sum of the CE concentrations; C: Stable carbon isotope signatures; D: Stable chlorine isotope signatures for PCE (blue lozenges) and TCE (red squares). The transect with highest degradation are indicated in green and purple with arrows on the map and colored dots on both the $\delta^{13}C$ and $\delta^{37}Cl$ plots

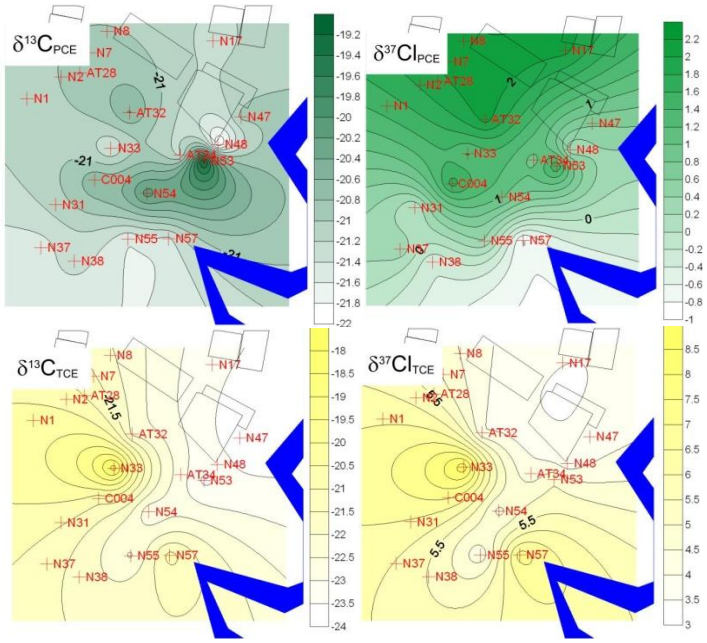

Figure 7. Spatial distribution of stable carbon and chlorine isotope signatures

Both C and Cl isotopes showed evidence of ongoing PCE transformation ($\delta^{13}C$ values -22.0 ± 0.1 to -19.1 ± 0.3‰; $\delta^{37}Cl$ values 0.23 ± 0.61‰ to 2.08 ± 0.68‰). Applying experimental carbon isotope fractionation factors (ε_C) yielded an estimated extent of PCE degradation from 14 to 78%, pointing to the existence of zones with high biodegradation activity. Furthermore, the 2D isotope framework allowed estimation of chlorine isotope enrichment factors ε_{Cl} (-6.2 to -0.8‰) and the determination of characteristic $\varepsilon_{Cl}/\varepsilon_C$ values for reductive PCE dechlorination at this field site (0.42-1.12). Last, $\delta^{37}Cl$ shifts correctly predicted a primary TCE contamination.

Overall, this investigation demonstrates that the new rapid analytical method for $\delta^{37}Cl$ determination of chlorinated compounds allows for the adoption of a dual-isotope approach, unraveling sources and extents of in-situ degradation of common groundwater contaminants such as CEs.

4.1.3.9 Computational chemistry – calculating the kinetic isotope effects (KIEs) of reactions and establishing a database for KIEs

The KIEs of reactions can be determined experimentally, as in the microbial and abiotic experiments of isoSoil, for use in field applications and science in general. However, deeper insights can be obtained by modeling the KIEs from known parameters. Furthermore, some applications of CSIA may not have the necessary empiric foundation based on experiments, and may therefore benefit from *in-silico* computed KIEs.

The QSIFR approach links information regarding the mechanism, rate-limiting steps, and transition state-structures of a particular reactant in a (bio)chemical reaction to structurally similar compounds. It should be noted that frequently the full analysis up to the transition state structure might not be important, e.g., it might be sufficient to identify the general type of the reaction that the pollutant underwent at the contamination spot. Such cases might be considered qualitative applications of the QSIFR approach, thus QSIFR in general may be considered either qualitative or quantitative depending on its application. Deliverable D7.2 lists the basic equations and common simplifications.

