

Comparison of the biodegradation of pharmaceuticals and biocides in water and soil systems

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Summary

The fate of organic chemicals in the environment is determined by both abiotic and biological processes and microbial degradation of chemicals is a key parameter for their environmental risk assessment. The majority of chemicals have been tested for ready biodegradability in aqueous systems (e.g. OECD 301/310 test) for regulatory purposes, whereas only few data exist for other environmental systems such as soil. This lack of data is mainly due to the high cost and complexity of the necessary simulation tests. Thus, it would be advantageous to extrapolate the biodegradability potential of chemicals from aqueous medium to soil.

Hence, we compared the fate of different environmentally relevant chemicals. The worldwide most applied herbicide, 2,4-D, and two environmentally relevant pharmaceuticals, the non-steroidal anti-inflammatory ibuprofen and the antibiotic ciprofloxacin were analysed as model compounds for their turnover in water and soil systems. Isotope labelled compounds (^{13}C , ^{14}C) were incubated in mineral medium (OECD test 301) and in an agricultural soil (OECD test 307). The results revealed the processes responsible for compound biodegradation including biotic and abiotic processes. The carbon redistribution into mineralisation, biomass and non-extractable residues (NER) formation during degradation was traced, allowing to establish a quantitative relationship between the degradation in the two systems. Moreover, to elucidate the potential effects of these compounds on the environment, those compounds that proved to be toxic to activated sludge microbial communities, were also tested for their toxicity towards soil microorganisms.

In the aqueous system, 85% of the initially applied ^{14}C -2,4-D and 68% of the ^{13}C -ibuprofen were mineralised within 28 days, indicating ready biodegradability. In soil, only 57% of 2,4-D and 45% of ibuprofen were mineralised. Parent compounds and metabolites decreased to < 2 % of the spiked amounts. In soil, 37% of the initially applied labelled 2,4-D and 30% of ibuprofen were recovered as NER, mainly in the form of biomolecules, e.g. amino acids and phospholipid fatty acids. In contrast, ciprofloxacin was recalcitrant to degradation and transformation in water systems. In soil, however, a

low but significant mineralisation was observed. The lower bioavailability of antibiotics in soil seems to reduce the compound's toxicity allowing its biodegradation. NER formation from ciprofloxacin was fast and independent of the microbial activity. Overall, the data suggest that NER formation from abiotic processes (e.g. sequestration of parent compounds) and from biogenic residues are competitive processes in soil.

Whereas based on their ready biodegradability and the high contribution of biomass residues to NER formation, 2,4-D and ibuprofen obviously are not hazardous for the environment; the data clearly demonstrated that ciprofloxacin is persistent, and strongly inhibits the microbial activity in the environment, e.g. activated sludge and soil bacterial communities. Thus, this compound is a hazardous pollutant for the environment and the ecosystem, and consequently much more attention needs to be given to contamination of soil by antibiotics, which often has been neglected.

In order to generate consistent data and provide a validated assessment of the environmental risk of a chemical, biodegradation tests in soil using compounds isotopically labelled in the most stable(s) position(s) of the molecule should be performed. In addition, the generally accepted concept of NER and the methodology for their determination need to be revised with respect to distinguishing the non-biogenic (potentially hazardous) and the biogenic (harmless) NER.

Nevertheless, simulation tests cannot always be implemented. For these cases some general rules for extrapolating results from water-based ready biodegradability tests to the biodegradation in soil systems can be deduced from the results of this study: i) mineralisation is higher in water than in soil for readily biodegradable and non-toxic compounds, ii) for compounds which are highly toxic towards microorganisms, the mineralisation and metabolisation is higher in soil systems because the reduced bioavailability in soil reduces their toxicity iii) lipophilic compounds tend to form NER and are less biodegraded in soil than in aqueous systems, iv) compound elimination with low mineralisation indicates formation of potentially hazardous NER, and v) high mineralisation accompanied by microbial biomass growth generally results in the formation of non-hazardous biogenic NER.

Zusammenfassung

Das Schicksal organischer Verbindungen in unserer Umwelt wird sowohl durch abiotische als auch biologische Prozesse (mikrobieller Abbau) bestimmt. Der biologische Abbau von Chemikalien stellt dabei einen Schlüsselparameter für die Abschätzung des Umweltrisikos dieser Chemikalien dar. Bisher erfolgten Untersuchungen auf rasche biologische Abbaubarkeit von Chemikalien auf regulatorischer Ebene zumeist in wässrigen Systemen (z.B. OECD-Testserie 301/310). Für andere Umweltsysteme, wie Böden, existieren hingegen nur wenige Daten. Die Ursachen hierfür liegen hauptsächlich in den hohen Kosten und der Komplexität der nötigen Simulationstests. Es wäre deshalb von großem Nutzen, die Ergebnisse der Untersuchungen zum Potential der biologischen Abbaubarkeit von Chemikalien in wässrigem Medium auf den Boden übertragen zu können.

Daher war es das Ziel der vorliegenden Arbeit das Abbauverhalten verschiedener Umweltchemikalien miteinander zu vergleichen. Modellhaft wurden das weltweit am häufigsten applizierte Herbizid, 2,4-Dichlorphenoxyessigsäure (2,4-D), und zwei umweltrelevante Pharmazeutika, das nicht-steroidale entzündungshemmende Ibuprofen und das Antibiotikum Ciprofloxacin, untersucht. Die isotopenmarkierten Verbindungen (^{13}C , ^{14}C) wurden in Mineralmedium (OECD-Test 301) und in einem landwirtschaftlich genutzten Boden (OECD-Test 307) inkubiert. Die Ergebnisse zeigten, welche Prozesse für den biotischen und den abiotischen Abbau der Verbindungen verantwortlich sind. Die Verteilung des Kohlenstoffs während des Abbaus auf die Mineralisierung sowie die Bildung von Biomasse und nicht-extrahierbaren Rückständen (NER) wurde im Detail verfolgt. Dies ermöglichte es, eine quantitative Beziehung zwischen dem Abbau in den beiden Systemen herzustellen. Um mögliche Effekte dieser Verbindungen auf die Umwelt zu erfassen, wurde für diejenigen Stoffe, die sich als toxisch für die Mikroorganismen im Belebtschlamm erwiesen, auch die Toxizität gegenüber Bodenmikroorganismen getestet.

Im wässrigen System wurden 85% des ursprünglich applizierten $^{14}\text{C}_6$ -2,4-D und 68% des $^{13}\text{C}_6$ -Ibuprofen innerhalb von 28 Tagen mineralisiert, was auf leichte biologische Abbaubarkeit schließen läßt. Im Boden wurden nur 57% des 2,4-D und 45% des Ibuprofens mineralisiert. Der Anteil der noch nicht metabolisierten Ausgangsverbindungen und der Stoffwechselprodukte verringerte sich auf < 2% der anfänglich zugegebenen Menge. 39% des zu Anfang in den Boden eingebrachten markierten 2,4-D und 32% des markierten Ibuprofens wurden als NER, hauptsächlich in Form von Biomolekülen, wiedergefunden. Im Gegensatz dazu war Ciprofloxacin im wässrigen System rezistent gegenüber Abbau und Umsetzung. Im Boden konnte jedoch eine geringe, aber signifikante Mineralisierung beobachtet werden. Die geringere Bioverfügbarkeit des Ciprofloxacins im Boden scheint dessen toxische Wirkung zu vermindern und so einen biologischen Abbau zu ermöglichen. Die Bildung von NER aus dieser Verbindung erfolgte schnell und unabhängig von der mikrobiellen Aktivität. Insgesamt zeigte sich, dass die Bildung von NER durch abiotische Vorgänge (z.B. Sequestrierung der Ausgangsverbindungen) und durch biogene Rückstände miteinander konkurrierende Prozesse im Boden darstellen.

Während 2,4-D und Ibuprofen aufgrund ihrer guten Abbaubarkeit und des hohen Anteils von Biomasserückständen in den NER offenbar wenig umweltgefährdend sind, zeigten die Daten deutlich, dass Ciprofloxacin persistent ist und die mikrobielle Aktivität, z.B. von Bakteriengemeinschaften in Belebtschlamm und im Boden, erheblich inhibiert. Infolgedessen stellt letztere Verbindung einen umweltgefährlichen Schadstoff dar. Dementsprechend sollte der Kontamination von Böden mit Antibiotika mehr Aufmerksamkeit zuteil werden als es bisher der Fall war.

Um einheitliche Daten zu gewinnen und eine validierte Abschätzung zum Umweltrisiko einer Chemikalie erbringen zu können, sollten Tests zur Bioabbaubarkeit in Böden mit Hilfe von Verbindungen, die an der stabilsten Position bzw. den stabilsten Positionen im Molekül durch Isotopen markiert sind, Anwendung finden. Des Weiteren bedürfen das gängige Verständnis von NER und die Methodik zu deren Bestimmung einer Überarbeitung in Bezug auf die Unterscheidung von nicht-biogenen (potentiell umweltgefährdenden) und biogenen (nicht schädlichen) NER.

Nichtsdestotrotz können Simulationstests nicht immer realisiert werden. Für diese Fälle konnten folgende generelle Richtlinien zur Anwendung von Testergebnissen zur leichten biologischen Abbaubarkeit in wässrigem Medium auf den biologischen Abbau in Bodensystemen aus den im Rahmen dieser Arbeit erhobenen Daten abgeleitet werden: i) die Mineralisierung für leicht bioabbaubare und nicht-toxische Verbindungen ist in Wasser stärker als im Boden, ii) für Verbindungen, die für Mikroorganismen stark toxisch sind, gilt, dass die Mineralisierung und Metabolisierung in Bodensystemen stärker ist, da die geringere Bioverfügbarkeit ihre Toxizität herabsetzt, iii) lipophile Verbindungen tendieren zur NER Bildung und werden im Boden weniger stark biologisch abgebaut als in wässrigen Systemen, iv) eine Elimination der Verbindungen, die mit einer geringen Mineralisierung einhergeht, weist auf die Bildung potentiell umweltgefährdender NER hin und v) eine starke Mineralisierung im Zusammenhang mit einem Anstieg der mikrobiellen Biomasse führt in der Regel zur Bildung von unschädlichen biogenen NER.

Introduction

1.1 Registration and environmental risk assessment of chemicals

Environmental sustainability is one of the main aspects in the conception of modern societies. Significant contributions to the identification, assessment and management of chemical stressors with legal outcomes have been made. In Europe, regulatory frameworks were established, e.g. the European Water Framework Directive, the European Soil Framework Directive, and recently, the European Regulation for Registration, Evaluation, Authorisation and Restriction of chemicals (REACH; Schäffer et al., 2009). Therefore, according to the actual European legislation, chemicals have to be tested to pass the registration system (REACH), the OECD Guidance for Industry Data Submissions on Plant Protection Products and their Active Substances (OECD, 2005) or environmental risk assessments (ERA) based on the European Medicines Agency (EMA) guideline 2006 for medicinal products (EMA, 2006) for a marketing authorisation.

These Environmental risk assessment guidelines e.g. for pharmaceuticals and pesticides, are based on a tiered system (EMA 2006; EEC, 2009), where the exposure of the environment to the compound is determined by predicted environmental concentrations that are calculated and threshold values determine if further investigation is required. When these values are exceeded, a second phase takes place, where information about the fate and effects in the environment is obtained and assessed. Under this step, toxicity, degradability and persistence of the substance and/or relevant metabolites are investigated. To this aim, ready biodegradability tests (screening test such as OECD 301 and 310 tests; OECD 1992, 2006) are conducted to estimate the fate of the substance in

waste water treatment plants (WWTP). Moreover, if a substance is not readily biodegraded and proved to potentially adsorb to sludge (high K_{oc}) it may reach the terrestrial environment with land spreading of sewage sludge, therefore also a fate and risk assessment in the soil environment should be conducted. To this aim, simulation tests for biodegradation (e.g. OECD 307) and toxicity tests are performed (EMEA, 2006).

Consequently, data on biodegradation is an essential part of this evaluation since this is one of the most important processes driving the persistence of chemicals in the environment. Standardised screening tests are used by industry, authorities and scientific institutions as mentioned above to characterise the degradation of chemicals in the environment. The OECD 301 guideline for ready biodegradability (OECD, 1992) is the most important group of internationally used screening tests for biodegradation. These tests are performed under aerobic conditions in liquid systems (test compound in growth medium inoculated with sewage sludge bacteria) and are considered to allow general predictions of the biodegradation behaviour of organic chemicals in both aquatic and terrestrial compartments (Guhl and Steber, 2006). If a compound is biodegraded to a higher extent than 60% of the initial amount within a 10-days window, it is classified as readily biodegradable. Ready biodegradability tests are stringent in providing a limited opportunity for biodegradation and therefore a positive result in such a test may indicate that the tested compound is biodegraded easily in the environment (De Bruin and Struijs, 1997). However, the implications of these tests for terrestrial ecosystems are limited and rather weak (Howard and Banerjee, 1984, Dörfler et al., 1996). As above described, simulation tests as the OECD 307 for soil (OECD, 2002) are used when more information about the compound's fate in soil is required, e.g. when the 301 test (OECD, 1992) pass level for ready biodegradability (60 % degradation in a 10-days window within the 28-days period of the test) was not fulfilled. This type of test provides more realistic estimates of biodegradation and fate of a chemical in this compartment.

1.2 Degradation of chemicals in the environment

Physicochemical properties like molecular structure, polarity, aqueous solubility, hydrophobicity (determined by the octanol-water partition coefficient, K_{ow}), lipophilicity

and volatility are controlling the behaviour of contaminants in the environment (Jones et al., 1996; Reid et al., 2000). In the environment, the pollutants are subjected to both abiotic and biotic degradation processes.

1.2.1 Abiotic degradation

The contribution of abiotic reactions on the transformation of chemicals in the environment is an important issue that has to be taken into consideration. In many cases, abiotic and biotic processes are complementary. For example for some herbicides, the first degradation step is an abiotic hydrolysis reaction, which is followed by microbial degradation of the produced intermediate (Neilson and Allard, 2008). The most important abiotic reactions are photochemical transformations and chemically mediated transformations as hydrolysis, oxidation and reduction. Photodegradation does occur in aquatic systems and also in terrestrial areas, mainly on the soil surface, e.g. mediated by humic matter (Zepp et al., 1981a,b). During hydrolysis, H^+ or OH^- originating from the dissociation of water attack the organic substrate, breaking an existent bond and forming a new one with the latter. Hydrolysis in the soil aqueous phase may be pH-moderated or pH-independent (Macalady et al., 1988; Yaron et al., 1996).

Abiotic degradation processes can be also catalysed by the surface of soil solid phase. Catalysis can be accomplished by a component of the surface or due to a third adsorbed species. For example, clay surfaces can catalyze many transformations, including hydrolysis, redox reactions, oligo- and polymerization, rearrangements, etc. Not only the type of the clay, but also the type of counter cation are important in such processes (Yaron et al., 1996).

Organic matter can catalyse chemical abiotic transformations, due to the presence of many reactive groups, which can enhance chemical changes in several organic substances, the strong reducing capacity of humic substances, and the presence of moderately stable free radicals in the fulvic acid and humic acid fractions (Stevenson, 1994; Yaron et al., 1996). Hydrolysis, solubilisation, and photosynthesis are catalysed processes that have been reported (Senesi and Chen, 1989). Moreover, metal oxides also have catalytic properties (Huang, 1990; Ruggiero et al., 1996). Besides, many chemical transformations can be initiated because soil surfaces are charged; the electrical field at

these surfaces can polarize or even dissociate solutes and solvents, and moreover it produces a concentration gradient of charged substances, which then in concentrated form may successfully react with organic solutes (Wolfe, 1990; Yaron et al., 1996).

1.2.2 Biodegradation

It is generally conceded that biotic reactions driven by microorganisms are of major significance for the fate and persistence of a pollutant in aquatic and terrestrial ecosystems (Neilson and Allard, 2008).

It is important to make a clear distinction between biodegradation and biotransformation. Aerobic biodegradation involves the breakdown of molecules either by biotransformation (primary biodegradation) into less complex metabolites or by mineralization (ultimate biodegradation) into inorganic chemicals (H_2O , CO_2). The extent and rate of biodegradation depend on many factors like O_2 , pH, temperature, moisture level or better water activity, microbial population, degree of adaptation, accessibility of nutrients, chemical structure of the compound, cellular transport properties, and chemical portioning in the growth medium (Leahy and Colwell, 1990; Singh and Ward, 2004). Biotransformation, in contrast involves only a restricted number of metabolic reactions, and the basic core of the molecule remains essentially intact.

It is important to consider however, that xenobiotics are not always degraded entirely to CO_2 because the microorganisms must channel a portion into the biosynthesis of essential molecules (anabolism) to enable growth and cell division. Moreover, many organisms degrade xenobiotics only in the presence of a suitable readily biodegradable molecule that supplies cell carbon and the energy for growth, known as co-metabolism (Neilson and Allard, 2008). Cometabolism is a fortuitous transformation of a molecule by an enzyme synthesised for another purpose (Hatzinger and Kelsey, 2005). Although cometabolism can lead to the accumulation of persistent intermediates, in many cases, the initial oxidative reaction produces readily degradable compounds that are then mineralised.

Microorganisms responsible for biodegradation may be divided in two major categories, based on their abilities to live under different environmental conditions. Oligotrophs are active under low concentrations of organic carbon and may be inhibited by high carbon

concentrations. Eutrophs on the contrary, are microorganisms that proliferate under high carbon concentrations and may be inhibited at low concentrations (Howard and Banerjee, 1984). Another classification, distinguish the r-strategist (or r-selected) that grow very quickly on simple and soluble substrates depending on the availability of their substrates, and K-strategists, that grow at slow rates on complex substrates (polymerised compounds). K-populations are expected to allocate more energy to extracellular enzyme production and defence from predation than to growth (Pepper et al., 1996; Fontaine et al. 2003).

Nevertheless, prior to the biodegradation of many compounds, a period of adaptation (lag phase), where the destruction of the chemical is not evident normally occurs. Different mechanisms have been proposed to explain this period. The explanations are related to, adaptation to new conditions, enzyme synthesis, proliferation of small populations, presence of toxicity, predation by protozoa (especially relevant in aqueous systems), appearance of new genotypes and diauxie (Alexander, 1994).

At high concentrations a compound can be degraded as a primary substrate with accompanying exponential growth of microorganisms, resulting in a sigmoid biodegradation curve that can be described by Monod kinetics. However, at low concentrations in the environment, where natural carbon substrates are degraded simultaneously, the compound cannot be used as a primary substrate and thus cannot support growth. In this case, biodegradation follows zero order kinetics (Alexander 1994; Ahtiainen et al. 2003).

As previously mentioned, toxicity is one of the main factors governing biodegradation. The concentration of toxic substances may preclude the microbial proliferation and metabolism. However, toxicity can be reduced by cell detoxification processes (e.g. efflux pumps), by synergism, when a second species is able to degrade the toxic compound that is inhibiting the first one (Alexander, 1994; Welp and Brümmer, 1999), by adaptation to the chemical e.g. induced antibiotic resistance and by sorption to solid surfaces.

Microbial populations degrading synthetic chemicals are subjected to a variety of physical, chemical, and biological environmental factors that influence their growth and activity. Therefore, the different environmental properties and characteristics have a

tremendous impact on the inhabitant populations, the rate of biochemical transformations and the identities and persistence of products of biodegradation. Moreover, it is not justified to assume that a compound that is degraded in one environment is going to be similarly transformed in another (Alexander, 1994).

1.2.2.1 Biodegradation in aqueous systems

In general, biodegradation in water systems results in the formation of biomass, metabolites and CO₂. Based on high bioavailability of the compound, the mass transfer from the compound to the cell is not significantly restricted in aquatic systems. Therefore, if the abiotic factors (temperature, O₂, pH, water activity, salinity, and nutrients) are not limiting, biodegradation is mostly dependant on the presence of degraders, the concentration of the compound and its toxicity (Katayama et al., 2010).

1.2.2.2 The soil environment

McBride (1994) stated “much of soil science is empirical rather than theoretical in practice. This fact is a result of the extreme complexity and heterogeneity of soils, which are impossible to fully describe or quantify by simple chemical or physical models”. Soils are natural bodies, whose lateral and vertical boundaries usually occur as gradients between mixtures of materials of atmospheric, geologic, aquatic, and/or biotic origin. These open systems are subjected to fluxes of energy (e.g., sunlight, wind) and matter (e.g., aqueous precipitation, erosion, deposition, and inputs of organic compounds from plants, other organisms like microorganisms and animals and finally human related activities). Therefore in certain extent the majority of waste products will end up in soil (Pepper et al. 1996). Moreover, the intrinsic complexity of soil derives from its nature as an assemblage of solid, liquid, gaseous, organic, inorganic, and biological constituents whose chemical composition and random three-dimensional structure have not been and cannot be completely characterized. In addition to physical complexity, the microbial (bacteria, fungi, algae, protozoa, and viruses) physiological processes in soil and their multitude of interactions are dauntingly complicated. The challenge of understanding *in situ* soil processes is the fact that abiotic reactions (e.g., precipitation, dilution, and hydrolysis) as well as the whole variety of biological processes (including the activities

of plants and animals) must be considered when attempting to understand soil biogeochemistry.

The solid phase

Normally, inorganic material makes up $\geq 95\%$ of the soil solid phase weight and only 1-5% of the solid phase is of organic origin. The inorganic fraction can be divided according to size into, sand, silt and clay. The relative proportion of each of these particles determines the soil texture. As also the mineralogical composition of the size fraction tends to differ, texture also affects the physical and chemical properties of the soil. The most common structural units in soil clays are layer silicates. Additionally, metal oxides (iron oxides and aluminium oxides), calcium carbonate and calcium sulphate are minerals that can be found in the clay fraction. Clay particles have a large reactive surface and are the most common soil particles carrying an electrical charge. They are the dominant factor in determining the properties of the soil (Pepper et al. 1996). The parameter cation exchange capacity (CEC) arises because of the negative charge associated with clay particles and is important in the physicochemical interactions with ionisable organic compounds.