Two data bases have been prepared, both searchable through the isoSoil web interface. The smaller one supplements the QSIFR program and contains data gathered for the pollutants of interest to isoSoil project which isotopic fate can be modeled using designed software. These include reactants on TCE degradation pathway, nitroaromatics, HCHs, and PAHs. The larger database is wider and contains over 50 pollutants with over 800 individual values of isotopic fractionation. This data base is planned to be further augmented with aim to eventually include all measured isotopic data connected with isotopic fractionation in physical, chemical, geochemical, biochemical and environmental processes. While majority of the data is retrieved from published resources, several systems have been modeled theoretically for the purpose of the project using the M05-2X DFT method expressed in the 6-31+G(d,p) basis set. This theory level has been chosen upon benchmarking a number of theory levels against experimental enthalpy of activation and isotopic fractionation of the reaction between TCE and permanganate.

4.1.3.10 isoSoil software – linking reactions and KIEs through a theoretical framework

A theoretical framework was needed to link the KIEs to the specific cases (actual reactions) to be studied, since the KIE is a general parameter. The theoretical framework is based on general equations for the evaluating isotopologue kinetics and isotope fractionation, which can be applied to any isotope system at very different degrees of kinetic complexity. In this approach, every isotopologue and isotopomer of an isotopic system is considered individually. Measured isotope signatures can be evaluated for bulk compound enrichment factors, and apparent kinetic isotope effects (and vice versa) according to the state-of-the art in environmental chemistry. In

addition, the chosen framework enables one to integrate information of position-specific isotope effects as obtained from the calculation of intrinsic KIEs by computational methods. Deliverable 7.1 provides the full background of isotopolog/isotopomer-specific equations and how isotope-related parameters are deduced.

4.1.3.11 isoSoil software – the kinetics simulator

The Kinetics Simulations module performs the calculations linking field and experimental data to KIEs, e.g. deriving a KIE from experimental data or using an existing KIE to estimate the progress of a reaction (i.e. the fraction remaining of a pollutant at a field site). Several concepts were tried out to reach the most efficient solution for the software. In spite of the fact that stochastic approach appears to be the best available, it failed in some cases, especially when solving chemical kinetics schemes where oscillations of concentrations of species at the very beginning of reaction progress are strongly undesirable. Such fluctuations have a large effect on the stability and reliability of the algorithm when solving for the reaction progress. In general, the accuracy of stochastic methods depends on the number of species used and can be improved that way especially for low values of reaction progress - but the computer power required in such a case is beyond the reach of well-equipped workstations or even servers. Therefore, the implemented algorithm connects both numerical (Runge-Kutta fourth order integration technique) and stochastic approaches as a consensus between accuracy, efficiency and limitations of both methods. This unique combination ensures that all species are still "counted" in the system, which is extremely important for kinetic isotope effects while all instabilities in low range of concentrations are no longer encountered.

4.1.3.12 isoSoil software – statistical optimization of sampling

The Site Preparation and Statistical Analysis module is an aid to plan the sampling for a CSIA campaign. Several components are non-conventional, providing simple, efficient and highly transparent statistical analysis of the sampling grid vs. quality requirements (e.g. the risk of not detecting a contaminant hotspot at a specific grid size). The module also contains the conventional tools, e.g. Kriging analysis. A back-end interpretor has been included for use of customized R code.

The following functionalities are implemented:

I. Site Preparation functionalities:
   1. Sampling project management
   2. Interface to Geo localization services and mapping solutions (Google maps used). The current (publically available map of all sampling fields is at URL address: http://app.isosoil.eu/Views/ProjectsMap.aspx
   3. Three methods for sampling strategies:
      a. Automated grid drawing
      b.Automated grid generation
      c. Unrestricted sampling (including data import from external files)
   4. Grid and sampling radius visualization
   5. Editing the sampling data (multiple layers allowed)

II. Statistical analysis:
   1. Gaussian 3D presentation of Sampling values
   2. Statistical Summaries (Five-Number Summary: The Sample Minimum, The First Quartile, The Median, The Mean, The Third Quartile, The Sample Maximum)
   3. Statistical Bubble Plot
   4. Kriging Interpolation
   5. Trend surfaces analysis (order 1 to 6)
   6. Normality tests
   7. Ability to define and perform Miscellaneous & custom calculations via easy to use interface to R statistical package

4.1.3.13 isoSoil software – tying it all together: the web interface

Four fundamental modules are comprised in the isoSoil software: the database, the QSIFR calculator, the kinetics simulator, and the statistical analysis. For all of the modules we have successfully designed and implemented both the computing (server-side) and visualizing (client-side) software modules. First and foremost the software implements a graphical, web-oriented interface to all the modules or algorithms as illustrated in the main software menu (Figure 8).

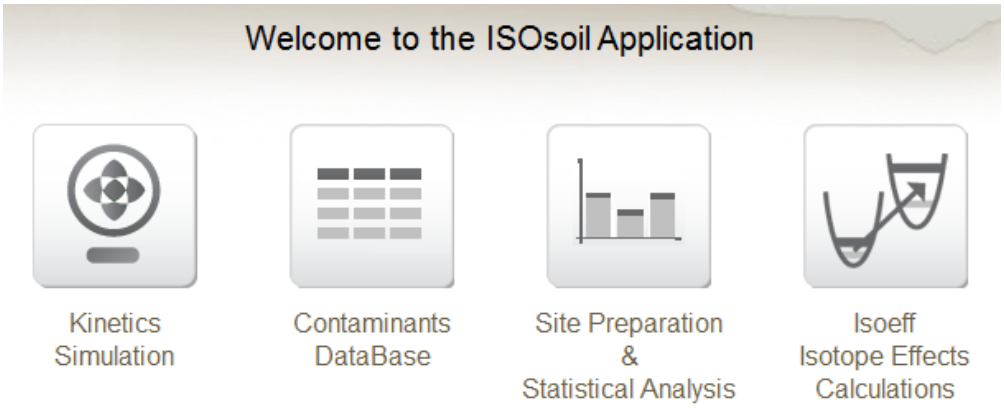

Figure 8. The main software meny with links to the four modules.

The software has been implemented as web application. The client side technology is Adobe Flex, the server side technology is Microsoft .NET. The system runs out of isoSoil server at MakoLab Data Center in Lodz, Poland. The access URL address of the system is: http://app.isosoil.eu/

4.1.3.14 Demonstrating the isoSoil concept and disseminating the foreground

All of the results and activities have been presented at conferences and other fora, both for the scientific and the more applied communities. More information can be found in the Periodic Report (Mo. 19-36). Several direct contacts have been made between the isoSoil consortium and agencies, institutes and companies. Clearly, there is a growing interest in CSIA and the isoSoil software. Future work should aim at providing the baseline services for CSIA, as the actual

analyses currently are difficult to purchase for the end users. One or two commercial enterprises exist that perform such tasks, but even they are in the development phase for e.g. $\delta^{37}Cl$ determinations, and $\delta^{2}H$ analyses of chlorinated solvents are not yet available anywhere. Furthermore, there are still very few experts available to interpret the data. Thus, the foreground of isoSoil (analytical methods, standard materials, user friendly software) has the potential to facilitate the development of that market.

The isoSoil project ended with two distinct demonstration/dissemination activities:

- webinars were given at two occasions to present and showcase the isoSoil software
- a field demonstration was launched at the SAP site to prove the completed isoSoil concept

Whereas the webinars were successful, and will be made available on the isoSoil software, the field demonstration was delayed due to complications in the software production. Samples have been taken and the analyses are completed. The results will be interpreted and published in a scientific journal together with the original data from the SAP site.

To conclude, isoSoil is now entering a post-project stage and is peaking in terms of productivity. Most experiments have just recently generated the desired isotope data, which will be interpreted and published during the next one or two years to come.

4.1.4 Potential impact, dissemination activities and exploitation of results

4.1.4.1 Potential impact

isoSoil has involved a consortium of both academia and enterprises to anchor the concept of CSIA in as many communities as possible, on a pan-European scale. This has not only been favourable for the dissemination activities, but also to increase the awareness of and interactions with other constellations that are active within CSIA (scientific and commercial networks). Several partners are now involved in new projects to further the knowledge and development of CSIA for environmental and chemical studies.

The development of analytical techniques for CSIA of several isotope systems, together with the generated data and the isoSoil software, has contributed to the possibilities of achieving precise and reliable site characterisation and monitoring design of contaminated land. First signs have already been seen for implementation of some of the isoSoil outcomes by commercial (e.g. for online determination of $\delta^{37}Cl$) and academic (e.g. use of the database for KIEs and the interpretation software) entities.