Soil organic matter (SOM) can be defined as the non-living portion of the soil organic fraction. It is a heterogeneous mixture of products resulting from microbial and chemical transformation of organic residues (Yaron et al., 1996). Even though SOM is normally a small part of the soil solid phase, it is of major importance in defining the physicochemical and surface properties of the media. SOM is composed of substances with known chemical structure such as amino acids, carbohydrates, lipids, polysaccharides, lignins etc. (nonhumic) and by humic substances. Humic substances are defined as high molecular weight complex stable macromolecules with no distinct physical or chemical properties and can be differentiated on the basis of solubility properties into humic acids (soluble in alkali, insoluble in acid), fulvic acids (soluble in alkali and acid) and humins (insoluble in alkali). This large colloidal complex may exhibit hydrophobic properties which govern the interaction with nonionic solutes. Moreover, the major structural components include fused aromatics rings, peptides and proteins, amino sugars and polysaccharides, pyrroles, polyphenolic chains and

unsaturated and saturated carbon. The main functional groups (COOH, OH, NH₂) can interact with carboxylic acids, amines, amides, phenols, hydroxyl, alkoxy, quinones, ethers and esters; and many of the xenobiotics entering soil have at least one of these functional groups (Hayes and Swift, 1978; Barraclough et al., 2005)

The gaseous and liquid phases

The soil atmosphere consists of the same gases as the atmosphere. However, due to soil respiration (by aerobic soil organisms) oxygen is depleted (19-20%) and carbon dioxide enriched (to around 1 %). This represents a variation compared to 21 % and 0.0035% present in the troposphere and derived by utilisation of oxygen by and the subsequent release of carbon dioxide (Pepper et al. 1996). Soil oxygen, an essential factor for the decomposition of organic compounds in soils, is mainly in the gas phase (soil pores) but also dissolved in the soil solution. The volume of the soil gas phase depends on soil porosity and soil moisture.

The liquid phase or soil solution has a composition and reactivity defined by the water entering the soil and is affected by fluxes of energy and matter originating from the adjacent soil solid phase, the biological system, and the atmosphere. Two liquid phase regions are distinguished in the soil, the near-surface water and the free water. The first one is the most important surface reaction zone of the porous system, because it controls diffusion of the reversibly sorbed (potentially mobile) fraction of the solute on the solid phase. The second one controls the water flow and solute transport in soils (Yaron et al., 1996).

Soil biota

Soil organisms are mainly viruses, eubacteria, actinomycetes, archaea, fungi, algae, protozoa and arthropods (Kästner, 2000). Each of them contributes to the overall biotic activity of the environment and this activity directly affects physical and chemical properties of the soil solid, liquid and gaseous phases. However, bacteria and fungi are the main drivers of biochemical transformation in soil and therefore have a crucial role on the fate and mitigation of many pollutants (Pepper et al. 1996).

Bacteria are predominant organisms (in terms of number) in soil and arthrobacter, streptomyces, pseudomonas and bacillus the most dominant. Bacteria are capable of rapid growth and reproduction, which occur by binary fission. Exchange of genetic material occurs by conjugation, transduction and transformation, resulting in an enormous versatility. These organisms can be classified by their mode of nutrition. Autotrophs obtain carbon from simple inorganic molecules (e.g. CO₂) and energy from light (photoautotrophs) or from the oxidation of inorganic substances (chemoautotrophs) while heterotrophs obtain carbon and energy from organic substances. Photoheterotrophs obtain energy from photosynthesis while chemoheterotrophs use organic molecules as energy source. In soil, chemoautotrophs and chemoheterotrophs predominate due to the lack of sunlight permeability in soils (Pepper et al., 1996). Aerobic organisms require oxygen as a terminal electron acceptor during respiration. Anaerobes live under absence of oxygen and use as electron acceptors nitrate, sulphate, ferric iron (Fe³⁺), CO₂ or humic acid (Alexander 1994; Neilson and Allard, 2008). Facultative anaerobic bacteria can grow in the presence or absence of oxygen, because they are capable of switching between aerobic respiration and fermentation. Microaerophiles (obligate aerobes that grow best at low oxygen tensions) are important in carrying out many aerobic processes in soil microenvironments in which O₂ levels are low (thoroughly decreased by decomposers, also in water saturated soil aggregates) (Killham and Prosser, 2007).

Due to their high diversity and metabolic versatility, bacteria are able to colonise different microenvironments in soil. They are normally found in micro and macro aggregates and also attached to soil particles (sand grains, clay) by extracellular polysaccharides providing protection against desiccation, predation, and toxic compounds (Haider and Schäffer, 2009). However, small pore sizes of soil microaggregates often prevent the entry of organisms. As a result, only a small portion of small pores can be invaded by bacteria (Yaron et al., 1996). Many bacteria are motile, but they can also move in soil through the bulk flow of water or attached to soil fungi, animals, or roots (Killham and Prosser, 2007; Furuno et al. 2010).

Microbial activity in soils is directly related to enzymatic reactions. Some enzymes are constitutive (routinely produced by cells) and others are induced by the presence of susceptible substrates. The enzyme profile of soil bacteria determines the range of

substrates being used. Both intracellular and extracellular enzymes are involved in biological reactions. However, the latter catalyse reactions of substrates (e.g. of the size of lignin or cellulose) which first have to be depolymerized outside the cell, before they can enter the cell and be further utilised. Often, a set of enzymes operates for specific substrate transformations (Killham and Prosser, 2007).

An ecological classification of soil bacteria distinguishes r-strategists bacteria which show quick growth and live in environments where easily degradable substrates are available and K-strategists, which are adapted to slowly grow on less favourable substrates (Pepper et al. 1996; Fontaine et al., 2003). Thus the availability and type of substrates and nutrients determine the diversity of microbial populations. Factors like bacterial versatility (genetic adaptation, capacity to form spores, capacity to cometabolize substrates, etc.) and the ability to form competitive microcolonies tend to enhance bacterial diversity (Killham and Prosser, 2007).

The largest amount of biomass in soil is represented by fungi; however they are numerically less prevalent than bacteria in most soils (especially in agricultural soils). Due to their extremely diverse enzymatic systems, they are very important in controlling the ultimate fate of organic compounds in soil (Pepper et al., 1996). Moreover, spreading their hyphae they represent a highway connecting chemicals and bacteria in the soil environment (Furuno et al., 2010).

1.2.2.3 Bioavailability, biodegradation and toxicity

Pollutants in soil will not be degraded if they are biologically unavailable. Bioavailability can be defined as “the amount of a chemical to be taken up or utilised by an organism/organisms in a defined time and environment” (Katayama et al., 2010). Dissolved and vaporised chemicals are usually completely bioavailable. However, if the chemical is in contact with sediments or particulate material the bioavailability in water may be reduced.

Bioavailability in terms of receptor is not an all encompassing term because it is organism and contaminant specific (Stokes et al., 2006).

Semple et al. (2004) distinguished the terms bioavailability and bioaccessibility (**Figure 1**). The second term implies that a constraint of time and/or space prevents the organism

from gaining access to the chemical. They define bioaccessible compound as that which is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical. However, the chemical may be either physically removed from the organism or become bioavailable only after a period of time (e.g. chemical occluded in SOM). Thus, bioaccessible cover what is actually bioavailable plus what is potentially bioavailable. According to this, routinely chemical techniques (exhaustive extraction methods) estimating the total concentration of contaminants in soil will over-estimate the bio-accessible/available fraction (Stokes et al., 2006). Nevertheless, since bioavailability is situation specific the total concentrations of the chemicals still need to be determined.

Bioavailability is affected by many factors, like properties of chemicals and soils, sorption, aging time in the soil, NER formation, climate, and the organism of concern (Katayama et al., 2010). It results from a series of dynamic processes including sorption/desorption, dissolution, diffusion, dispersion, convection, and uptake. However, the most significant interaction between soils and chemicals affecting bioavailability is sorption, followed by aging and non-extractable residue formation. Thus, bioavailability of a compound can be reduced if the compound is sorbed to soil particles, entrapped within the soil matrix, or dissolved in a nonaqueous solvent (Hatzinger and Kelsey, 2005). Moreover, since compounds generally have to be in an aqueous phase to be biodegraded, sorption often increases the resistance of pollutants to microbial attack. Sorption or more exactly adsorption is the process of adhesion of a compound to the surface of soil particles. Several mechanisms like charge-transfer, ionic and hydrogen bonding, ligand exchange, van der Waals forces and hydrophobic bonding are responsible for the adsorption of a chemical to soil particles (Khan, 1987; Pignatello, 1989; Gevao et al., 2000). After a given period of time, equilibrium of the chemical takes place between the sorbed and aqueous phase, and the extent of sorption at the equilibrium is quantified by the sorption coefficient (K_d). Clay minerals and soil organic matter provide most of the sites in soil to which chemicals can sorb. Therefore, positively charged compounds (cations) are often sorbed to clay minerals and non polar molecules tend to associate with organic matter by adsorption and absorption. Consequently, properties of the chemicals like water solubility, vapour pressure, molecular size, K_{ow} ,

and the charge of the molecule greatly affect sorption. Moreover, adsorption rates are also affected by soil properties as OM, pH, moisture, metal hydrous oxides, clay minerals, and CEC (Katayama et al., 2010).

Aging of compounds in soil refers to the decrease of bioavailability due to long-term contamination (increased contact time between chemical and soil) of a soil and is a result of chemical reactions, e.g. resulting from covalent bonding with humic acids after sorption inside micropores, and slow chemical diffusion of the pollutant into soil micropores (Pignatello and Xing, 1996; Dec and Bollag, 1997; Katayama et al., 2010). Thus, it mainly represents a slow sequestration phenomenon in comparison to adsorption, which is a short-term and reversible process (Gevao et al., 2000). Aging makes chemicals less available for uptake by organisms, less likely to exert toxic effects, less susceptible to biodegradation (Alexander 2000) and contributes to the irreversibility of the binding process.

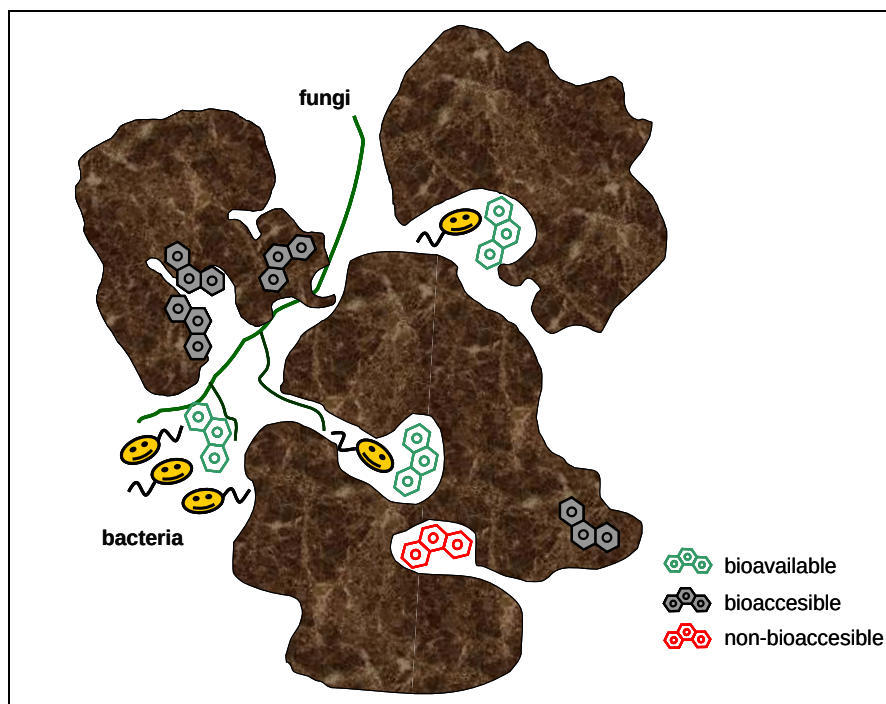


Figure 1. Scheme of contaminant bioavailability at the soil microscale. Prevalently, only a fraction of contaminants is bioavailable to degrading organisms in heterogeneous soils. A substantial part is only bioaccessible, denoting that the compound is physically or temporally constrained but could become bioavailable, e.g. by aggregate destruction and humic matter degradation. Contaminants can also be occluded and, hence, are non-bioaccessible (cf. legend).

Bioavailability and its relation to toxicity and biodegradation

The interaction between chemicals and microorganisms in soil is controlled by many processes, the mass transfer of a chemical to the microorganism, the uptake (absorption) and its transport within the organism to site of biological response (Bosma et al. 1997, Katayama et al., 2010; **Figure 2**). The processes A,B, C and D relate to bioavailability and E to metabolic processing or exerting a toxic effect (Ehlers and Luthy, 2003). Further processes, such as sorption to the outer surface of the organisms, still complicate the fate of the chemical, but are not considered here. Biochemical activity and toxicokinetics refers to the quantitative transport of chemicals to receptors (enzymes, organs, etc.) within the organism. Therefore, bioavailability is the product of interactions among soil, chemical and organism while biochemical activity and toxicokinetics mostly depend on the interaction chemical-organism. Thus, bioavailability is a determinant of effective exposure of an organism to a chemical and is directly related to the toxicity and biodegradability of chemicals in soils (Katayama et al., 2010).

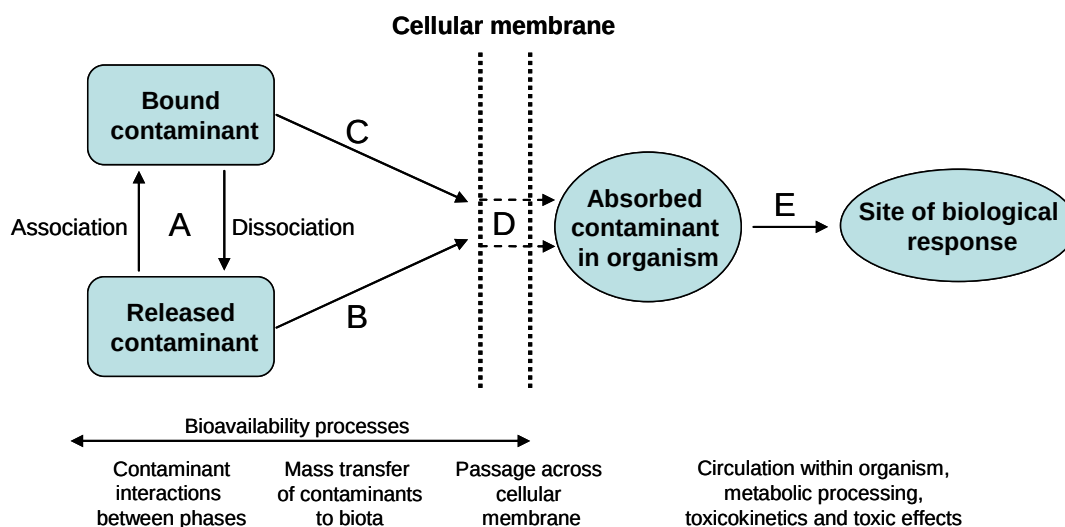


Figure 2. Bioavailability processes. Individual physical, chemical and biological interactions that determine the exposure of organisms to chemicals associated with soils and sediments. A, ageing, binding, and release of compound to a (more) labile state; B, transport of labile, soluble or dissolved compound to biological membrane; C, transport of bound compound to biological membrane; D, uptake across a physiological membrane; E, incorporation into a living system. Note: (i) A, B and C can occur internally or externally to an organism. The National Research Council (NRC) report defines A, B, C and D to be bioavailability processes, but not E, because soil/sediment no longer play a role (NRC, 2003). (Adapted from Ehlers and Luthy, 2003; Semple et al., 2007)

Bioavailability for biodegradation is a consumptive process, essential for catabolism and anabolism, and promoted by the target organism (e.g. positive chemotaxis) and will be dominant when mass transfer is slower than the degradation capacity, meanwhile, bioavailability for toxic effects is undesired and thus avoided by the target organism (e.g. negative chemotaxis; Semple et al., 2007). Induced toxicity by a pollutant is determined by mass transfer and the kinetics of the detoxification mechanisms (Sikkema et al., 1995; Ehlers and Luthy, 2003). In other words, high toxicity is a result of a faster mass transfer than the elimination mechanisms capacity. Moreover, bioaccumulation of a toxicant is a non-consumptive process, which occurs within tissues that are often inaccessible to normal elimination mechanisms, and depend much stronger on the bioaccessibility of the compound (Semple et al., 2007).

The ecotoxicity of a chemical in soil is determined by its bioavailability and toxicokinetics and therefore both have to be taken into account in the context of risk assessment of a compound.

1.2.2.4 Microbial degradation in soil

As previously mentioned, many abiotic factors control the microbial degradation of contaminants. However, moisture level or better defined water activity of soil is of special importance in this compartment. Water activity can range from 0.0 to 0.99 in soils, in contrast to aquatic systems, where it is stable at 0.98. This parameter plays a key role in controlling the degradation rate of pollutants in soil, such as hydrocarbons and pesticides (Leahy and Colwell, 1990; Han and New, 1994). As the moisture content (or water activity) decreases there is an increase in the duration of the lag phase, a significant decrease in microbial metabolic activity (Orchard and Cook, 1983), a reduction in the specific growth rate and decrease in the maximum community size and thus, a reduction in the biodegradation rate of compounds.

Microbial activity in soils is also directly related to enzyme activity. Soil enzymes are not only associated with the microbial biomass since they are often found entrapped in soil organic and inorganic colloids (extracellular enzymes) (Paul and Clark, 1989). Extracellular enzymes are excreted out of the cell to degrade high molecular weight

substrates. By their activity, microorganisms control the availability and cycling of nutrients such as carbon, nitrogen, sulphur and phosphorus.

Several types of kinetics describe the microbial transformation in soil: zero-order kinetics where the rate of transformation is not affected by the concentration of the substrate, first-order kinetics, in which the rate of transformation directly depends on the substrate concentration and hyperbolic reaction kinetics, in which the rate of transformation approaches a maximum with time (Yaron et al. 1996).

The main type of microbial reactions occurring in soil are oxidation, hydroxylation, N-dealkylation, β -oxidation, decarboxylation, ether cleavage, oxidative coupling, aromatic ring-cleavage (type of bond, specific substituent, position and number determine susceptibility to cleavage), heterocyclic ring cleavage, sulfoxidation, reduction (e.g. of double or triple bonds, of nitro groups, sulfoxide reduction and reductive dehalogenation), hydrolytic reactions, and synthetic reactions (conjugation and condensation). The last two types of reaction are usually involved in the degradation of toxic molecules (Yaron et al. 1996).

Studies from the last decades revealed that chemicals in the soil environment are not completely bioavailable (Alexander 1995; Gevaio et al., 2003). Thus, biodegradability, toxicity and efficacy of xenobiotics are dependent on their bioavailability (Katayama et al., 2010). Moreover, it is generally accepted that sorption and aging reduces the bioavailability of a compound. Hydrophobic compounds may partition into SOM or water-air interfaces and hydrophilic compounds may adsorb to minerals (Harderlein and Schwarzenbach, 1993) and, therefore, desorption must precede biodegradation. However, there is increasing evidence that also sorbed contaminants can be biodegraded by attached cells (Park et al. 2001; Schnürer et al., 2006). Furthermore, sorption to SOM was shown to increase the degradation rates of 2,4-D and its related metabolites (Benoit et al. 1999). In this case, the concentration of these compounds was higher at the surface of particles than in the aqueous phase, meaning that bioavailability was higher on the particle surface than in soil solution. Biodegradation enhancement is also described when metal hydrous oxides are used as catalysts; a situation in which degradation rates are higher for chemicals in the sorbed state (Katayama et al., 2010).

Sludge application to agricultural soil has an impact on biodegradation processes. It can increase the soil's capacity to degrade pollutants (adding available carbon, nutrients and potential degraders) in case of easily degradable compounds. However, it can also increase the persistence of compounds by decreasing their bioavailability and by limiting their abiotic degradation (Sánchez et al. 2004; Debosz et al., 2002).

As previously mentioned, one of the main factors affecting biodegradation is toxicity. In soil, toxicity as previously discussed in section 1.2.3.1 is directly proportional to bioavailability and therefore can be reduced by mechanism reducing the compounds bioavailability.

1.2.2.5 Non-extractable residues

The most accepted and widely used definition of non-extractable residues was proposed by the Applied Chemistry Division, Commission on Pesticide Chemistry of the International Union of Pure and Applied Chemistry (IUPAC): non-extractable residues (sometimes referred to as "bound" or "non-extracted" residues) in plants and soils are defined as chemical species originating from pesticides, used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues. These non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products (Roberts, 1984). Other authors reported that non-extractable residues represent compounds in soils, plants, or animals which persist in the matrix in form of parent substance or its metabolite(s) after extraction. The extraction method must not substantially change the compounds themselves or the structure of the matrix (Führ et al., 1998). The amount of NER extracted depends on the extraction methods, nature of the compounds to be extracted and the soil properties, therefore high variability is found in the available data (Barriuso et al., 2008).

NER formation is normally considered as a process contributing to pollutant dissipation decreasing the pollutant bioavailability. Consequently, the decreased availability implies an increase in the persistence of the compound (Barriuso et al., 2008). The current EU regulation on pesticide NER is to treat NER as persistent compounds (Craven, 2005).

In general, NER formation can derive from parent compound, metabolites, biomass (biogenic residues) or CO₂ fixation (Capriel et al., 1985; Kästner et al., 1999; Berns et al., 2005; Miltner et al., 2005; Jablonowski et al., 2009; Miltner et al., 2009). NER formation kinetics can be divided into 3 steps (Barriouso et al., 2008): (i) rapidly or flash formed NER, which correspond to the extractability at the beginning of incubation; (ii) “formation step”, where a plateau is quickly reached or, at low rates of NER formation, is never reached; and (iii), the “maturation stage”, which corresponds to the fate of NER when the formation rate decreased. Three situations are possible in the last case, a plateau is reached and NER remain stable during time; a low formation with continuous incorporation of new residues; NER decreases if the release rate is higher than the formation rate.

Covalent bonding (ester, ether, carbon-carbon or carbon-nitrogen) mediated by chemical, photochemical or enzymatic reactions is reported to be the main mechanism of coupling between the nonextractable molecule and soil (Dec and Bollag, 1997; Kästner et al. 1999; Gevaio et al., 2000; Kästner and Richnow, 2001). The main mechanisms of sorption and aging were already reviewed in section 1.2.3 (bioavailability).

Factors governing NER formation are the molecular properties of the compound, e.g. chemicals possessing free reactive groups (phenyl, aniline), or hydroxyl and amino groups tend to produce large proportions of NER (Helling 1975; Bollag et al., 1980; Winkelmann and Klaine 1991; Benoit et al., 1999). Likewise, the formation of NER, increased with compound molecular weight, Kow and Koc (Northcott and Jones, 2001). In contrast, compounds having a large number of electronegative substituents (such as halogens) tend to form lower amounts of NER than similar compounds with fewer substitutions (Scheunert et al., 1985).

Soil properties affecting NER formation are mostly soil biological activity and the amount of SOM. In general, both are shown to enhance NER formation (Kruger et al., 1997; Kästner et al., 1999; Rice et al., 2002, Barriouso et al., 1997). Moreover, soil water content, temperature and pH are also factors influencing NER formation (Barriouso et al., 2008).

The availability and release of NER has been largely studied. Mobilization of NER may be important because NER become bioavailable and can have ecotoxicological

implications. The release of NER can be for example a consequence of physicochemical and microbial reactions, changes in bioavailability and plant or earthworm uptake (Barriouso et al., 2008). Furthermore, NER residues were shown to be directly mineralised by the soil microflora (Roberts et al., 1981; Gerstl et al., 1985).

1.3 Comparison of biodegradation in water and soil

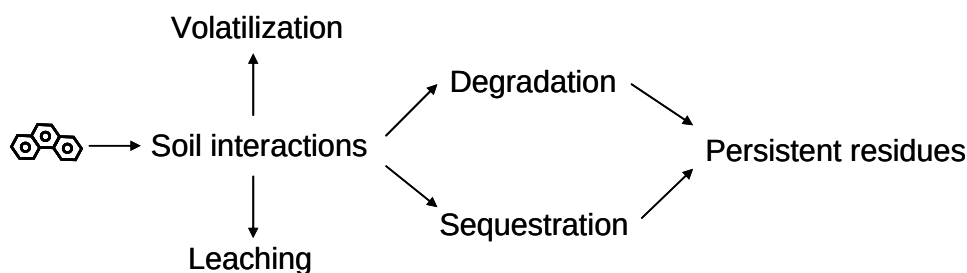


Figure 3. Fate of organic contaminants in soil (adapted from Stokes et al., 2006).

In general, it is difficult to compare degradation in two different environmental systems. In this case, the factors affecting degradation in water and soil are different as discussed previously (section 1.2.2). A contaminant entering the soil is subjected to numerous processes which determine its fate or persistence, including volatilization, leaching or degradation (**Figure 3**). For example, factors that are different in these systems are sunlight irradiation, which only penetrates the top few millimetres of the soil surface; water activity, which can vary considerably in soil (from 0.00 to 0.99), in contrast to aqueous systems (Leahy and Colwell, 1990); soil is mainly composed of particles (% of sand, silt and clay); limited bioavailability in soil (Pepper, 1996; due to sorption, NER formation etc.); differential microbial activity, e.g. reduced bacterial motility; and difference in nutrient contents, e.g. carbon. It was reported that the organic carbon content in the environment determines compound biodegradation in such systems, high organic carbon content can even strongly reduce the degradation of readily degradable compounds in low concentrations (Ahtiainen et al. 2003).

Furthermore, in aqueous systems, the concentration of a chemical in the media may be proportional to total concentration, however in soils, bioavailability is more complex. In

terms of toxicity, soil ecotoxicity testing has been developed using the tools coming from freshwater environments, such as effective concentration (EC_{50}) and therefore maybe less applicable in soils (Stokes et al., 2006). To overcome these incompatibilities, the concept of predicted environmental concentrations (PEC) and predicted no-effect concentration (PNEC) has been implemented. The approach generates a simple quotient $PEC/PNEC$ for hazard and risk assessment that regulators can use for managing the risk of the chemicals, e.g., by limiting their amounts to be used and application mode (Stokes et al., 2006).

Therefore, mass balances of the biodegradation of compounds are not easily transferable from aqueous based studies to soil systems, mainly due to aging, NER formation in soil and to the difference in the microbial activity between water and soil that will considerably reduce the compound mineralisation. As considered here, the differences in the mass balance of the biodegradation of a hypothetical easily degraded compound in water and soil are schematised in **Figure 4**.

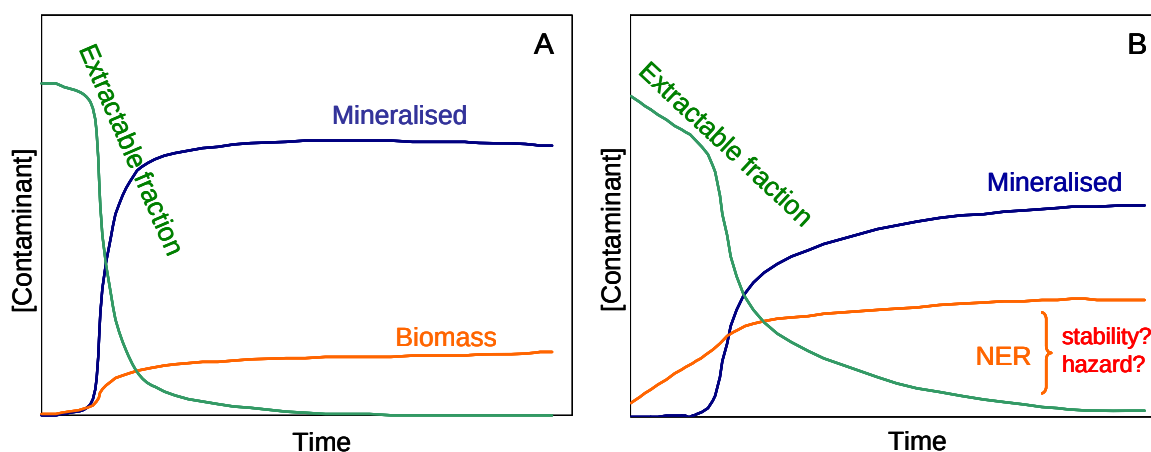


Figure 4. Conceptual mass balance of an easily degradable compound in water (A) and soil (B). In water-sediment systems, formation of NER in sediment has to be considered, similarly to the soil system (B).

For most organic chemicals mass balances in soil, rapid sorption takes place, generating a labile fraction (easily desorbable). This fraction, depending on the extraction method used, includes the easily extractable/bioavailable/degradable fraction (**Figure 5**). The remaining compound in soil can be divided in two portions, the strongly bound or recalcitrant which is not readily bioavailable but may be extracted with certain solvents,

and the irreversibly bound or non-extractable fraction (Jones et al., 1996; Reid et al., 2000; Macleod et al., 2001; Semple et al., 2001; Stokes et al., 2006). The stability of the recalcitrant fraction is highly important in terms of toxicity (**Figure 4**).

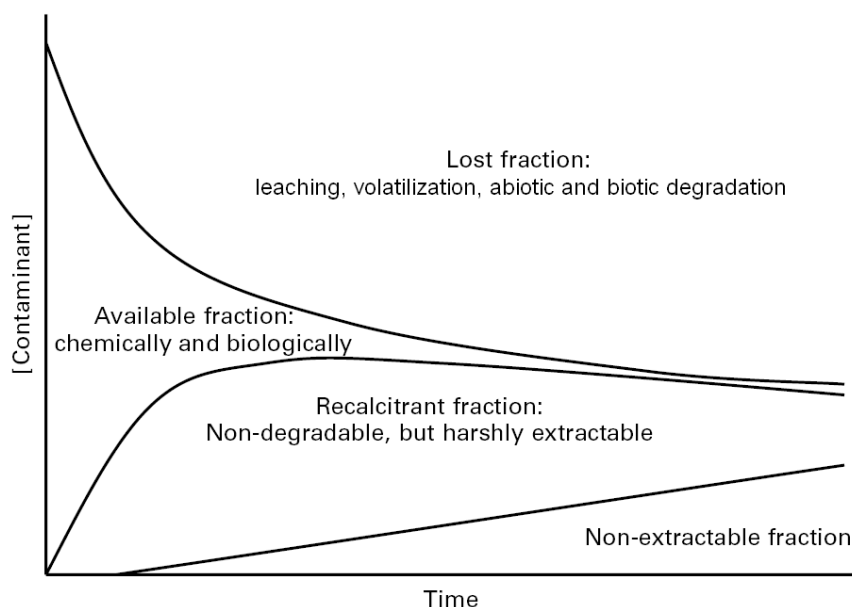


Figure 5. Temporal changes in organic contaminant fractions in soil. (source: Stokes et al., 2006)

Possibilities for prediction of biodegradability

As previously mentioned, ready biodegradability data is a key parameter for environmental risk assessment. Information about prediction and interpretation of ready biodegradability data is growing; complete guidance and reviews are available (Howard and Banerjee 1984, Boethling et al. 1995, Tunkel et al. 2000, Boethling et al 2009). However, it remains unclear how transferable these data are to other environmental compartments such as soil. Ready biodegradability tests generally use high chemical concentrations, standardised nutrient salts media, and the chemical as the sole source of carbon and energy. These arbitrary, but not necessarily realistic, assumptions considerably compromise the predictive power of these tests for real environmental compartments (Ahtiainen et al., 2003). For a realistic estimate of biodegradation and the fate of a chemical in soils, simulation tests such as the OECD 307 (OECD, 2002) are

much more appropriate. Biodegradation in soil is a complex process as mentioned above and cannot be described adequately by short-term experiments and simple models (Dörfler et al 1996). Moreover, NER can only be detectable, quantifiable and identifiable (only in case of using stable isotopes) by using isotope tracers, which limits the use of data coming from ready biodegradability tests or from experiments using unlabelled compounds (Kästner et al., 1999; Richnow et al., 1999).

Environmental degradation half-lives have not yet been determined for most of the commercially available chemicals (Aronson et al., 2006). Most of the chemical biodegradation data available come from aqueous biodegradability screening tests (Boethling 1995, Aronson et al. 2006) and much less data from tests simulating the soil compartment are available (Struijs and Van den Berg, 1995). In particular, information about transformation products and non-extractable residues formation is missing (Boethling et al. 2009). The reason for this lack of data is the complexity and diversity of soils, which results in higher efforts in time, technical equipment and cost (e.g. labelled compounds) for soil tests. A possible solution which allows eliminating the need for simulation tests while still setting up realistic estimates for other environmental compartments such as soil would be to transfer the abundant biodegradation data from aqueous systems. This, however, requires that a proper extrapolatory transfer function to the soil system be developed. The reasons why the comparison of biodegradability in water and soil is crucial and the advantages and disadvantages when doing the above mentioned estimation are summarised in **Figure 6**.

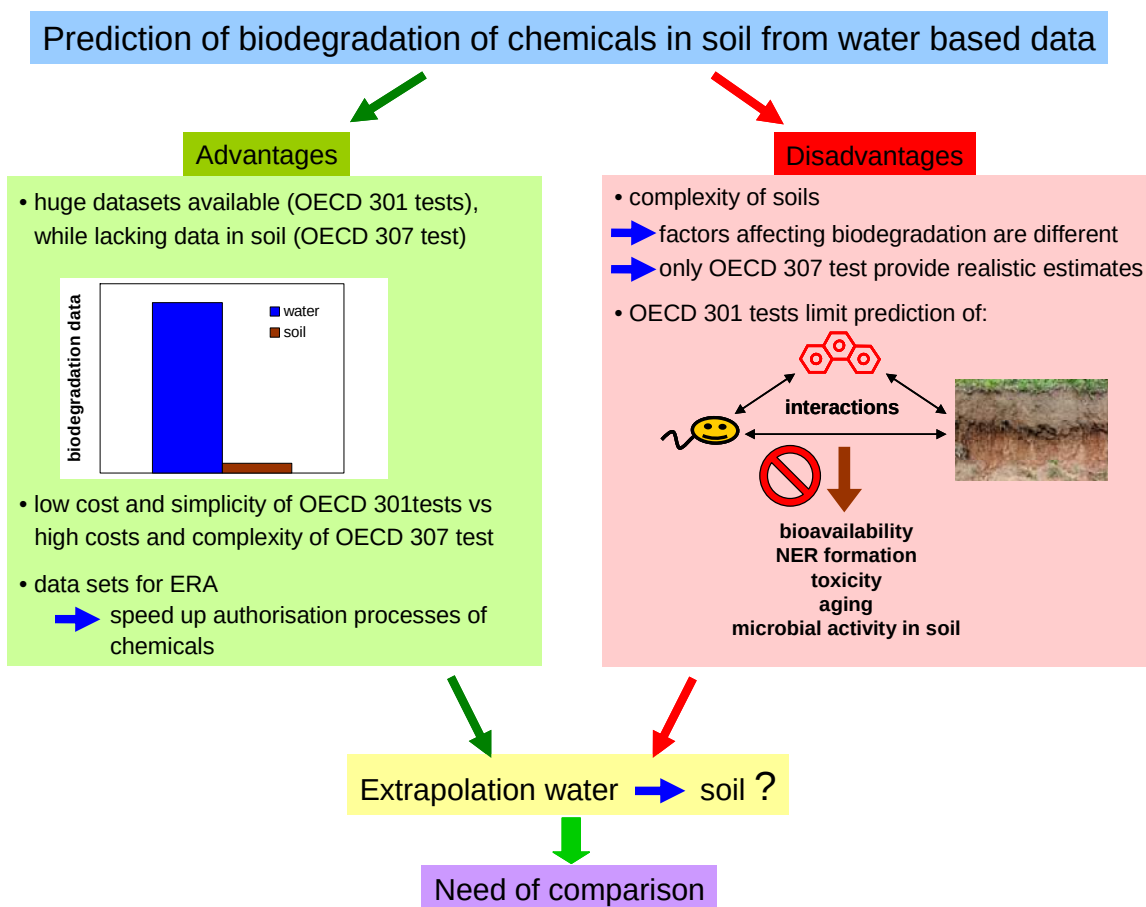


Figure 6. Advantages and disadvantages of the extrapolation of biodegradability data from aqueous to soil systems.

1.4 Aims of the study

Due to the amount of biodegradation data coming from aqueous systems and the contrasting dearth amount of corresponding data in soil, it would be advantageous to predict the fate of a compound in terms of biodegradation and toxicity in the complex soil system, based on data from aqueous experiments. Hence, the overall aim of this work was to directly compare the microbial biodegradation of environmentally relevant compounds in aqueous and soil systems and to elucidate the potential effects of these compounds on the environment. The comparison was performed using stable-isotope (^{13}C) or radio-labelled (^{14}C) 2,4-dichlorophenoxyacetic acid (2,4-D), ibuprofen and ciprofloxacin and the OECD ready biodegradability test 301B (OECD, 1992) (using some modifications included in the updated version, the OECD 310 test; OECD 2006)

and the OECD 307 test (OECD, 2002) for biodegradation studies in soil, in order to produce a data basis for extrapolating biodegradation data obtained in water based tests to the soil environment. The results will be used as a basis for the development of a conceptual approach for predicting the environmental fate of the three model compounds in the two different environmental systems, providing some general rules for using ready biodegradability data obtained from aqueous systems in order to estimate biodegradation in soil.

Specific objectives of this study were:

- 1) To address the carbon redistribution during degradation under biotic and abiotic conditions and obtain a detailed mass balance including mineralisation, parent compound, transformation products or metabolites, and formation of biomass and non extractable residues. The determination was carried out following the OECD 301B test for aqueous systems and the OECD 307 test for soil.
- 2) To estimate the toxic effects of each compound on the activated sludge and soil microbial communities. Inhibition tests following the activated sludge microbial activity in the presence of an easily biodegradable substance and the tested compound were performed in mineral medium. In soil, the inhibition of the microbial soil respiration after the addition of the tested compound was assessed. Moreover, the induced changes in the microbial community and the induced appearance of antibiotic resistance genes were analysed by molecular biology methods.

1.5 Model compounds

The study was performed with isotope labelled model compounds of pesticides, pharmaceuticals and antibiotics. Criteria for the choice of the model compounds were:

- high production and related high consumption,
- ubiquitous occurrence in the environment
- potential risk ($PEC/PNEC > 1$),
- availability of ready biodegradability data. The selection covered a wide range of biodegradation extents.

1.5.1 2,4-dichlorophenoxyacetic acid (2,4-D)

Pesticides are applied to crops worldwide at a rate of around four millions tons/year (Zhang et al., 2004). More than 500 different formulations of these compounds are being applied in the environment and create hazards in the environment (Gavrilescu, 2005). 2,4-D (CAS:94-75-7) is a phenoxy herbicide (**Figure 7**) which is potentially toxic to humans (Boivin et al., 2005) and its production exceeds 100.000 tons per year (Merini et al., 2008). It has a low molecular weight ($221.04 \text{ g mol}^{-1}$), a Log K_{ow} of 0.18 at pH 7 (indicating low hydrophobicity and thus sorption potential of 2,4-D), K_d for sorption to soil of $0.4 \text{ litre kg}^{-1}$ (indicating low sorption), limited volatilisation, high solubility in water (600 mg L^{-1}) (Barriuso et al., 1997; Technical Factsheet on: 2,4 -D, www.epa.gov/ogwdw/pdfs/factsheets/soc/tech/24-d.pdf).

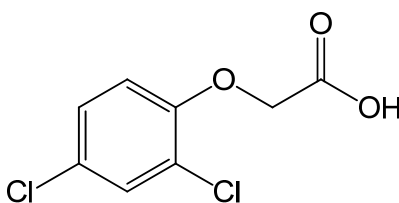


Figure 7. Chemical structure of 2,4-D.

2,4-D has a low to moderate persistence (DT_{50} 5-59 days; Villaverde et al., 2008). Microbial degradation of 2,4-D has been extensively described (Fulthorpe et al., 1996; McGowan et al., 1998; Vieubl  Gonod et al., 2003; Lerch et al., 2009a). Even though 2,4-D is regarded as readily biodegradable in aqueous systems (Nyholm et al., 1992; EPA, 2010;), residues are detected in surface water (IFEN, 2004). Biotic degradation of 2,4-D in soils implies the cleavage of the ether linkage or the loss of the acetic acid side chain (Foster and Mckercher, 1973; Chaudhry and Huang 1988, Roberts et al., 1998) resulting in the formation of 2,4- dichlorophenol (2,4-DCP) and other phenolic metabolites (e.g. chlorohydroquinone), which are further degraded by cleavage of the phenyl ring (Smith and Aubin, 1991; Roberts et al., 1998). Abiotic degradation can also participate in the degradation of phenoxy herbicides by hydrolysis or photolysis (Cresp n et al., 2001). In soil biodegradation experiments, half of the 2,4-D initial concentration was mineralised after 8 days, and the other half remained as NER (Lerch et al., 2009a).

Consistently, mineralisation in biotic incubations has been reported to be around 50%-65% of initially applied isotope labelled compound and no mineralisation was observed under abiotic conditions (Benoit and Barriuso, 1997; Vieublé Gonod 2003; Boivin et al., 2005; Lerch et al., 2009a). Moreover, this compound was reported to be biodegraded even in pristine soils (Fulthorpe et al., 1996). 2,4-D has been reported to be used by microorganisms as carbon and energy sources, or biodegraded co-metabolically (Soulas, 1993). High amounts of extractable residues (80-90%) have been reported directly after its application, thus sorption did not reduce dramatically 2,4-D degradation, although extractability decreased with time (Benoit and Barriuso, 1997; Boivin et al., 2005). Biodegradation of 2,4-D may be partial and the main metabolite (2,4-DCP) is an important contributor to NER formation mainly due to binding to SOM (Soulas and Fournier, 1981; Benoit and Barriuso, 1997; Boivin et al., 2005; Lerch et al., 2009b). NER amounts are reported to be in the range of 10%-60% and less than 10% under abiotic conditions (Barriuso et al., 1997; Benoit and Barriuso, 1997; Boivin et al., 2005; Lerch et al., 2009a). The stability of 2,4-D derived NER is dependent on aging, but even aged NER are bioavailable (Boivin et al., 2005; Lerch et al., 2009b). In summary, many studies about 2,4-D biodegradation and NER formation are available, however, a detailed understanding of their formation and qualitative analyses of their chemical structure are still missing.

1.5.2 Pharmaceuticals

The occurrence of pharmaceuticals in the environment has, over the recent years, become recognised a major issue in environmental sciences (Richardson and Bowron, 1985; Halling-Sørensen et al, 1998; Ternes, 1998; Dauton and Ternes, 1999; Beausse et al., 2004; Carlsson et al., 2006; Cooper et al., 2008).