It remains to be seen how far the isoSoil foreground will be applied to field investigations, but the potential is there to improve the effectiveness of remediation and mitigation by using CSIA. We believe that the isoSoil "toolbox" is a step in that direction. A few brief points illustrate this:

- Analytical methods have been developed for the historically challenging measurements of $\delta^{37}Cl$ and $\delta^{2}H$ in organochlorine compounds
- Microbial experiments have been performed to generate the necessary baseline data for putting CSIA of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{37}Cl$ to work in field applications of contaminated soil and groundwater
- Abiotic experiments have been carried out to better be able to characterize and understand biotransformation reactions of the selected contaminants
- Multi-dimensional CSIA has been applied in several independent field studies, showing the increased resolution of this approach compared to the conventional single-dimensional studies
- One of the few field studies deploying 3-dimensional CSIA has been conducted within isoSoil
- A software package has been produced, offering extensive capabilities for planning CSIA sampling, interpreting data and accessing isotope fractionation factors, thus facilitating CSIA use by experts and non-experts
- The concept of CSIA has been presented and introduced to a wide range of audiences involved in remediation and/or monitoring of contaminated land and groundwater

This, combined with the extensive publishing of results that has just started, forms the foundation of the legacy of isoSoil.

4.1.4.2 Dissemination activities

The isoSoil community has been active in reaching out to both the scientific community and potential end-users, both inside and outside the EU with >50 presentations/contacts made. The highest success rate is within the scientific community, where CSIA is currently being explored and developed at high speed. Especially the developments in analytical methods have attracted interest, e.g. the online method for $\delta^{37}Cl$ determinations in organic compounds using gas-chromatography quadrupole mass spectrometry (Aeppli et al., Analytical Chemistry, 2010). The software package has also attracted interest, as shown by the webinar attendance list and communications at meetings (e.g. within the Marie Curie ITN project CSI:Environment).

Presentations have been made at e.g. environmental consultant firms based on contacts made, but agencies and institutes are easier to access. This stems from the novel character of CSIA; although it is widely recognized that CSIA holds great potential for investigations of polluted soil and water, the lack of available analytical services holds back the realization of this potential. We note that several enterprises have inquired about the $\delta^{37}Cl$ online method and that the commercially available services may expand rapidly. In this perspective, $\delta^{2}H$ and $\delta^{13}C$ analysis has reached a more mature state and are easier to purchase for non-analytical entities.

4.1.4.3 Exploitation of results

Most of the isoSoil foreground will not be exploited *per se*, but it will be put to use by the consortium and, potentially, other users within the academia and commercial sector. All foreground except the code for the isoSoil software and some chemical standard materials will be disseminated through scientific publications.

The exploitable foreground pertaining to the software and standard materials is as follows:

- The statistical code in R (Anders Jönsson, IVL, and Mirek Sopek, MLAB) is used to optimize sampling for contaminant analysis. The code is executed through the web interface produced by partner 11 - MLAB, using a back-end translator from R to code that is amenable in the applications. These components are already implemented and may be commercialized by Jönsson/Sopek at will.

- The software for kinetics simulations is owned by partner FQS, Poland, and is currently implemented in the webinterface by partner MLAB. The kinetics simulations engine, as a standalone component, is the property of FQS and may be commercialized either as a part of the isoSoil software package or as one component of another framework.

- The chemical standards for $\delta^{37}Cl$ measurements are used in the method by partner SU (Aeppli et al., 2010). This standard may be shared with academic entities or sold to commercial entities. Methods developed to produce new standards may be used by SU or disseminated to others to facilitate the $\delta^{37}Cl$ analysis. Further research is needed to develop the methods for production of new standards, and this may be performed during 2012-2013 by partner SU.

4.1.5 Contact details

The project website will be running for the duration of 2012: www.isoSoil.eu

Software tools can be tried for free at: app.isoSoil.eu (contact Mirek Sopek at sopekmir@makolab.pl for login credentials)

Further information can be obtained by e-mailing to the isoSoil coordinator group at Stockholm University, Sweden:

Charline Wiegert, administrative manager - charline.wiegert@itm.su.se
Dr. Henry Holmstrand, scientific manager - henry.holmstrand@itm.su.se
Prof. Örjan Gustafsson, project coordinator - orjan.gustafsson@itm.su.se