Pharmaceutical compounds are developed with the objective of performing a biological effect and they can reach the environment by release of urine and feces to sewage, discharges from sewage treatment plants (since pharmaceutical substances and their metabolites are not completely removed or degraded during sewage treatment or storage [Richardson and Bowron, 1985; Halling-Sørensen et al, 1998]), leaching from landfills, release from pharmaceutical industries, livestock activities and application of sewage

sludge, manure or treated waste water to agricultural land (Daughton and Ternes, 1999; Boxall et al., 2003; Pedersen et al. 2005; Thiele-Bruhn, 2003; Topp et al. 2008). Therefore, these environmental pollutants are relevant not only for freshwater environments but also for soil, but their fate and effects in the soil ecosystem are widely unknown (Picó and Andreu 2007).

1.5.2.1 Ibuprofen

Ibuprofen (CAS:15687-27-1) is a nonsteroidal antiinflammatory, analgesic and antipyretic drug. Its global annual production is about several kilotons constituting the third-most used drug in the world (Buser et al., 1999). It is a non-prescription medicine having a high therapeutic dose (600-1200 mg/day) and being excreted to a significant degree (70%-80% of the therapeutic dose) as parent compound or metabolites (Mills et al., 1973). Ibuprofen is a racemic compound ([RS]-2-[4-[2-methylpropyl]phenyl]propanoic acid), composed of the inactive (R)-(-)-ibuprofen and the active (S)-(+)-enantiomer, with the R enantiomer being more persistent in the environment (Buser et al., 1999). This propionic acid derivative (**Figure 8**) has a relatively low molecular mass of 206.3 g mol^{-1} and a moderate solubility of 21 mg L^{-1} in water (Yalkowsky and Dannenfelser, 1992), a $\log K_{ow}$ of 2.5 at pH 6 (Avdeef et al., 1998), and a K_d in soil between 1.52 L kg^{-1} and 64 L kg^{-1} depending on the soil characteristics (Kreuzig et al., 2003; Xu et al., 2009), indicating its moderate sorption potential.

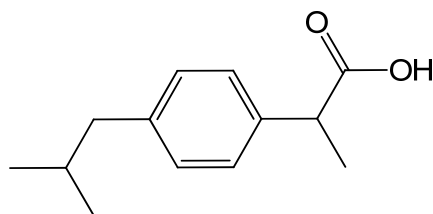


Figure 8. Chemical structure of ibuprofen

Its main metabolites are hydroxyibuprofen, carboxyibuprofen and carboxyhydratropic acid (Zwiener et al., 2002). The metabolic degradation pathways of this compound are not well understood. Side chain hydroxylation and deacylation before ring cleavage were

reported (Zwiener et al., 2002; Murdoch and Hay, 2005). The propionic acid moiety is removed followed by the dioxygenation of the ring.

Stuer-Lauridsen et al. (2000) determined a PEC/PNEC ratio >1 , this means that ibuprofen may represent a risk for the environment. Moreover, it has been proposed as an environmental priority hazardous substance in the parliament of the European Union (Carlsson et al. 2006).

Controversial results for ibuprofen biodegradation are reported in the literature. Richardson and Bowron (1985) classified ibuprofen as inherently degradedable, whereas Quintana et al. (2005) reported no degradation within 28 days when ibuprofen was the sole C source. Moreover, ibuprofen was readily degraded ($> 95\%$) in waste water treatment plant and had a half-life of 20 days in lake water (Buser et al., 1999). Concentrations up to $168 \mu\text{g L}^{-1}$ have been reported in the influent of a waste water treatment plants. In general, ibuprofen is eliminated mainly by biodegradation, with removals between 38% to more than 98% in WWTPs (Kimura et al., 2007; Clara et al., 2005; Joss et al., 2005; Castiglioni et al., 2006; Gómez et al., 2007; Jones et al., 2007; Smook et al., 2008; Miège et al., 2009), while sorption to sludge appears to be negligible (Ternes et al., 2004). However, most of the studies determine dissipation of the compound, thus its exact removal mechanism cannot be fully elucidated. Nevertheless, this pharmaceutical is still detected at high concentrations in effluents of WWTP ($28 \mu\text{g L}^{-1}$ [Gómez et al., 2007]) and in sewage sludge (246–750 ng/g of dry weight of dewatered municipal biosolids [Edwards et al., 2009; McClellan and Halden, 2010]). Thus, application of sewage sludge (biosolids) on agricultural fields as a fertiliser may introduce ibuprofen into soils (Edwards et al., 2009).

Few biodegradation studies are available in soil. Half-lives between 1 and 6 days depending on soil characteristics were reported, indicating low persistence (Xu et al., 2009). Degradation rates were negatively correlated with clay and OM content. Moreover, degradation rates were 34-fold faster in biotic systems than in the abiotic ones, indicating the important contribution of microbial activity to the overall degradation. In another study using ^{14}C -labelling in the methyl group ($^{14}\text{C}_3$ -ibuprofen) of the molecule, mineralisation extents of 38% in clayey silt and of 48% in silty sand soils after 100 days were reported (Kreuzig et al. 2003; Richter et al., 2007). NER formation was fast,

reaching a maximum at day 4, when the parent compound was still available, and then decreased at a very slow rate. At the end, NER amounts corresponded to 50% and 35%; extracted residues to 12% and 28% of the applied radioactivity, respectively, for the clayey silt and the silty sand soil.

To summarise, microbial degradation of ibuprofen in soil is not well studied. Data from studies using ring labelled compounds are missing, although they would be needed for a realistic estimation of its mineralisation, its associated NER formation and the hazard related to them.

1.5.2.2 Ciprofloxacin

Antibiotics are used in human and veterinary medicines to treat and prevent bacterial infections (Thiele-Bruhn, 2003; Boxall et al., 2003). They are designed to be highly stable during time and therefore not refractory to biodegradation and to act effectively even at low doses. In the last years, the concern about potential ecological impacts of antibiotics increased because they may affect key ecosystem processes due to the potential negative effects they can exert on microorganisms having crucial roles in these processes, like nutrient regeneration, carbon and nitrogen cycles and pollutant degradation (Ollivier et al., 2010). However, no regulation exists on concentration limits of the compounds in the different environmental compartments (Picó and Andreu, 2007).

One of the most prescribed and prevalent human antibiotics found in the environment is the fluorquinolone ciprofloxacin (CIP; **Figure 9**), which is active against a broad spectrum of Gram-negative and Gram-positive bacteria (Davis et al. 1996; Beausse et al., 2004). Ciprofloxacin (CAS: 85721-33-1; 1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-yl-quinoline-3-carboxylic acid) has a molecular weight of 331.3 g mol⁻¹ and exist as a cation, zwitterion, and/or anion under environmentally relevant pH conditions (Vasudevan et al., 2009). It has a log K_{ow} of -1.1 at pH 7.4 (Takács-Novák et al., 1992), a low aqueous solubility (50 mg L⁻¹), a log K_d of 4.3 to sludge (Golet et al., 2003) and K_d to soil within the range of 106-50000 L kg⁻¹ depending on soil characteristics and pH (Vasudevan et al., 2009).

It is frequently detected in the environment (Kümmerer et al. 2000). It is also the main metabolite of enrofloxacin, a commonly used veterinary fluorquinolone (Picó and Andreu

2007). 45 - 62% of the administered dose of ciprofloxacin in humans is excreted unmetabolized via the urine and 15% - 25% via feces (Golet et al.2003).

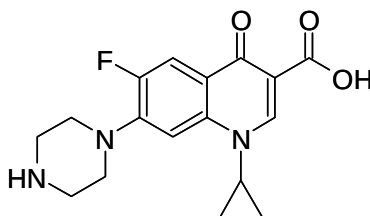


Figure 9. Chemical structure of ciprofloxacin

Ciprofloxacin concentrations in the environment range from ng L^{-1} to mg L^{-1} . Larsson et al. (2007) reported concentrations of up to 31 mg L^{-1} in the effluents of a wastewater treatment plant for pharmaceutical industries in India, which are higher than the maximum therapeutic human plasma levels. These levels were orders of magnitude above the EC_{50} toxicity values for microorganisms, plants, invertebrates and aquatic organisms (Larsson et al., 2007; Carlsson et al., 2009). The discharge load of CIP corresponded to 45 kg of the antibiotic per day. Furthermore, Halling-Sørensen et al. (2000) estimated environmental loadings in Europe of 186.2 tonnes/year. Ciprofloxacin is considerably eliminated in wastewater treatment (80-90%), mainly by sorption to sludge (Picó and Andreus. 2007), which stabilises the substance. Therefore, digested sludge contains ciprofloxacin residues (around 3 mg kg^{-1} ; Golet et al., 2003). In soil, the concentrations range from 0.37 mg kg^{-1} to 0.45 mg kg^{-1} (Golet et al. 2002; Martinez-Carballo et al. 2007), underlining the ecotoxicological relevance of ciprofloxacin in soil. In particular, soil can act as a reservoir of this (and other) antibiotics (Rooklidge, 2004). Ciprofloxacin is not readily biodegradable (Kümmerer et al., 2000) and as reported before, strongly sorbs to soil (Uslu et al, 2008; Picó and Andreu 2007), mostly by cation exchange (Vasudevan et al. 2009). Nevertheless, biodegradation of ciprofloxacin by the brown rot fungi *Gloeophyllum striatum*, *Mucor ramannianus* and *Pestalotiopsis guepini* has been reported (Wetzstein et al., 1999; Parshikov et al., 1999; Parshikov et al., 2001). Four degradation pathways are described, with different sites of initial attack by hydroxyl radicals. Oxidation, decarboxylation, defluorination, acetylation, hydroxylation may lead

to the cleavage of the heterocyclic core and the piperazine ring of CIP, and to the elimination of its antibacterial activity. Ciprofloxacin is also photodegraded (Burhenne et al., 1999) with a half-life of 13 ± 2 min in surface water (Lam et al. 2003). Moreover, the antibiotic can be completely mineralised in 4 hours by photoinduced degradation (Calza et al., 2008). Ciprofloxacin transformations that significantly affect the antibiotic properties involve two parts of the molecule, the piperazinic moiety and the quinolone moiety.

Environmental risk assessments of ciprofloxacin determined PEC/PNEC values above the trigger value, implying a possible environmental hazard and the need of assessing the occurrence and behaviour of CIP in the environment, particularly in sludge-treated soils (Halling-Sørensen et al., 2000; Golet et al., 2003). In addition, the effects of ciprofloxacin on microbial communities in wastewater, stream water, marine and salt marsh sediment were studied thoroughly (Halling-Sørensen et al., 2000; Kümmerer et al., 2000; Maul et al., 2006; Naslund et al., 2008, Cordova-Kreylos et al., 2007). It showed high potency against activated sludge bacteria and reduced algal diversity at environmentally relevant concentrations (Halling-Sørensen et al., 2000; Wilson et al., 2003). However, nothing is known about its effects on soil microbial communities (Picó and Andreu, 2007), and standardized studies on its degradation in soil, e.g. OECD 307 tests (OECD, 2002), have not been reported. This information is needed to estimate the fate of these compounds and to perform accurate risk assessments.

Exposure of bacteria in the environment can contribute to spreading antibiotic resistance to pathogens (Daughton and Ternes, 1999). Fluorquinolone-resistant *Campilobacter jejuni* was found in poultry husbandry (Gaunt and Piddoc, 1996). Moreover, it has been reported that ciprofloxacin is genotoxic and induces horizontal transfer of resistance genes even at low concentrations (5 to $10 \mu\text{g L}^{-1}$; Beaber et al., 2004). Furthermore, antibiotics in sewage can inhibit the microbiota of WWTP (Al-Ahmad et al. 1999; Halling-Sorensen, 2001) and thus reduce the waste water treatment efficiency. Composting is used to degrade organic contaminants such as pesticides, PAHs, PCBs in sewage sludge before its application to soils (Xia et al, 2005). However, the implications for degradation of this process as well as the fate of the antibiotics themselves remain

unclear. We hypothesize that the antibiotic ciprofloxacin is not degraded in water and soil and that it can pose a risk for the environment.

To conclude, due to its properties and characteristics ciprofloxacin is a pharmaceutical of high environmental concern.

Materials and methods

2.1 Chemicals and materials

All chemicals were analytical or reagent grade. Chemicals and materials were obtained from VWR (Darmstadt, Germany) or Sigma-Aldrich (Munich, Germany) if not specified otherwise.

Chemicals

2-Hydroxyibuprofen (chemical purity 99.8%) was purchased from LGC GmbH (Luckenwalde, Germany), $^{13}\text{C}_6$ -2,4-dichlorophenoxyacetic acid (2,4-D) and $^{13}\text{C}_6$ -ibuprofen (both with 99 at% ^{13}C and 98% chemical purity) from Alsachim (Illkirch, France), $^{14}\text{C}_6$ -2,4-D (10 mCi/mmol, ≥ 98 at% of ^{14}C) from Hartmann Analytik (Braunschweig, Germany), sodium acetate- $^{13}\text{C}_2$ (99 atom% ^{13}C) from Cambridge Isotope Laboratories Inc. (Andover, USA), and sodium acetate-U- ^{14}C (50 mCi/mmol, ≥ 98 atom% ^{14}C) from Biotrend GmbH (Cologne, Germany). Ciprofloxacin hydrochloride (99% purity) was purchased from Biotrend Chemicals (Zurich, Switzerland) and [2- ^{14}C] ciprofloxacin (radiochemical purity 99.4 %; specific activity 20 mCi mmol $^{-1}$) from Hartmann Analytic GmbH (Braunschweig, Germany). The positions of the label in the studied chemicals are shown in **Figure 10**.

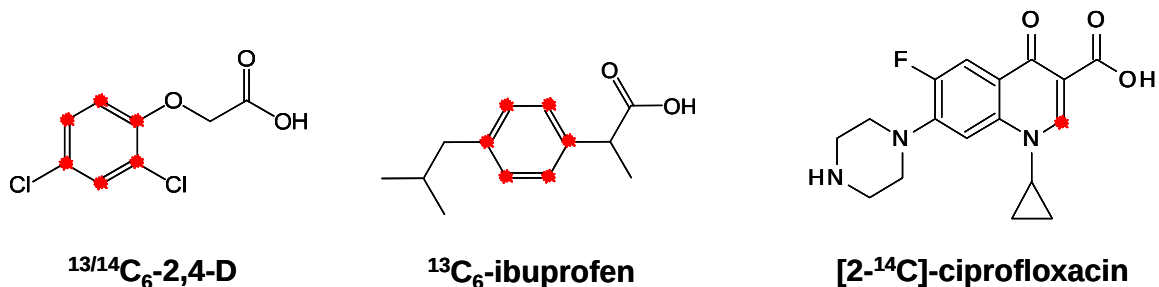


Figure 10. Positions of the label in the studied molecules

2.2 Incubations in aqueous media

In the present study, biodegradation experiments in aqueous systems were performed according to the OECD 301B (OECD, 1992) and to an updated version of this guideline, the OECD guideline 310 (OECD, 2006) test. The main differences between these guidelines are the methods for determining the amount of CO₂ in the gas phase and the one dissolved in the media. For 2,4-D and ciprofloxacin (¹⁴C labelled) mineralisation was followed by the determination of radioactivity in the trapped CO₂. In the case of ¹³C₆-ibuprofen, mineralisation was followed by determining the total inorganic carbon (IC) or CO₂ (by GC-MS) and the label in the trap for CO₂ after acidification.

2.2.1 Mineral medium

The standard mineral medium (MM) was prepared according to the OECD 301/310 test (OECD, 1992, 2006). MM components and their final concentrations are presented in **Table 1**. The final pH of MM was adjusted to 7.4 (± 0.2). The tested chemicals were added as the sole C source at a final concentration of 20 mg L⁻¹ per system.

Table 1. MM components for OECD 301B experiments

Component	Concentration
KH ₂ PO ₄	85 mg L ⁻¹
KHPO ₄	217.5 mg L ⁻¹
Na ₂ HPO ₄ ·2H ₂ O	334 mg L ⁻¹
NH ₄ Cl	5 mg L ⁻¹
CaCl ₂ ·2H ₂ O	36.4 mg L ⁻¹
MgSO ₄ ·7H ₂ O	22.5 mg L ⁻¹
FeCl ₃ ·6H ₂ O	0.25 mg L ⁻¹

2.2.2 Experimental setup

Four different incubations in triplicates were performed for 2,4-D and ciprofloxacin and six for ibuprofen:

- 1) MM spiked with ¹⁴C₆-2,4-D, ¹³C₆-ibuprofen or [2-¹⁴C] ciprofloxacin,
- 2) sterilised MM with the labelled test compound (sterile control, providing abiotic degradation),

- 3) MM with labelled acetate (positive control),
- 4) MM with labelled acetate and the unlabelled test compound (inhibition test),
- 5) MM with unlabelled ibuprofen (control), only for ibuprofen,
- 6) non-amended MM (blank), only for ibuprofen.

Control samples provided the information on the natural abundance of ^{13}C in the medium after addition of unlabelled ibuprofen. The abiotic system was performed to assess the degradation in the absence of biotic processes. Each treatment was inoculated with diluted fresh activated sludge (adjusted to 10 mg L^{-1} of suspended solids) from a municipal wastewater treatment plant (Klärwerk Rosental, Leipzig, Germany). For abiotic incubations, the MM inoculated with activated sludge was autoclaved (Systec Autoclave, Wettberg, Germany) once at 121°C for 20 min before spiking.

$^{14}\text{C}_6$ -2,4-D was dissolved in acetone, $^{13}\text{C}_6$ -ibuprofen and sodium acetate- $^{13}\text{C}_2$ in MM. [2- ^{14}C] ciprofloxacin was dissolved in alkaline MilliQ water (pH 9) and mixed with unlabelled ciprofloxacin hydrochloride dissolved in MilliQ water. The pH of the solution was adjusted to 7.8 before spiking.

For 2,4-D and ciprofloxacin, the initial radioactivity was 10 kBq per system. 300 mL of the spiked MM was incubated in 500 mL Schott bottles in the dark at 20°C ($\pm 2^{\circ}\text{C}$) for 28 or 29 days. Samples were every 3 days flushed with humidified and CO_2 -free air in order to provide the O_2 necessary for microbial respiration. The CO_2 in the gas leaving the bottles was trapped in 20 mL 1 M NaOH (**Figure 11**). The biotic incubations were destructively sampled after 3, 6, 20 and 28 days in the 2,4-D experiment, after 6, 13, 20 and 28 days in the ibuprofen experiment and after 12 and 29 days in the ciprofloxacin experiment. Abiotic incubations were destructively sampled after 20 and 28 days for 2,4-D, after 28 days for ibuprofen, and after 12 and 29 days for ciprofloxacin.

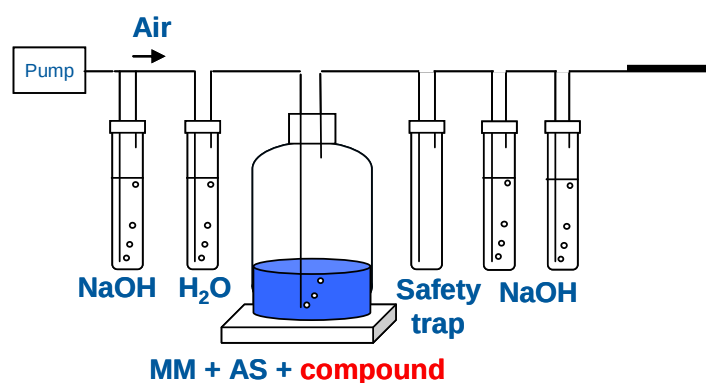


Figure 11. The OECD 301B incubation experiment with semi-continuous aeration

2.3 Soil incubation experiments

To characterise and obtain a detailed mass balance of the biodegradation of our model compounds in an agricultural soil, we followed the OECD 307 test (OECD, 2002).

2.3.1 Soil

The soil used was collected from the A horizon of a Haplic Chernozem from the agricultural long-term experiment “Statischer Düngungsversuch” in Bad Lauchstädt, Germany (Blair et al., 2006). The plot has been cultivated with crop rotation, fertilised with farmyard manure (30 tons/ha every second year), and cultivated with a sugar beet - summer barley - potato - winter wheat crop rotation since 1902. The soil characteristics are presented in **Table 2**.

Table 2. Characteristics of the agricultural soil used in this study (Körschens et al., 2000)

Parameter	Content
Clay	21%
Silt	68%
Sand	11%
Total N	0.17%
TOC	2.1%
pH	6.6
WHC	37.5%

2.3.2 Experimental setup

The soil was slightly dried, sieved to 2 mm and pre-incubated for 3 days at 20°C before spiking. In case of ibuprofen and ciprofloxacin, it was amended with stabilised sewage sludge (corresponding to 5 t/ha which is the maximum sewage sludge load allowed in a three-year period in Germany) from a local wastewater treatment plant (Klärwerk Rosental, Leipzig, Germany) to mimic the major route of entry of pharmaceuticals into soil (Thiele-Bruhn 2003).

Four different incubations were performed in triplicates for 2,4-D and ibuprofen and two for ciprofloxacin:

- 1) Soil with the ^{13}C or ^{14}C -labelled test compound,
- 2) sterilised soil with the test ^{13}C or ^{14}C -labelled test compound (sterile control),
- 3) soil with the unlabelled compound (control), only for 2,4-D and ibuprofen,
- 4) non-amended soil (blank), only for 2,4-D and ibuprofen.

Blank and control samples provided the information on the natural abundance of ^{13}C in the soil and the abundance of ^{13}C in the soil after addition of the unlabelled compound, respectively. The abiotic system was performed to assess the abiotic contribution to biodegradation in soil. In order to obtain sterile conditions in these systems, soil or soil inoculated with stabilized sludge was autoclaved (Systec Autoclave, Wettenberg, Germany) three times within 3 days, at 121°C for 20 min before spiking.

^{13}C -labelled and unlabelled 2,4-D were dissolved in acetone and ibuprofen in acetonitrile. Ciprofloxacin was dissolved as described in section 2.2.2.

2,4-D and ibuprofen were first added to 10 % of the total amount of soil and the solvent evaporated to avoid killing microorganisms. Then the spiked soil was thoroughly mixed with the remaining 90% with a pastry blending machine (Kenwood Chef premier, New Lane, U.K.). The final concentration of each chemical in soil was adjusted to 20 mg kg⁻¹ of soil. For ciprofloxacin, the radioactivity added was 10 kBq per system. The water content of the soil was adjusted to 60% of its maximum water holding capacity (WHC). Finally, 40 gram of soil for 2,4-D and ibuprofen, were weighed in 1000 mL Duran glass bottles sealed with Teflon-lined caps. For ciprofloxacin, 20 gram of soil were weighed in 500 mL Duran glass bottles sealed with Teflon-lined caps. The bottles were incubated in

the dark at 20°C ($\pm$ 2°C) and subjected to intermittent aeration with humidified and CO₂-free air in order to aerobic conditions. The CO₂ in the gas leaving the bottles was absorbed in two consecutive traps with 40 ml 1 M NaOH. The process scheme for the soil incubation system (**Figure 12**) is basically the same as the one used for the aqueous systems (**Figure 11**).

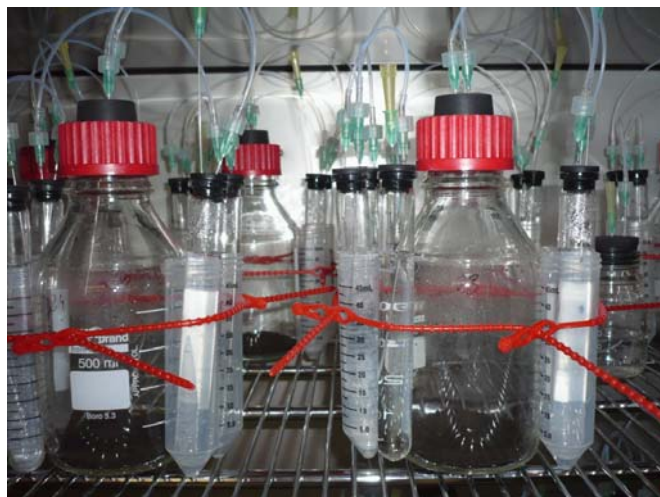


Figure 12. Soil incubation experiments according to OECD 307 test.

Incubation periods and sampling days are shown in **Table 3**. The bottles were destructively sampled and the soil was analysed to determine extractable residues, metabolites, and NER.

Table 3. Incubation periods and sampling days for soil experiments.

Compound	Incubation period (days)	Sampling biotic (day)	Sampling abiotic (day)
2,4-D	64	0, 2, 4, 8, 16, 32, 64	0, 32, 64
Ibuprofen	90	0, 2, 7, 14, 28, 59, 90	0, 28, 90
Ciprofloxacin	93	0, 17, 32, 60, 93	0, 32, 93

2.4 Mass balance and analytical procedures

One of the main objectives of this study was to provide a detailed mass balance of the labelled C in each system.

The following fractions were determined in the MM experiments: CO₂, labelling in MM (including parent compound and its metabolites) and label incorporation into total biomass (SS).

The following fractions were determined in soil experiments: CO₂, solvent-extractable fraction (including parent compound and its metabolites) and total NER.

2.4.1 Mineralisation

CO₂ in the experiments with ¹³C label was determined periodically by measuring total inorganic carbon in the NaOH traps using a Shimadzu TOC-5050 Total Organic Carbon Analyser (Duisburg, Germany) and confirmed by GC-MS using an Agilent 7890A GC (Agilent Technologies, Germany) equipped with a HP-5MS column (30 m × 250 µm × 0.25 µm; Agilent Technologies, Germany). The GC was run isothermally at 45°C. The injector was set at 45°C and the He flow was 1.9 mL min⁻¹. A standard solution of NaHCO₃ 1.6 g L⁻¹ was prepared in de-ionised water and 5 different dilutions representing different amounts of C (from 0.4 µmol to 0.04 µmol) were prepared. 2 ml of the NaOH traps were acidified with 400 µl phosphoric acid (85%) in sealed 15 ml crimp cap vials. Head space samples (100-250 µl) were analysed by GC-MS and GC-C-IRMS. The isotopic composition (atom %) of CO₂ was determined by GC-combustion-isotope ratio mass spectrometry (GC-C-IRMS; Finnigan MAT 252, Thermo Electron, Bremen, Germany, coupled to Hewlett Packard 6890 GC, Agilent Technologies, Germany) equipped with Porabond Q-HT Plot FS column (50 m × 0.32 mm × 5 µm; Chrompack, Middelburg, Netherlands).

CO₂ was separated isothermally from other gases at a temperature of 40°C, the oxidation was at 940°C and the reduction oven at 640°C as described by Hermann et al. (2010). For all ¹³C samples, the isotopic composition was expressed relative to Vienna PeeDee belemnite (VPDB; Coplen et al. 2006).

For ¹⁴C-experiments, ¹⁴CO₂ in the NaOH traps was determined by liquid scintillation counting (LSC) with UltimaGold scintillation cocktail and a Wallac 1414 scintillation counter (Perkin Elmer Wallac GmbH, Freiburg, Germany). 1 ml of NaOH was mixed with 10 ml of UltimaGold scintillation cocktail and measured by LSC (5 min of counting time, chemiluminescence correction).

2.4.2 Label in MM and in suspended solids (SS)

MM samples were filtered over 0.22 μm cellulose filter to determine the amount of label in SS. Filtered (dissolved label only) and unfiltered (dissolved + suspended label) samples were measured either by LSC for radiolabeled samples or by Elemental Analyser-Combustion-Isotope Ratio monitoring Mass Spectrometry (EA-C-IRMS) with a EA-C-IRMS; Finnigan MAT 253 (Thermo Electron, Bremen, Germany) coupled to Euro EA 3000, Eurovector, Milano, Italy) for samples from the ^{13}C -labeled experiments. The temperature of the oxidation oven was 1020°C and the one of the reduction oven was 650°C. In addition, crude extracts from soil before purification by SPE were also analysed for their C content and the isotopic composition.

2.4.3 Extractable residues in soil

Extractable residues for mass balance determination were extracted by Accelerated Solvent Extraction (ASE) for the three model compounds and determined by either EA-C-IRMS or LSC. Due to their similar structure, 2,4-D and ibuprofen were extracted with the same extraction method, whereas ciprofloxacin was extracted by ASE using a different extraction method from the one of the other compounds. Moreover, CIP was sequentially extracted by sonication for sorption mechanism studies.

Soil extractions of 2,4-D and ibuprofen residues by ASE

Soil extractions were performed using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA) equipped with 11 ml stainless steel extraction vessels (modified from Radjenović et al., 2009). Five grams of soil were mixed with diatomaceous earth (Hydromatrix™, Varian Associates, Inc., Palo Alto, USA) and with 20 μg of the internal standard 2-methyl-4-chlorophenoxyacetic acid (MCPA) for quantitative analyses. The samples were extracted with methanol-water (1:1, v/v) at the following operating conditions: extraction temperature, 100 °C; extraction pressure, 100 bar; preheating period, 5 min; static extraction period, 10 min; number of extraction cycles, 3; solvent flush, 150% of the cell volume; nitrogen purge, 150 s. A subsample of 1 ml of the extract (crude extract) was evaporated and thereafter used for ^{13}C analysis; the remaining sample

was diluted with MilliQ water until the solvent content was <5 % for purification and chemical analysis (see section 2.4.5).

Exhaustive sequential extraction of ciprofloxacin

Ciprofloxacin residues were extracted in a first step by exhaustive sequential extraction using 5 different extraction solvents to optimise an extraction protocol, but due to the high effort and time required, ASE extractions (see below) were performed for mass balance purposes. Nevertheless, the data obtained was used for the study of binding mechanisms of ciprofloxacin to soil. Two grams of soil samples corresponding to time 0, were weighed into 15 ml centrifugation tubes. Five different extraction solvents were tested. Three of them were already described in the literature and two are proposed in this study: 0.2 M KOH/acetonitrile (3:1 v/v) (Wetzstein et al., 2009), 50 mM H₃PO₄/acetonitrile (1:1 v/v) (Golet et al. 2002), acetone/water/NH₃ (2:1:1 v/v) (Turiel et al. 2006), acetone/0.1 M KOH (1:1 v/v) and acetone/0.2 M KOH (1:1 v/v). Three ml of extraction solvent were added to each tube and then the tube was sonicated in an ultrasonic bath (Elma Hans Schmidbauer GmbH & Co. KG, Singen, Germany) at 35 KHz for 30 minutes at ambient temperature. Soil and solvent were separated by centrifugation at 3000 g for 12 min at 4°C. The organic solvent used during the first extraction was removed from the tube after centrifugation and the volume determined with a microliter syringe. The residual soil pellet was re-extracted until no additional radioactivity could be measured by LSC.

In addition, the extracted soil was re-extracted with pressurized steam in a commercial solid-sample extractor (Europiccola-professionel, LaPavoni, San Giuliano, Italy, modified with Teflon sealings) with approximately 120 ml of steam at 1 bar pressure, to simulate maximum water leaching.

Soil extractions of ciprofloxacin residues by ASE

Ciprofloxacin residues for mass balance determination were extracted using an ASE 200 accelerated solvent extraction system (Dionex, Sunnyvale, CA) equipped with 33 ml stainless steel extraction cells. Five grams of soil were mixed with Hydromatrix™ in the extraction cell. The samples were extracted with a solvent mixture of 63% ethyl acetate,

25% methanol and 3% ammonium hydroxide at the following operating conditions: extraction temperature, 100 °C; extraction pressure, 120 bar; preheating period, 5 min; static extraction period, 30 min; number of extraction cycles, 5; solvent flush, 50% of the cell volume; nitrogen purge, 120 s. A subsample of the extract was removed for ^{14}C analysis and the remaining sample was diluted with MilliQ water until < 5% solvent content for purification and chemical analysis.

2.4.4 Non-extractable residues in soil

Between 2 mg and 10 mg of pre-extracted air-dried soil sample were analysed for NER by ^{13}C analysis by means of EA-C-IRMS, for 2,4-D and ibuprofen..

In the case of ciprofloxacin, the determination of the initial total radioactivity in soil and the ^{14}C label in non-extractable residues after extraction was performed as follows: 1 g of soil sample was air dried at 40°C and combusted in a biooxidizer (Biological oxidizer OX 500, Zinsser Analytic, Frankfurt, Germany) at 900°C according to Weiß et al. (2004). The CO_2 produced during combustion was absorbed in Oxysolve 400 (Zinsser Analytic GmbH, Frankfurt, Germany) and analysed by LSC (Perkin Elmer Wallac GmbH, Freiburg, Germany).

2.4.5 Chemical analyses

The identification of the parent compounds and their metabolites in a sample was done by comparison of their retention times with those of an authentic standard.

2.4.5.1 2,4-D and ibuprofen determination and their metabolites

MM and diluted soil extracts were acidified to pH 2 and purified by solid phase extraction (SPE). The SPE cartridges (CHROMABOND® EASY, 200 mg; Macherey Nagel, Düren, Germany) were conditioned with 5 ml of methanol and 5 ml of de-ionised water. After the conditioning, the sample was passed slowly through the column applying a slight vacuum. The SPE column was then washed with 10 ml of de-ionised water and dried under vacuum using a vacuum pump (KNF Neuberger, Frankfurt, Germany) for 20 min. The columns were eluted twice with 5 mL of methanol-acetonitrile mixture (1:1, v/v) for 2,4-D and its metabolites and with 5ml of methanol-tetrahydrofuran (1:1, v/v) for

ibuprofen. A subsample of the purified extract samples (purified extract) was used for ^{13}C analysis.

The extracts were evaporated under a gentle stream of nitrogen. Dried samples were silylated with 40 μl acetonitrile and 80 μl bis-trimethylsilyltrifluoroacetamide (BSTFA) for 10 min at 60 $^{\circ}\text{C}$, cooled down to room temperature and finally transferred into a GC vial. Target chemicals were identified and quantified by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A GC (Agilent Technologies, Germany) equipped with a HP-5MS column (30 m $\times$ 250 μm $\times$ 0.25 μm ; Agilent Technologies, Germany) and coupled to an Agilent 5975C quadrupole mass spectrometer (Agilent Technologies, Germany), using the temperature program of Zwiener et al. (2002): initial temperature 60 $^{\circ}\text{C}$ (1.5 min), heat to 120 $^{\circ}\text{C}$ (0 min) at 20 $^{\circ}\text{C}/\text{min}$, to 160 $^{\circ}\text{C}$ (0 min) at 4 $^{\circ}\text{C}/\text{min}$ and finally to 260 $^{\circ}\text{C}$ (5 min) at 16 $^{\circ}\text{C}/\text{min}$. The injector was set at 260 $^{\circ}\text{C}$, the transfer line was held at 300 $^{\circ}\text{C}$ and the He flow was set to 1.5 mL min $^{-1}$.

The isotopic compositions of the parent compound and its metabolites were determined by GC-C-IRMS (Finnigan MAT 253 coupled to Trace GC, Thermo Electron, Bremen, Germany). The compounds were separated on a BPX-5 column (50 m $\times$ 0.32 mm $\times$ 0.5 μm ; SGE International; Darmstadt, Germany) with the following temperature program: 60 $^{\circ}\text{C}$ (2 min), 160 $^{\circ}\text{C}$ (0 min) at 20 $^{\circ}\text{C}/\text{min}$, 260 $^{\circ}\text{C}$ (0 min) at 6 $^{\circ}\text{C}/\text{min}$, 300 $^{\circ}\text{C}$ (5 min) at 20 $^{\circ}\text{C}/\text{min}$.

2.4.5.2 Ciprofloxacin determination and its metabolites

Between 2 and 50 μl of filtered MM samples over 0.22 μm cellulose filter were spotted on silica gel plates (20 x 20 cm Silica gel 60 F₂₅₄, Merck, Darmstadt, Germany) and analysed by Thin Layer Chromatography (TLC) developed with a mobile phase composed of dichloromethane, methanol, 2-propanol, and 25% NH_3 (3:3:5:2) (Sukul et al., 2009). Identification was carried out at 254 nm with reference ciprofloxacin. The radioactivity on the TLC plates was determined with a Linear TLC Analyser LB284 (Berthold GmbH & Co KG, Bad Wildbad, Germany) with standard deviations <5% as described by Weiß et al. (2004).

The diluted soil extracts were acidified to pH 3 and purified by SPE as described by Uslu et al., (2008). Samples were passed through a 500 mg anion-exchange (MAX) cartridge

(Waters, Taunton, USA) stacked on top of a 500 mg hydrophilic–lipophilic balance (HLB) cartridge (Waters, Taunton, USA). Before sample application, the columns were conditioned with 3 mL of methanol and 3 mL of pH 3 de-ionised water. After sample application, the columns were washed with 10 mL of de-ionised water and dried under vacuum using a vacuum pump (KNF Neuberger, Frankfurt, Germany) for 15 min. The columns were eluted with 5 mL methanol/NH₃ 6%. The solvent was evaporated under nitrogen and the samples resuspended in the mobile phase of the subsequent analysis.

Ciprofloxacin was quantified by reversed phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a Thermo Fisher Surveyor HPLC-system (Thermo Fisher Corporation, USA) equipped with a Phenomenex Luna PFP(2) column (150 x 2 mm, 3 µm particle size; Aschaffenburg, Germany). 10 µL samples were separated with the following gradient program (solvent A: 1 mM ammonium acetate and 0.1% HCOOH in water, solvent B: 0.1% HCOOH in methanol): 90% A for 2 min, followed by a linear gradient to 50% A over 23 min, and to 100% B within the next 1 min. Subsequently, the column was rinsed with 100% B for 5 min, and then the system was returned to its initial condition (90% A) within 1 min where it was held for 5 min before the next run was started. The mobile phase flow rate was 0.3 mL min⁻¹. The column temperature was kept at 26°C. The mass spectra were acquired using a TSQ Quantum Ultra AM triple quadrupole mass spectrometer (Thermo Fisher Corporation, USA) equipped with a HESI-II ion source (Ion Max) operating in positive mode. Nitrogen was both the drying and the nebulizer gas, and argon (1.5 bars) was the collision gas. The capillary temperature for the TSQ Quantum was 250°C and the vaporizer temperature 350°C. The MS/MS parameters (tube lens, collision energy) were optimized in continuous flow mode for maximum sensitivity for product ions, and the two most sensitive SRM (Selected Reaction Monitoring) transitions were determined for each molecule (for instrument parameters and SRM data for ciprofloxacin, see **Table 4**).

Table 4. SRM data, retention time, LOD and LOQ of ciprofloxacin

Compound	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Collision Energy (eV)	LOD ($\mu\text{g L}^{-1}$)	LOQ ($\mu\text{g L}^{-1}$)
Ciprofloxacin	19.07	332.1	314.1	-19	5	15
		332.1	288.0	-17		

Ciprofloxacin related metabolites were identified by electrospray ionization high resolution mass spectrometry (ESI-HR-MS) with an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA) as described by Sukul et al. (2009). Nitrogen was used as sheath gas (5 arbitrary units) and helium served as the collision gas. The spectrometer was operated in positive mode (1 spectrum s^{-1} ; mass range: 50-1000) with nominal mass resolving power of 60000 at m/z 400 and with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation. Bis(2-ethylhexyl)phthalate (m/z = 391.284286) was used as an internal calibration standard. The spectrometer was equipped with a Dionex HPLC system Ultimate 3000 consisting of auto-sampler (injection volume 0.5 μL) and Flow Manager, pump, and UV detector (λ , 254 nm). The separations were performed by using a Phenomenex Gemini C18 column (3 μ , 0.3 x 150 mm) (Torrance, CA, USA) with H_2O (+ 0.5% HCOOH) (A) / acetonitrile (+ 0.1% HCOOH) (B) gradient (flow rate 4 $\mu\text{L}/\text{min}$). Samples were analyzed by using a gradient program as follows: 90% A isocratic for 4 min, linear gradient to 50% A over 10 min, and to 100% B during another 2 min, after 100% B isocratic for 10 min, the system returned to its initial condition (90% A) within 1 min, and was equilibrated for 7 min before injection of the next sample.

2.5 Inhibition of microorganisms by ciprofloxacin

In addition to the inhibition test recommended in the OECD guideline 301 (OECD, 1992), the inhibition of *Pseudomonas putida* mt-2 and of soil microbial communities by the antimicrobial effect of ciprofloxacin was studied.

2.5.1 Inhibition study in pure culture

The EC₅₀ for *Pseudomonas putida* mt-2 in pure culture was determined as described by Heipieper et al. (1995). The strain was cultivated in the mineral medium described in **Table 5** with succinate as sole carbon source. Cells were grown in 100 ml shake cultures in a horizontally shaking water bath at 30°C and 145 rpm. An inoculum from an overnight culture (5 ml) was transferred to 100 ml fresh medium. After 4 hours of growth, ciprofloxacin was added. Cell growth was measured by monitoring the turbidity (OD_{560nm}) of the cell suspensions using a spectrophotometer (Lambda 2 Spectrophotometer, Perkin Elmer, Rodgau, Germany). Growth inhibition caused by the antibiotic was measured by comparing the differences in growth rates μ between cultures amended with ciprofloxacin with that of a control culture as described by Keweloh et al. (1989). For better comparability between different cultures, the growth rates of the cultures grown with the antimicrobial are given as a percentage of the growth rate in the control cultures (Eq. 1).

$$\text{Eq. 1: inhibited growth (\%): } \frac{\mu 1 (+ \text{ ciprofloxacin})}{\mu 0 (\text{control})} \times 100$$

Table 5. Mineral medium for *Pseudomonas putida* mt-2

Component	Concentration
Na ₂ HPO ₄ x 2 H ₂ O	7 g L ⁻¹
KH ₂ PO ₄	2.8 g L ⁻¹
NaCl	0.5 g L ⁻¹
NH ₄ Cl	1 g L ⁻¹
HCl	0.005 %
MgSO ₄ x 7 H ₂ O	0.1 g L ⁻¹
FeSO ₄ x 7 H ₂ O	0.01 g L ⁻¹
MnSO ₄ x H ₂ O	0.005 g L ⁻¹
ZnCl ₂	0.0064 g L ⁻¹
CaCl ₂ x 2 H ₂ O	0.01 g L ⁻¹
BaCl ₂ x 2 H ₂ O	0.0006 g L ⁻¹
CoSO ₄ x 7 H ₂ O	0.0004 g L ⁻¹
CuSO ₄ x 5 H ₂ O	0.0004 g L ⁻¹
H ₃ BO ₃	0.0065 g L ⁻¹
EDTA	0.01 g L ⁻¹
di-sodium succinate	4 g L ⁻¹

2.5.2 Inhibition studies in soil

To study the effects of ciprofloxacin on soil microbial activity (by means of soil respiration), on the composition of the soil microbial community (by terminal restriction fragment length polymorphism [T-RFLP] analysis using universal primers for the 16S rDNA), and on the induction of antibiotic resistance, similar soil incubations as the ones described before (section 2.3.2) were performed with ciprofloxacin hydrochloride at different concentrations (0 mg l⁻¹, 0.2 mg l⁻¹, 2 mg l⁻¹ and 20 mg l⁻¹) each in triplicate in a Sapromat® E BOD Measuring Unit (H+P Labortechnik, Oberschleissheim, Germany). The experiment lasted for 113 days and soil respiration measurements were constantly recorded during the first 77 days of the experiments. Due to technical problems with the equipment no more soil respiration data could be obtained thereafter. 1 g of soil samples were taken on days 3, 14, 29, 65 and 113 days of incubation for T-RFLP analyses and detection of ciprofloxacin resistance genes.

Soil DNA extraction, T-RFLP analyses and detection of resistance genes

Total soil DNA was extracted from 0.5 g of soil using the UltraClean® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA).

T-RFLP analyses of the extracted DNA were performed as described by Bombach et al. (2010). 16S rDNA was amplified by PCR with the primers 27f (Lane, 1991) and 1378R (Heuer et al., 1997). The reaction conditions for the 25 µL reaction (Hot Start Taq PCR master mix Qiagen, 0.5 µM of each primer [final concentration], and 1 µL template DNA) were: 15 min at 95 °C, 32 cycles of 30 s at 94 °C, 30 s at 52 °C, 1.2 min at 72°C, and a final extension for 10 min at 72 °C. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany), and quantified by a NanoDrop ND-100 device (NanoDrop Technologies. USA). Sixty ng of purified PCR products were digested with 10U of MspI (Fermentas, Germany) in a total volume of 10 µL at 37°C overnight. Digestion was stopped at 65°C for 20 min, followed by precipitation with 25 µL absolute ethanol and 1 µL 3 M sodium acetate (pH 4.8). The digests were purified with 300 µL of ethanol 70%, the pellet dried and mixed with 20 µL HiDi and 0.4 µL GeneScan-500 ROX Sixe Standard (Applied Biosystems, Carlsbad, USA). The samples were denaturated at 95°C for 10 minutes and immediately transferred

to ice. Finally the samples were loaded on an ABI3100 genetic analyser (Applied Biosystems Carlsbad, USA). T-RFs were analysed using the GENEMAPPER software version 3.7 (Applied Biosystems, Carlsbad, USA). T-RFs smaller than 50 bp were excluded from the analysis.

Resistance genes *qnrA*, *qnrB* and *qnrS* (Cattoir et al. 2007) were amplified with specific primers and analysed using the PCR reagents described above using the PCR program: 10 min at 95 °C, 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 45 s at 72°C, and a final extension for 10 min at 72 °C. 5 µL of the PCR products were analysed in a 2% agarose gel.

2.6 Data analyses and statistics

Labelled isotope tracers (^{13}C and ^{14}C) were employed to investigate biodegradation of the model compounds and to obtain an accurate and detailed mass balance of their degradation in aqueous media and soil. In aqueous media, the mineralised fraction, C-label in medium and in SS, and the extractable fraction (including parent compounds and their metabolites) were determined. In soil experiments, the mineralised fraction, the extractable fraction (including parent compounds and their metabolites), and the NER (including biogenic residues) were determined. Each fraction was presented as the percentage of the initially applied C-labelled compound to an experiment and quantified at each sampling date. From these data, the recovery was calculated, to determine the dissipation kinetics and a complete mass balance. Moreover, the application of C-labelled compounds in biodegradation studies allows identifying metabolites and analysing biogenic residues using sophisticated analytical techniques such as GC-MS and LC-MS (Richnow et al., 1999).

However, ^{13}C is also naturally present in soil (~ 1%); consequently blank (without compound application) and control (amended with ^{12}C -labelled compound) samples are needed for the correction of ^{13}C abundance in a soil amended with the tested ^{13}C -labelled compound. Likewise, ^{13}C abundance in the aqueous experiments was also corrected by the blank and control samples. The amount of ^{13}C in each fraction described above was estimated according to Lerch et al. (2009a).

All experiments were performed in triplicate and the results are presented as means with standard deviation. The error bars represent the standard deviation of these triplicates.

To visualise the changes caused by ciprofloxacin on the soil microbial communities, non-metric Multidimensional Scaling Analysis (MDS) were performed using the Bray-Curtis distance and Jaccard index measure on the T-RFLP data (Rees et al. 2004). A two-way PERMANOVA was used to test between groups and ANOSIM within treatment differences of the T-RFLP data results. Statistical tests were conducted with PAST (Hammer et al., 2001). Differences were regarded as statistically significant if $P < 0.05$.

Results

The biodegradation and fate of C labelled 2,4D, ibuprofen and ciprofloxacin was studied in aqueous and soils systems. The aim was to develop a conceptual approach of the environmental fate of the three model compounds in the two different environmental systems, providing some general rules for using biodegradability data from ready biodegradability test in aqueous medium for predicting biodegradation in soil. The main focus was to obtain a detailed mass balance of the degradation in both systems and also to assess the effects of these compounds in the environment.

3.1 Biodegradation of 2,4-D

3.1.1 Aqueous media (OECD 301B test)

Mineralisation of $^{14}\text{C}_6$ -2,4-D in MM consisted of 3 phases (**Figure 13**). An initial lag phase, from day 0 to day 12, characterised by very low degradation rates ($7.8 \mu\text{g day}^{-1}$), indicating adaptation of the degrading microbes to the introduced chemical. The degradation rate was highest ($489 \mu\text{g day}^{-1}$) during the second phase, from day 12 to day 20. During the third phase, from day 20 to 28, the degradation rate decreased to $136 \mu\text{g day}^{-1}$. At the end of the incubation, 85.0% of $^{13}\text{C}_6$ -2,4-D was mineralised. In contrast, no mineralisation was observed under abiotic conditions, highlighting the low significance of abiotic processes under these conditions.

Acetate was readily biodegraded and the herbicide at the applied concentration (20 mg kg^{-1}) barely inhibited acetate mineralisation, suggesting that 2,4-D and its degradation products are only slightly toxic for the microbial community of activated sludge. After 28 days, the incorporation of the label in SS was higher in the abiotic incubations (23.7%;

Table 6) than in the biotic ones (3.9%) indicating that biodegradation is responsible for this difference. Recoveries ranged between 83 and 101% (**Table 6**).

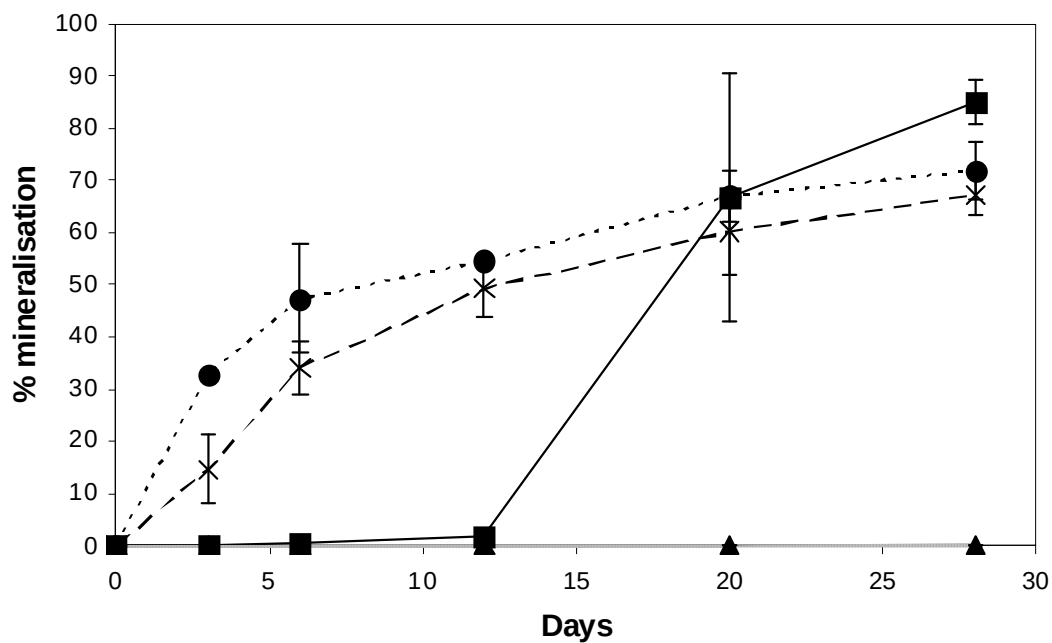


Figure 13. Mineralisation of $^{14}\text{C}_6$ -2,4-D and ^{14}C -acetate in mineral medium according to the OECD test 301B protocol. (●) Acetate (control), (X) inhibition of acetate degradation, (■) 2,4-D biotic, (▲) 2,4-D abiotic. Percentages refer to the total radioactivity applied.

Table 6. Mass balance from 2,4-D degradation in mineral medium (% of initially applied ^{14}C)

	Time (days)	% of initial ^{14}C			Recovery
		Mineralisation	^{14}C in medium ^a	^{14}C in suspended solids	
Biotic	3	0.208 ($\pm$ 0.04)	100.1 ($\pm$ 0.81)	n.a.	100.3 ($\pm$ 0.82)
	6	0.346 ($\pm$ 0.04)	99.9 ($\pm$ 0.73)	n.a.	100.6 ($\pm$ 0.72)
	20	66.8 ($\pm$ 23.8)	25.4 ($\pm$ 22.9)	n.a.	92.2 ($\pm$ 4.41)
	28	84.9 ($\pm$ 4.28)	5.3 ($\pm$ 0.38)	3.91 ($\pm$ 0.38)	93.8 ($\pm$ 3.70)
Abiotic	20	0.029 ($\pm$ 0.01)	105.6 ($\pm$ 2.42)	n.a.	105.6 ($\pm$ 2.43)
	28	0.040 ($\pm$ 0.02)	97.4 ($\pm$ 3.76)	23.7 ($\pm$ 5.27)	98.0 ($\pm$ 3.76)
Acetate	3	32.9 ($\pm$ 0.20)	42.4 ($\pm$ 15.9)	n.a.	86.6 ($\pm$ 4.17)
	6	47.3 ($\pm$ 5.02)	34.3 ($\pm$ 7.70)	n.a.	82.8 ($\pm$ 2.39)
	20	66.0 ($\pm$ 4.86)	23.5 ($\pm$ 3.06)	n.a.	90.4 ($\pm$ 7.92)
	28	71.9 ($\pm$ 5.49)	16.4 ($\pm$ 4.01)	9.72 ($\pm$ 0.50)	88.9 ($\pm$ 8.46)
Inhibition	3	14.6 ($\pm$ 6.66)	81.4 ($\pm$ 2.21)	n.a.	96.1 ($\pm$ 3.43)
	6	34.2 ($\pm$ 5.16)	56.5 ($\pm$ 1.65)	n.a.	90.7 ($\pm$ 6.41)
	20	60.2 ($\pm$ 8.16)	29.5 ($\pm$ 9.23)	n.a.	89.6 ($\pm$ 10.1)
	28	67.2 ($\pm$ 3.65)	27.0 ($\pm$ 1.74)	9.51 ($\pm$ 3.72)	94.1 ($\pm$ 5.46)

^a includes ^{14}C in suspended solids

n.a: not analysed

values in brackets ($\pm$) represent the standard deviation of the average of triplicates

3.1.2 Soil (OECD 307 test)

In comparison to the results in water, the mineralisation of $^{13}\text{C}_6$ -2,4-D under biotic conditions in soil proceeded in two phases (**Figure 14A**). 2,4-D mineralisation started quickly, without a pronounced lag phase and at relatively high rates ($30 \mu\text{g day}^{-1}$). The activity was high until day 8, when 30 % of the compound was mineralised. Finally, during the second phase (day 8 to 64) the mineralisation rate decreased ($4 \mu\text{g day}^{-1}$). By the end of the incubation 58% of the compound was mineralised.

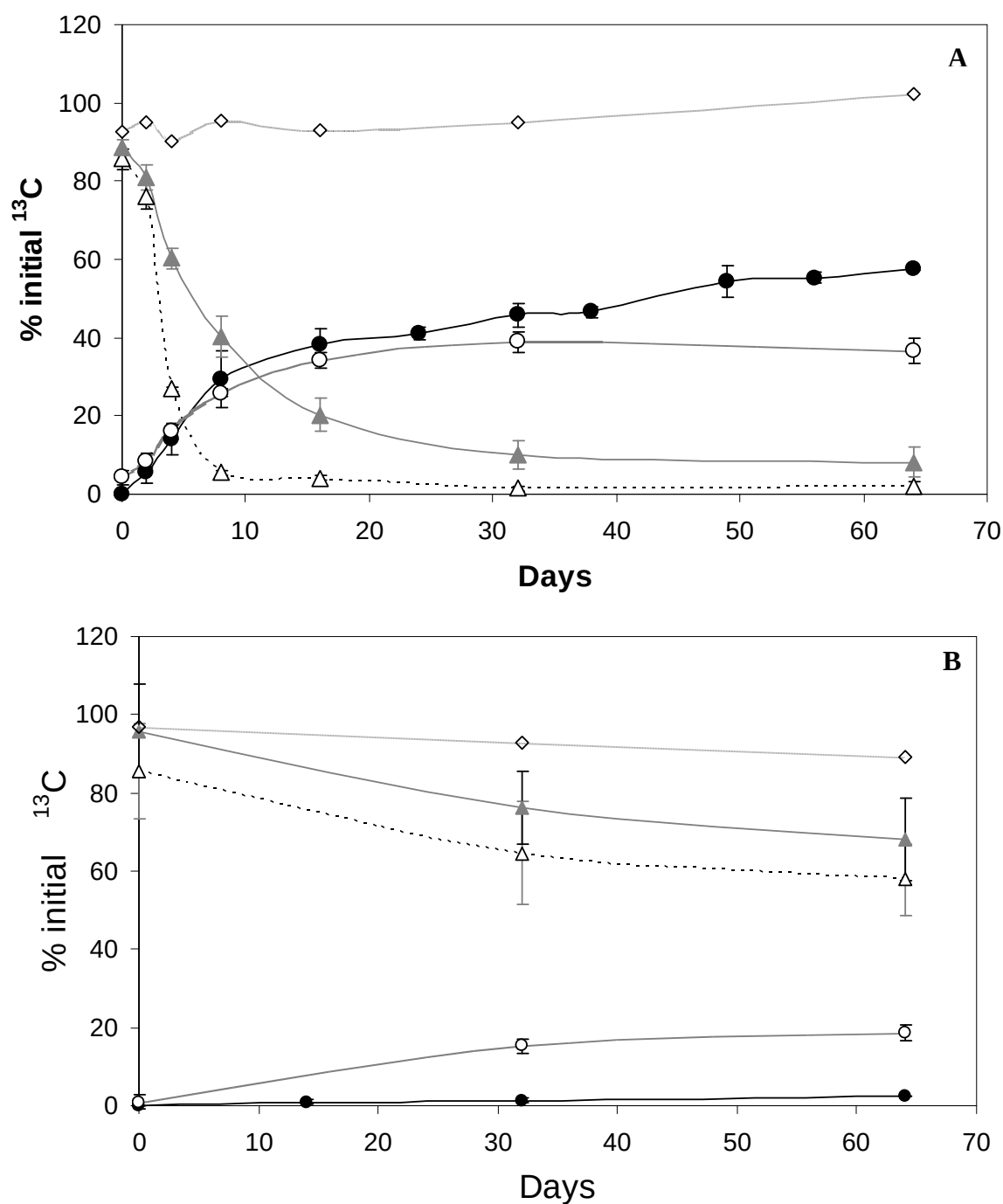


Figure 14. Degradation of $^{13}\text{C}_6$ -2,4-D in soil under biotic (A) and abiotic conditions (B) according to the OECD test 307 protocol. ($\bullet$) Mineralisation, ($\blacktriangle$) extractable amount before purification, ($\triangle$) extractable amount after purification, ($\circ$) non-extractable residues and recovery ($\diamond$). Percentages refer to the total ^{13}C -label applied.

^{13}C -label in the crude extract (before purification by SPE) decreased rapidly with time. At the beginning of the incubation, 88% of the initial ^{13}C amount could be extracted. The extractability decreased to 40% after 8 days and to 8% after 64 days. In addition, the amount of ^{13}C -label remaining in the purified extract, which mainly corresponds to the parent compound and metabolites, decreased even faster than the crude extract, from 86% at the beginning to 5.6% at day 8 and to 2 % at the end of the incubation. These results are consistent with the GC-MS analysis of 2,4-D and its metabolites in the purified extracts (**Table 7**). Only the parent compound and its metabolite 2,4-dichlorophenol (2,4-DCP) were detected, whereas chlorohydroquinone was never detected. 2,4-DCP was detected only until day 4, which is consistent with the reported rapid formation of metabolites by biotic or abiotic reactions in soil (Boivin et al. 2005).

Low amounts of NER were determined at the beginning of the incubations, indicating limited sorption of 2,4-D to soil. However, a rapid increase in the biotic incubations (from 4.3% to 26%) was observed until day 8 (**Figure 14A**), whereas the level remained stable from day 16 onward. At the end of the experiment, NER accounted for 37% of the initially added ^{13}C label.

In the abiotic systems (**Figure 14B**), mineralisation was low (2.5% of the initial ^{13}C). NER amounted to 18.6% of the applied ^{13}C at the end of the incubation, much less than in the biotic systems. On day 0, the crude extractable fraction accounted for 95% of the applied ^{13}C . This fraction decreased to 68% at the end. Moreover, crude and purified extracts were not significantly different. This and the lower amount of NER in these systems demonstrate the importance of microbial activity for NER formation. 2,4-D was also transformed abiotically: 11% of the applied $^{13}\text{C}_6$ -2,4-D was found as $^{13}\text{C}_6$ -2,4-DCP after 32 and 64 days (**Table 7**). Recoveries for biotic and abiotic incubations ranged between 89 and 102% (**Table 7**).

Table 7. Mass balance, parent compound and metabolite from 2,4-D degradation in soil (% of initially applied ^{13}C and % of initial $^{13}\text{C}_6$ -2,4-D)

		% of initial ¹³ C ^a					% of initial ¹³ C ₆ -2,4-D ^b	
	Time (days)	Mineralisation	¹³ C in crude extract	¹³ C in purified extract	NER	Recovery	2,4-D	2,4-DCP
Biotic	0	0	88.6 (± 2.11)	85.6 (± 2.80)	4.25 (± 1.80)	92.8 (± 5.38)	100	n.d.
	2	5.79 (± 2.99)	80.9 (± 3.32)	76.2 (± 3.30)	8.53 (± 1.82)	95.2 (± 14.2)	94.3 (± 7.03)	21.5 (± 0.52)
	4	14.0 (± 3.91)	60.3 (± 2.55)	26.8 (± 0.64)	16.0 (± 1.81)	90.3(± 7.96)	30.4 (± 2.19)	7.42 (± 0.48)
	8	29.4 (± 7.28)	40.1 (± 5.17)	5.58 (± 0.55)	25.8 (± 1.02)	95.4 (± 4.89)	2.28 (± 0.20)	n.d.
	16	38.4 (± 3.97)	20.3 (± 4.22)	4.18 (± 0.54)	34.3 (± 1.99)	92.9 (± 24.3)	1.22 (± 0.24)	n.d.
	32	45.7 (± 2.92)	10.2 (± 3.63)	1.44 (± 0.58)	38.9 (± 2.64)	94.9 (± 9.73)	2.54 (± 3.16)	n.d.
	64	57.6 (± 0.33)	8.2 (± 3.78)	2.07 (± 1.10)	36.5 (± 3.19)	102.3 (± 6.97)	0.52 (± 0.05)	n.d.
Abiotic	0	0	95.8 (± 12.0)	85.6 (± 2.80)	1.00 (± 1.79)	96.8 (± 13.8)	100	n.d.
	32	1.38 (± 0.55)	76.2 (± 9.21)	64.6 (± 13.1)	15.3 (± 1.81)	92.9 (± 11.6)	81.0 (± 0.67)	11.2 (± 2.13)
	64	2.45 (± 0.16)	68.1 (± 10.4)	57.8 (± 9.27)	18.6 (± 1.89)	89.2 (± 12.4)	75.6 (± 5.19)	10.9 (± 1.32)

^a 100% is equal to initially added ^{13}C ^b 100% corresponds to initially measured amount of $^{13}\text{C}_6$ -2,4-D

n.a: not analysed

n.d: not detectable

values in brackets ($\pm$) represent the standard deviation of the average of triplicates

3.2 Biodegradation of ibuprofen

3.2.1 Aqueous media (OECD 301B test)

The biotic mineralisation of $^{13}\text{C}_6$ -ibuprofen in MM followed sigmoidal kinetics. A clear lag phase from day 0 to day 6 was observed, in which the mineralisation rate was low ($0.16\ \mu\text{g day}^{-1}$), indicating adaptation of the microorganisms to ibuprofen. Between day 6 and 13, the mineralisation rate was high ($300\ \mu\text{g day}^{-1}$; **Figure 15**). In the last phase, the degradation rate decreased to $126\ \mu\text{g day}^{-1}$, but mineralisation continued until the end of the experiment. Incorporation of the ^{13}C -label into suspended solids was high until day 6 most probably due to sorption of ibuprofen to suspended sludge particles and also to incorporation of it into biomass (**Table 8**). The proportion of ^{13}C in suspended solids decreased with time, indicating that ibuprofen is adsorbed reversibly to the activated sludge particles or can be degraded in the sorbed state (Kimura et al. 2007). Consistent with the long lag phase, no ibuprofen-derived metabolites were found in the biotic incubations until day 20 (**Table 8**).

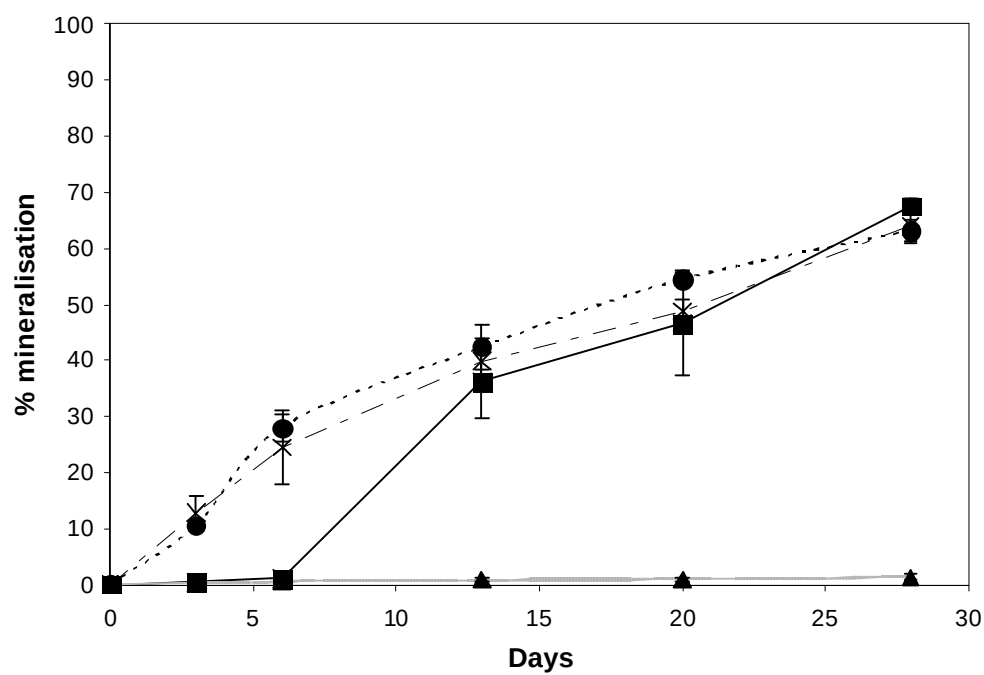


Figure 15. Mineralization of $^{13}\text{C}_6$ -ibuprofen and $^{13}\text{C}_2$ -acetate in mineral medium according to the OECD test 301B protocol. (●) acetate (control), (x) inhibition of acetate degradation, (■) ibuprofen biotic, (▲) ibuprofen abiotic. Percentages refer to the total ^{13}C -label applied.

Table 8. Degradation mass balance from ibuprofen in mineral media (% of initially applied ^{13}C and % of initial $^{13}\text{C}_6$ -ibuprofen)

	Time (days)	% of initial $^{13}\text{C}^a$				% of initial $^{13}\text{C}_6$ -ibuprofen ^b	
		Mineralisation	^{13}C in medium	^{13}C in suspended solids	Recovery	Ibuprofen	2-hydroxyibuprofen
Biotic	0	0	n.a.	n.a.	n.a.	100	n.d.
	6	1.18 ($\pm$ 0.05)	78.6 ($\pm$ 15.6)	53.0 ($\pm$ 17.5)	79.8 ($\pm$ 15.7)	76.4 ($\pm$ 4.85)	n.d.
	13	36.3 ($\pm$ 6.52)	52.0 ($\pm$ 31.2)	40.5 ($\pm$ 32.6)	88.3 ($\pm$ 31.2)	0.169($\pm$ 0.18)	n.d.
	20	46.7 ($\pm$ 9.47)	40.2 ($\pm$ 18.8)	30.6 ($\pm$ 20.7)	86.9 ($\pm$ 18.8)	5.93 ($\pm$ 2.51)	0.022 ($\pm$ 2.65E-3)
	28	67.5 ($\pm$ 1.38)	18.0 ($\pm$ 3.20)	9.10 ($\pm$ 4.90)	85.5 ($\pm$ 4.58)	0.015 ($\pm$ 0.02)	0.005 ($\pm$ 6.46E-4)
Abiotic	0	0	n.a.	n.a.	n.a.	100	n.d.
	28	1.33 ($\pm$ 0.59)	107.6 ($\pm$ 8.82)	23.5 ($\pm$ 9.01)	108.9 ($\pm$ 9.41)	68.7 ($\pm$ 3.12)	n.d.
Acetate	28	63.2 ($\pm$ 1.88)	30.9 ($\pm$ 10.1)	17.0 ($\pm$ 11.3)	94.1 ($\pm$ 12.0)		
Inhibition	28	63.9 ($\pm$ 3.08)	22.5 ($\pm$ 6.80)	22.5 ($\pm$ 6.77)	86.4 ($\pm$ 9.84)		

^a 100% is equal to initially added ^{13}C ^b 100% corresponds to initially measured amount of $^{13}\text{C}_6$ -ibuprofen

n.a: not analysed

n.d: not detectable

values in brackets ($\pm$) represent the standard deviation of the average of triplicates

In contrast to the biotic experiments, the abiotic systems showed a low mineralisation (1.3% after 28 days; **Figure 15**), and 23.5% of the applied $^{13}\text{C}_6$ -ibuprofen was found adsorbed to suspended solids at the end of the incubation (**Table 8**). This result is consistent with the ^{13}C -label sorbed to SS in the beginning of the biotic incubation, where microbial activity was not relevant.

The acetate degradation curve and the inhibition test curve were not significantly different, indicating that ibuprofen did not inhibit the microbial activity at the applied concentration. Recoveries for each treatment were between 80% and 109% (**Table 8**).

3.2.2 Soil (OECD 307 test)

Biotic mineralisation of $^{13}\text{C}_6$ -ibuprofen in soil started rapidly, without a clear lag phase, and consisted of four phases (**Figure 16A**). From day 0 to day 14, the mineralisation rate was relatively low ($6.1 \mu\text{g day}^{-1}$). From day 14 to day 22, degradation proceeded relatively fast ($12 \mu\text{g day}^{-1}$). After day 22, the degradation rate decreased to $3.5 \mu\text{g day}^{-1}$ and from day 71 onwards, no relevant degradation was observed. Overall, ibuprofen degradation kinetics did not follow a sigmoidal time course, suggesting limited degradation without or with poor microbial growth at the beginning of the incubation (first 15 days). At the end of the experiment, 45.2% of the applied ^{13}C -label has been mineralised.

The amount of ^{13}C -label in the crude extracts decreased from 95.6% of the initially applied one on day 0 to 13.4% on day 90. Again, the amount of ^{13}C -label in the purified extracts decreased faster than in the crude extract. On day 0, the parent compound and metabolites in the purified extract accounted for 81.0% of the initially added amount, on day 28 for 9.7% and remained very low from day 59 onwards (around 1%; **Table 9**). Also the GC-MS data show that ibuprofen decreased fast until day 28 and remained very low until the end of the experiment (0.3%).

At the beginning of the incubation only 2.7% of the applied ^{13}C -label was adsorbed to soil (**Figure 16A**), thus indicating a low sorption affinity of ibuprofen to the soil particles. NER increased gradually until day 28, and then remained constant at around 30% of the initially added ^{13}C -label until the end of the experiment.

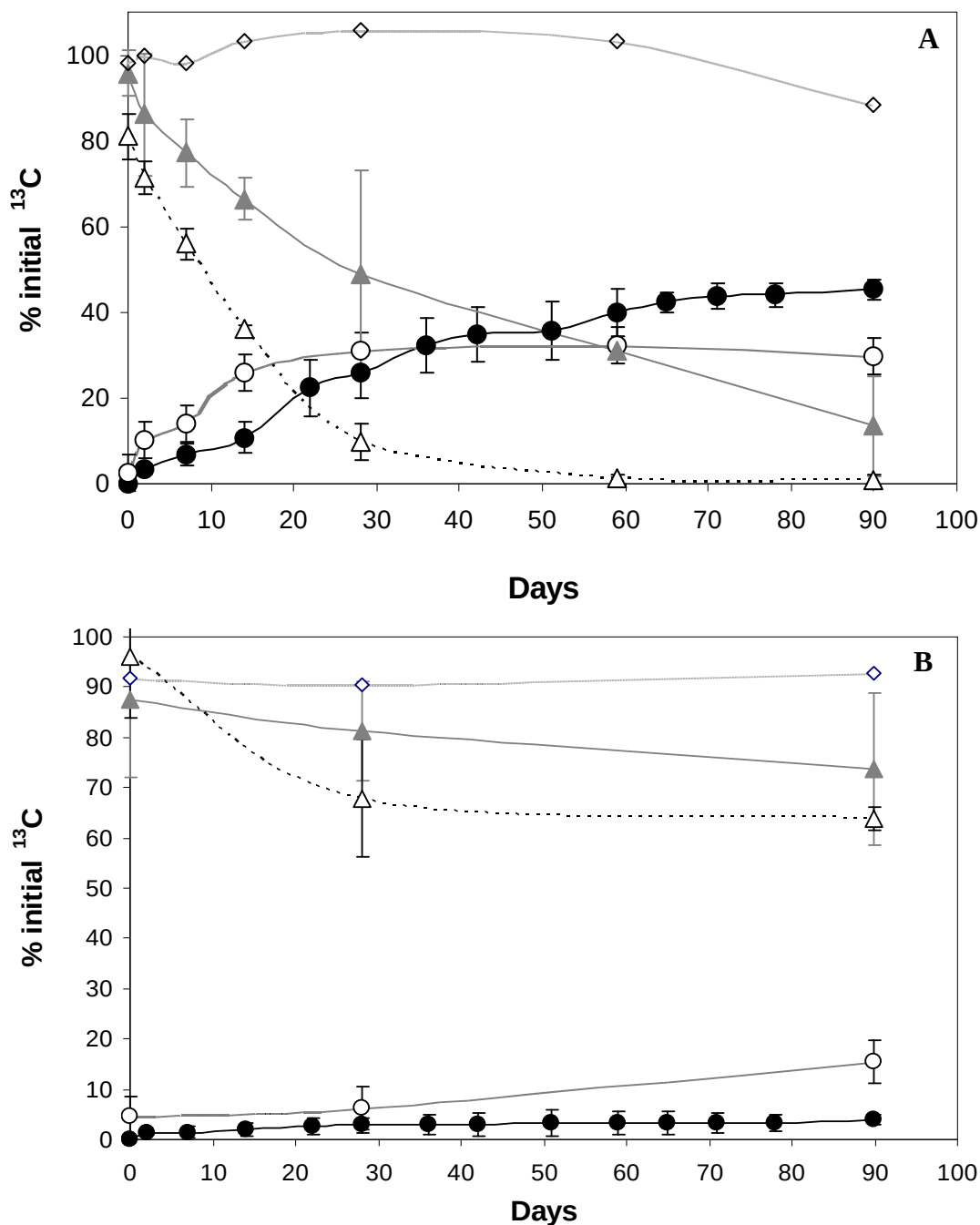


Figure 16. Degradation of $^{13}\text{C}_6$ -ibuprofen in soil under biotic (A) and abiotic (B) conditions according to the OECD test 307 protocol. (●) Mineralisation, (▲) extractable amount before purification, (△) extractable amount after purification, (○) non-extractable residues and (◇) recovery. Percentages refer to the total ^{13}C -label applied.

Under abiotic conditions, total mineralisation accounted for 3.9% of the initially added ^{13}C -label at the end of the incubation, and only 15.4% of the applied ^{13}C was found in NER (**Figure 16B**). Crude and purified extracts were not significantly different and no

metabolites were detected (**Table 9**), indicating an irrelevant contribution of abiotic processes in the biodegradation of ibuprofen in soil. Recoveries ranged between 88 and 105% of the applied ^{13}C for biotic and abiotic incubations.

Table 9. Degradation mass balance from ibuprofen in soil (% of initially applied ^{13}C and % of initial $^{13}\text{C}_6$ -ibuprofen)

		% of initial $^{13}\text{C}^a$				% of initial $^{13}\text{C}_6$ -ibuprofen ^b		
	Time (days)	Mineralisation	^{13}C in crude extract	^{13}C in purified extract	NER	Recovery	Ibuprofen	2-hydroxyibuprofen
Biotic	0	0	95.6 ($\pm$ 5.38)	81.0 ($\pm$ 5.38)	2.67 ($\pm$ 4.22)	98.3 ($\pm$ 5.38)	100	n.d.
	2	3.36 ($\pm$ 1.21)	86.1 ($\pm$ 14.2)	71.4 ($\pm$ 3.81)	10.3 ($\pm$ 4.25)	99.2 ($\pm$ 14.2)	91.0 ($\pm$ 9.10)	1.13 ($\pm$ 0.21)
	7	6.81 ($\pm$ 2.58)	77.2 ($\pm$ 7.96)	56.0 ($\pm$ 3.67)	14.0 ($\pm$ 4.24)	98.0 ($\pm$ 7.96)	58.0 ($\pm$ 10.2)	1.30 ($\pm$ 0.37)
	14	10.6 ($\pm$ 3.59)	66.4 ($\pm$ 4.89)	36.0 ($\pm$ 1.04)	26.0 ($\pm$ 4.27)	103.1 ($\pm$ 4.89)	26.6 ($\pm$ 2.98)	6.16 ($\pm$ 0.81)
	28	26.0 ($\pm$ 5.92)	48.9 ($\pm$ 24.3)	9.73 ($\pm$ 4.41)	31.0 ($\pm$ 4.27)	105.8 ($\pm$ 24.3)	2.66 ($\pm$ 1.65)	0.502 ($\pm$ 1.16)
	59	39.9 ($\pm$ 5.47)	31.0 ($\pm$ 0.48)	1.10 ($\pm$ 0.91)	32.2 ($\pm$ 4.25)	103.1 ($\pm$ 9.73)	0.602 ($\pm$ 0.06)	n.d.
	90	45.2 ($\pm$ 2.16)	13.4 ($\pm$ 11.6)	0.80 ($\pm$ 1.33)	29.6 ($\pm$ 4.25)	88.2 ($\pm$ 11.6)	0.502 ($\pm$ 0.11)	n.d.
Abiotic	0	0	87.3 ($\pm$ 15.1)	96.0 ($\pm$ 12.1)	4.48 ($\pm$ 4.23)	91.8 ($\pm$ 15.1)	100	n.d.
	28	2.89 ($\pm$ 1.21)	81.2 ($\pm$ 9.88)	67.9 ($\pm$ 11.6)	6.30 ($\pm$ 4.22)	90.4 ($\pm$ 9.88)	82.3 ($\pm$ 10.9)	n.d.
	90	3.86 ($\pm$ 0.88)	73.6 ($\pm$ 39.0)	63.7 ($\pm$ 2.23)	15.3 ($\pm$ 4.30)	92.9 ($\pm$ 4.30)	91.6 ($\pm$ 26.4)	n.d.

^a 100% is equal to initially added ^{13}C ^b 100% corresponds to initially measured amount of $^{13}\text{C}_6$ -ibuprofen

n.d.: not detectable

values in brackets ($\pm$) represent the standard deviation of the average of triplicates

3.3 Biodegradation of ciprofloxacin

3.3.1 Aqueous media (OECD 301B test)

No mineralisation of [2- 14 C]-ciprofloxacin under biotic or abiotic conditions was observed in the aqueous system over 29 days of incubation (**Figure 17**), and only the parent compound was detected by TLC analyses (**Figure 18**) at that time. Consistent with this, the radioactivity in the MM remained relatively constant over time, and the radioactivity in SS was similar for abiotic and biotic incubations (**Table 10**). Ciprofloxacin is therefore recalcitrant to degradation in aqueous media. Recoveries ranged between 96% and 106% of the applied 14 C for biotic and abiotic incubations.

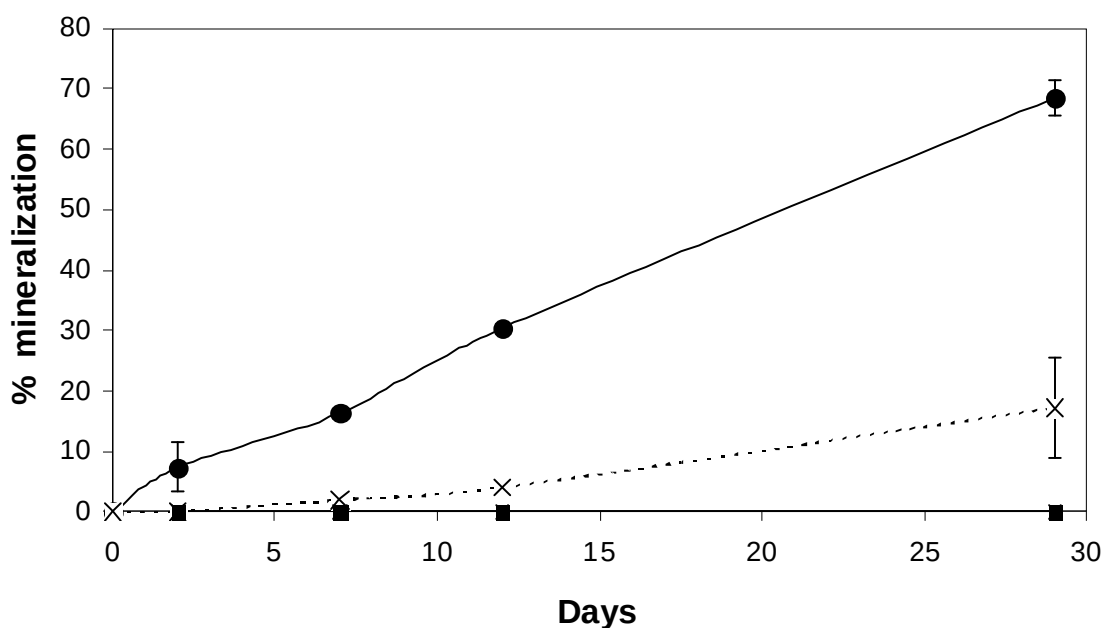


Figure 17. Mineralization of [2- 14 C]-ciprofloxacin and U- 14 C-acetate in mineral medium according to the OECD test 301B. (●) acetate alone (control), (x) acetate degradation in presence of unlabeled CIP (inhibition test), (■) ciprofloxacin biotic and abiotic. Percentages refer to the total radioactivity applied.

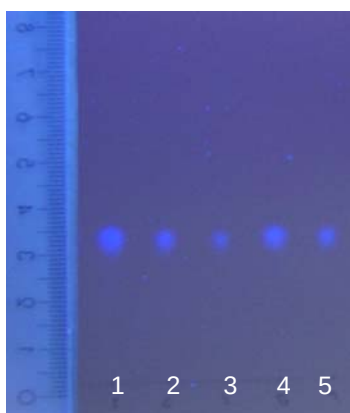


Figure 18. TLC analysis of ciprofloxacin in mineral medium: CIP standard (1), biotic replicates (2,3), abiotic replicates (4,5).

Table 10. Degradation's mass balance from ciprofloxacin in mineral media (% of initially applied ^{14}C)

	Time (days)	% of initial ^{14}C			Recovery
		Mineralisation	^{14}C in medium	^{14}C in suspended solids	
Biotic	0	0	100 (± 2.79)	n.a.	100 (± 2.79)
	12	0	106.4 (± 5.64)	n.a.	106.4 (± 5.64)
	29	0	95.8 (± 3.57)	6.57 (± 4.37)	95.8 (± 3.57)
Abiotic	0	0	100 (± 0.83)	n.a.	100 (± 0.83)
	12	0	106.1 (± 1.96)	n.a.	106.1 (± 1.96)
	29	0	106.2 (± 1.96)	10.0 (± 3.47)	106.2 (± 1.96)
Acetate	0	0	100 (± 3.15)	n.a.	100 (± 3.15)
	12	30.4 (± 0.61)	n.a.	n.a.	n.a.
	29	68.5 (± 2.92)	15.4 (± 8.85)	n.a.	83.9 (± 11.8)
Inhibition	0	0	100 (± 10.6)	n.a.	100 (± 10.6)
	12	4.02 (± 1.13)	n.a.	n.a.	n.a.
	29	17.2 (± 8.25)	66.1 (± 5.93)	n.a.	83.3 (± 14.2)

n.a: not analysed

To test the effect of ciprofloxacin on the general activated sludge microbial activity in aqueous systems, the inhibition of acetate mineralisation by ciprofloxacin was analysed. Without the antibiotic, acetate mineralization started immediately, and after 29 days, 70% of the acetate was mineralized (**Figure 17**). However, in the presence of ciprofloxacin, acetate was slowly degraded and almost no microbial activity was detected until day 5 (lag phase). At the end of the incubation, mineralization was inhibited by 75% compared

to the control without ciprofloxacin (**Figure 17** and **Table 14**). Due to the high toxicity of ciprofloxacin observed in these systems, further investigations of ciprofloxacin effects on the soil microbiota were carried out (Section 3.2.3).

3.3.2 Soil (OECD 307 test)

3.3.2.1 Mass balance and metabolite identification

In soil, a low but significant mineralization which corresponded to 0.9% of the added [2- ^{14}C]-ciprofloxacin was observed at the end of the incubation (**Figure 19**). The contribution of biotic and abiotic processes to the overall mineralisation seemed to be approximately equal. Mineralisation in soil proceeded in two phases, with a first phase until day 6, where the mineralisation rate was relatively high (around $0.03\% \text{ day}^{-1}$), followed by a second one of low and constant mineralisation rate ($0.008\% \text{ day}^{-1}$).

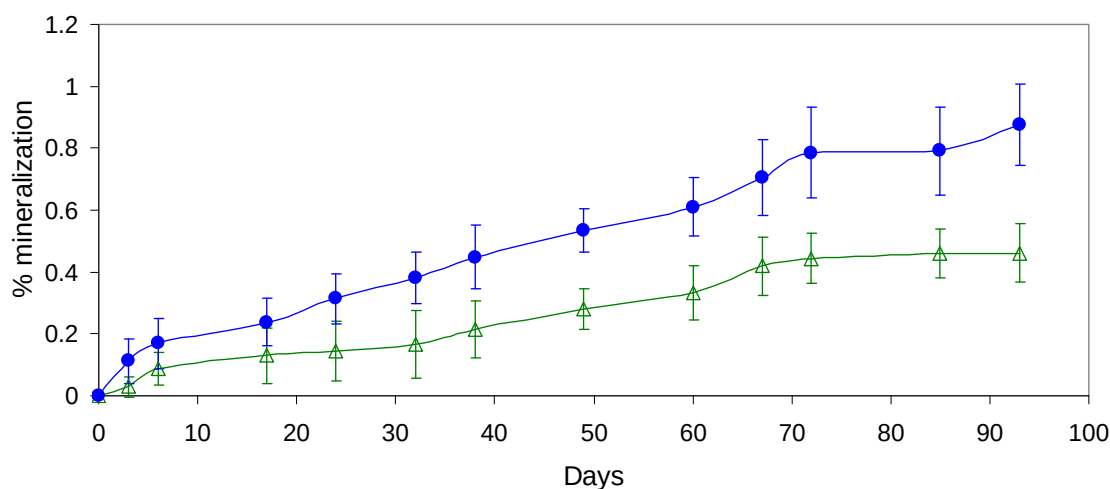


Figure 19. Mineralization of [2- ^{14}C]-ciprofloxacin in soil under (●) biotic and (△) abiotic conditions. Percentages refer to the total radiocativity applied.

The extractability of [2- ^{14}C]-ciprofloxacin related radioactivity decreased over time. At the beginning of the incubation, 39% of the initially added ^{14}C -label was extracted in the biotic incubations (**Figure 20A**). The extractability decreased to 12% after 93 days. In the abiotic systems, 46% of the initially applied radioactivity was extracted on day 0. Again,

the extractability decreased to 15% after 93 days (**Figure 20B**). Overall, the extractable fraction of ciprofloxacin-derived radioactivity was slightly higher in the abiotic systems.

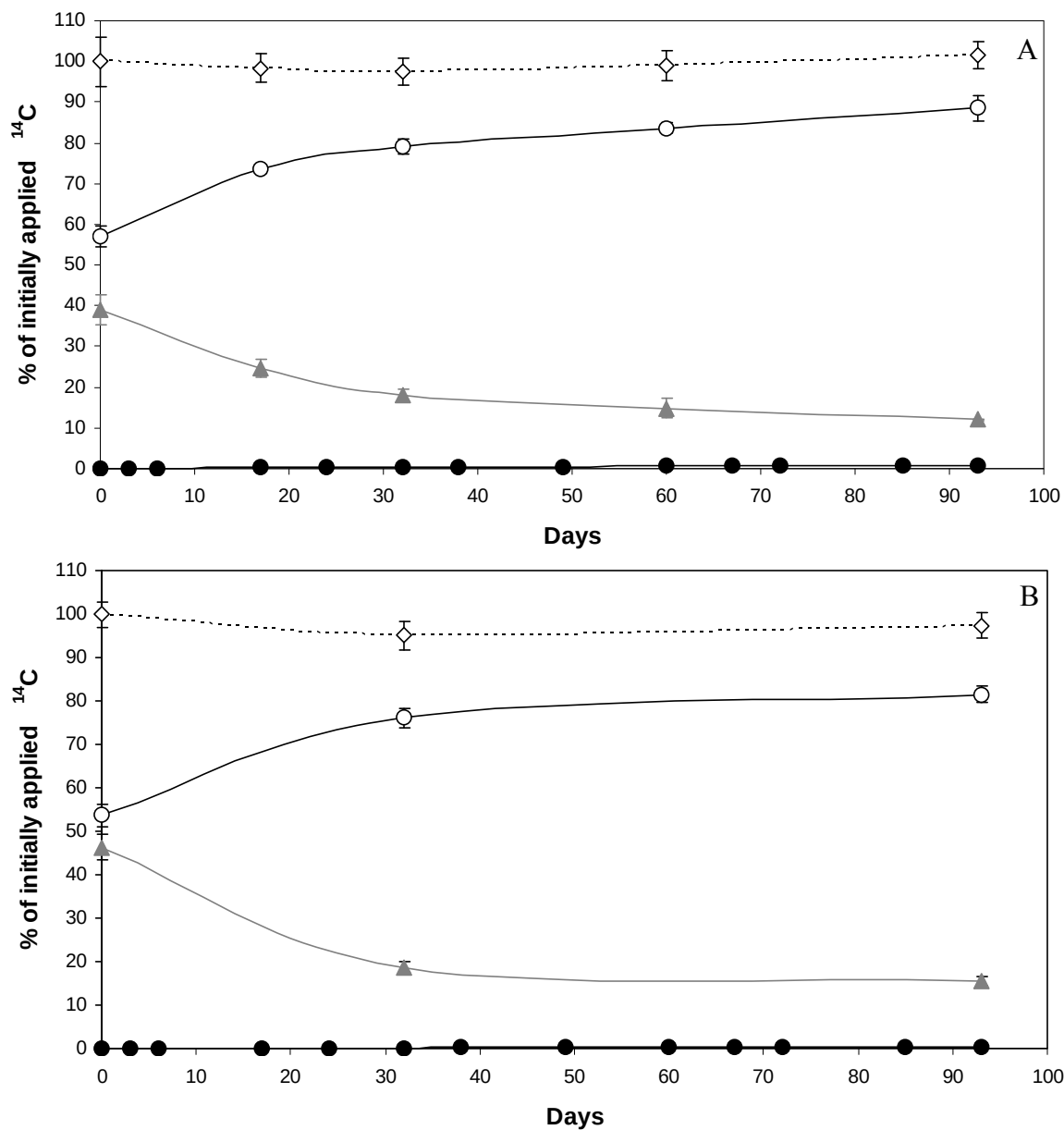


Figure 20. Degradation [2-¹⁴C]-ciprofloxacin in soil under biotic (A) and abiotic conditions (B). (●) mineralisation, (▲) extractable amount, (○) non-extractable residues and (◇) recovery.

In chemical analyses by LC-MS of the purified extracts we observed that the concentration of ciprofloxacin in the extracts declined over time in both biotic and abiotic

incubations (**Table 11**), which is consistent with ^{14}C extractability (**Figure 20**). The concentration decline was more pronounced in the biotic incubations (10.5 % of initial ciprofloxacin concentration on day 93) than in the abiotic one (25.2% of initial ciprofloxacin concentration on day 93). Three ciprofloxacin derived metabolites (F9, F6 and M311) were found by ESI-HR-MS analyses (**Table 11**, **Table 12**) at all the sampling times (including time 0) under biotic and abiotic conditions. The metabolites F6 and F9 were already described in biotic and abiotic degradation experiments of ciprofloxacin (Wetzstein et al., 1999; Calza et al., 2008). However, the unknown metabolite M311 was not previously reported in the literature and the information obtained from its analysis was not sufficient to propose a chemical structure.

In general, the amounts of the 3 metabolites were similar in both experiments, only F9 was more abundant under the biotic ones (**Table 11**). Therefore, it seems that abiotic processes are dominant in the transformation of ciprofloxacin in soil.

Table 11. Ciprofloxacin and metabolites relative abundance (M311, F6 and F9) in purified soil extracts

	Time (days)	% of initial $^{14}\text{C}_2$ -ciprofloxacin ^a			
		Ciprofloxacin	M311	F6	F9
Biotic	0	100 ($\pm$ 13.1)	5.13 ($\pm$ 0.92)	4.70 ($\pm$ 0.92)	3.28 ($\pm$ 0.50).
	17	38.7 ($\pm$ 0.98)	10.5 ($\pm$ 2.76)	6.49 ($\pm$ 1.26)	13.8 ($\pm$ 6.10)
	32	45.3 ($\pm$ 13.1)	2.72 ($\pm$ 1.19)	1.85 ($\pm$ 0.14)	3.01 ($\pm$ 0.26)
	60	20.8 ($\pm$ 1.30)	8.38 ($\pm$ 1.47)	1.48 ($\pm$ 0.25)	3.23 ($\pm$ 0.87)
	93	10.5 ($\pm$ 2.87)	6.16 ($\pm$ 0.20)	0.674 ($\pm$ 0.18)	4.43 ($\pm$ 2.73)
Abiotic	0	100 ($\pm$ 5.82)	17.4 ($\pm$ 1.52)	8.12 ($\pm$ 2.63)	4.14 ($\pm$ 0.49)
	32	44.3 ($\pm$ 3.38)	5.27 ($\pm$ 0.85)	1.51 ($\pm$ 0.46)	0.916 ($\pm$ 0.09)
	93	25.2 ($\pm$ 7.65)	12.8 ($\pm$ 3.23)	0.854 ($\pm$ 0.04)	0.793 ($\pm$ 0.04)

^a 100% corresponds to initially measured amount of ciprofloxacin

values in brackets ($\pm$) represent the standard deviation of the average of triplicates

NER at the beginning of the experiment accounted for 57% and 54% of the applied radioactivity for biotic and abiotic systems, respectively. This fraction, increased over time to reach 88% and 81% of the initially added ^{14}C -label on day 93 in the biotic and abiotic incubations, respectively. Nevertheless, after a rapid increase, the NER formation

slowed down from day 30 onwards, but still continues constantly until day 93 (**Figure 20**). These results demonstrate that ciprofloxacin strongly sorbs to soil and aging increases the amount of NER in soil. In addition, due to the similar characteristic of the biotic and abiotic degradation we conclude that the microbial contribution to the fate of ciprofloxacin in soil is of minor importance. Recoveries for biotic and abiotic incubations ranged from 93 to 101% (**Table 13**).

Table 12. Accurate masses $[M+H]^+$ and chemical structures of ciprofloxacin (332 m/z) and metabolites F9, 7-Amino-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid (263 m/z); F6, 1-Cyclopropyl-7-(1-piperazinyl)-6-fluoro-1,4-dihydro-8-hydroxy-4-oxo-3-quinolinecarboxylic acid (348 m/z) and M311 (311 m/z) in soil (collaboration with Dr. Lamshöft, INFU TU Dortmund; structures from Wetzstein et al., 1999).

Compound	$[M+H]^+$ [m/z] (experimental)	$[M+H]^+$ [m/z] (theoretical)	Calculated Formula	Chemical structure
CIP	332.14042	332.14050	$C_{17}H_{19}N_3O_3F$	
F9	263.08260	263.08265	$C_{13}H_{12}N_2O_3F$	
F6	348.13540	348.13541	$C_{17}H_{19}N_3O_4F$	
M311	311.12787	not available	$C_{14}H_{13}O_4N_3$	not determined

Table 13. Degradation's mass balance from ciprofloxacin in soil (% of initially applied ^{14}C)

		% of initial ^{14}C			
	Time (days)	Mineralisation	^{14}C in extract	Bound residues	Recovery
Biotic	0	0	39.0 ($\pm$ 3.56)	53.6 ($\pm$ 2.55)	92.6 ($\pm$ 6.10)
	17	0.234 ($\pm$ 0.08)	24.5 ($\pm$ 2.24)	73.6 ($\pm$ 1.15)	98.3 ($\pm$ 3.47)
	32	0.380 ($\pm$ 0.08)	18.1 ($\pm$ 1.46)	79.0 ($\pm$ 1.75)	97.5 ($\pm$ 3.32)
	60	0.610 ($\pm$ 0.10)	14.8 ($\pm$ 2.44)	83.7 ($\pm$ 1.12)	99.1 ($\pm$ 3.66)
	93	0.875 ($\pm$ 0.13)	12.0 ($\pm$ 0.13)	88.5 ($\pm$ 3.09)	101.4 ($\pm$ 3.35)
Abiotic	0	0	46.3 ($\pm$ 2.93)	53.6 ($\pm$ 0.02)	99.9 ($\pm$ 2.95)
	32	0.166 ($\pm$ 0.11)	18.7 ($\pm$ 1.08)	76.1 ($\pm$ 2.20)	95.0 ($\pm$ 3.39)
	93	0.461 ($\pm$ 0.09)	15.5 ($\pm$ 1.00)	81.4 ($\pm$ 1.87)	97.4 ($\pm$ 2.97)

3.3.2.2 Sorption of ciprofloxacin

In order to standardise and optimise the extraction of ciprofloxacin from soil, sequential extractions of ciprofloxacin from day 0 soil samples were performed using different extraction methods. After 17 extraction steps of exhaustive extraction, only 60% of the total radioactivity applied could be extracted using the identified most efficient extraction solvent which was the mixture acetone/KOH (**Figure 21**). No more significant radioactivity was extracted in the following steps (less than 0.5 % of the initially applied ^{14}C -label). This method was efficient for extracting ciprofloxacin and its residues from soil. However, it required a lot of effort and time; thus, it was decided to carry out the extractions for mass balance purposes using ASE. Nevertheless, these results allowed inferring the sorption mechanisms of ciprofloxacin to soil. In comparison to acetonitrile, acetone is more polar and destroys soil aggregates more efficiently. It therefore increases the solubility of ciprofloxacin and/or the organic matter the compound is associated with. In addition, the low performance of this mixture when adding ammonia may be due to the formation of an unstable colloid that complicated the separation of the supernatant and the soil pellet. The darker colour of the acetone/KOH extract compared to the others indicates that the recovery of ciprofloxacin increases with co-extraction of humic substances, which bind the antibiotic (Rosliza et al., 2009). These results confirm that ciprofloxacin is strongly sorbed to soil, in particular to soil organic matter. In addition, no radioactivity was mobilized by re-extracting the extracted soil with pressurized steam,

indicating a low remobilization potential and the absence of low-molecular-weight breakdown products of polymeric humic fractions (Weiss et al., 2004), and that the extraction with acetone/KOH can be regarded as exhaustive.

When comparing these results with the extractability by ASE, we observe that the extractability by sequential extractions with acetone/1M KOH (1:1) was higher (~ 60 compared to ~ 40% of the initially applied radioactivity) than the one by ASE using the mixture 63% ethyl acetate, 25% methanol and 3% ammonium. The difference between these fractions presumably represents the amount of ciprofloxacin strongly bound to organic matter (humic substances) that wasn't efficiently extracted by ASE. These observations illustrate how NER estimation is determined by the extraction method used and its efficiency.

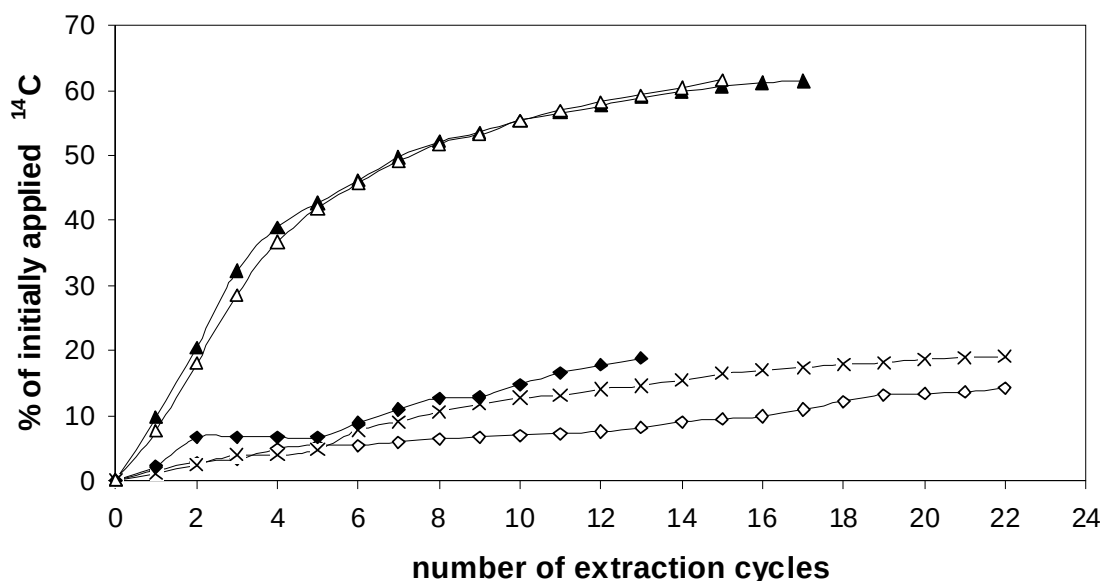


Figure 21. Sequential extractions of ciprofloxacin with different solvents from soil samples. (◇) acetonitrile/0.2 M KOH, (×) acetonitrile/50 mM H₃PO₄, (◆) acetone/NH₃/ 0.1 M KOH, (▲) acetone/0.2 M KOH and (△) acetone/0.1 M KOH.

3.3.3 Toxicity studies of ciprofloxacin

3.3.3.1 Effects on activated sludge and soil microbiota

In order to evaluate the effects of different concentrations of ciprofloxacin on soil microorganisms, we used soil respiration as an indicator of microbial activity. Soil

respiration was significantly inhibited by ciprofloxacin (**Figure 22**). This effect was particularly pronounced at the beginning of the incubation and did not depend on the concentration of ciprofloxacin in the range tested (**Table 14**). Moreover, the inhibition of soil respiration by ciprofloxacin was lower than the mineralisation of acetate in the aqueous system (**Figure 17**), and also decreased with time (**Table 14**). After 2 days of incubation, the soil respiration was reduced by 72%, as opposed to only around 35% at the end of the experiment.

Overall, the microbial activity was thus strongly inhibited in both aqueous systems and soil. Even though concentrations in the soil solution were much higher than in MM (**Table 14**), it seems that the toxic effect of ciprofloxacin was stronger in aqueous media than in soil. This may be due to the higher diversity of microorganisms in soil than in activated sludge. More important, however, seems to be the reduced bioavailability of the toxicant in soil due to its strong sorption, which potentially reduces its toxicity. Furthermore, in the concentration range studied here, the extent of ciprofloxacin toxicity did not depend on its concentration (**Figure 22**) showing the efficiency of the antibiotic. The decrease of inhibition in soil during the time of incubation can be explained by aging of the compound and by adaptation of the microorganisms to the antibiotic.

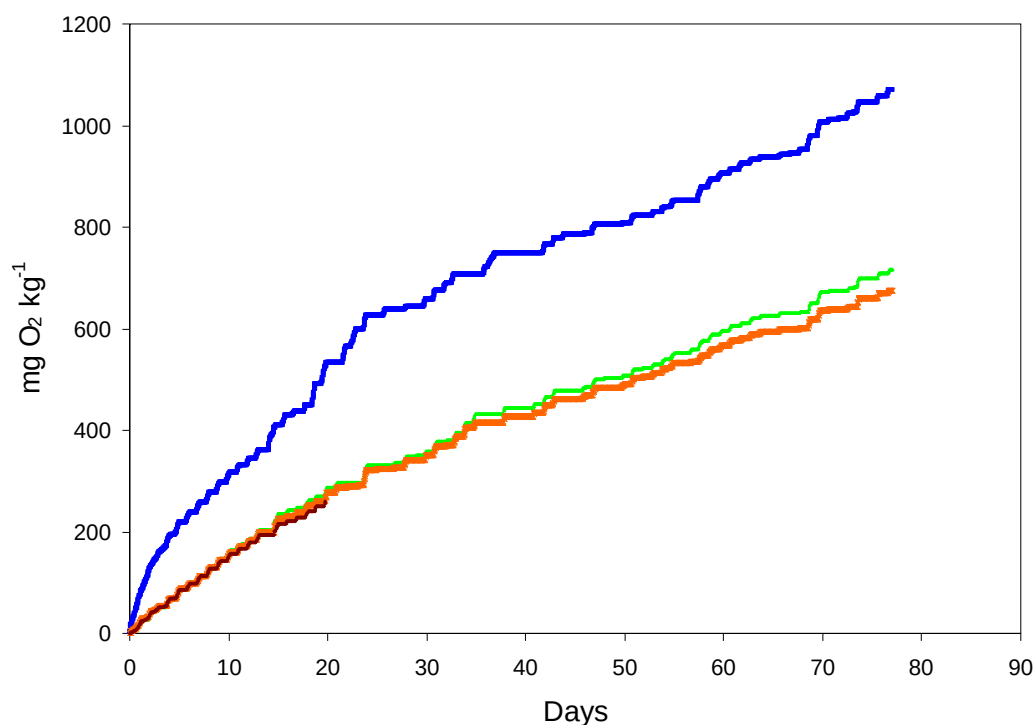


Figure 22. Soil respiration (Inhibition test) of ciprofloxacin-spiked soil samples. (—) 0 mg kg⁻¹ CIP, (—) 0.2 mg kg⁻¹ CIP, (—) 2 mg kg⁻¹ CIP, (—) 20 mg kg⁻¹ CIP.

Table 14. Microbial activity inhibition in soil and water at different concentrations and times

Time (days)	% of inhibition (compared to control)			
	Soil			MM
	0.2 mg kg ⁻¹ CIP	2 mg kg ⁻¹ CIP	20 mg kg ⁻¹ CIP ^a	20 mg l ⁻¹ CIP
2	70.9 (±12.0)	69.1 (±3.78)	71.5 (±15.6)	99.1 (± 4.03)
7	56.4 (± 3.02)	56.7 (± 3.77)	56.8 (± 3.23)	87.8 (± 0.81)
12	45.9(± 1.19)	47.0 (± 2.14)	48.3 (± 1.33)	86.8 (± 1.72)
20	46.9 (± 4.14)	49.0 (± 4.78)	50.4 (± 7.99)	79.4 ^c
end ^b	33.1 (± 1.49)	36.8 (± 4.06)	n.a	74.9 (±11.2)

n.a: not assessed

^a the concentration in the soil solution corresponded to 45.8 mg·l⁻¹ and to 12.5 mg·l⁻¹ on days 0 and 77, respectively.

^b end of the experiment was on day 29 for water and on day 113 for soil

^c estimated by linear interpolation

values in brackets (±) represent the standard deviation of the average of triplicates

To have a detailed overview of the progression of the inhibition, we calculated the soil respiration rates within the incubation time (**Figure 23**). As commonly observed in soil

incubation experiments, the soil respiration rates decreased over time due to the depletion of C sources reaching a starvation level. It was during the first month of incubation, when the inhibition of soil respiration was most evident. In the treatments with the antibiotic, the activity remained relatively constant, close to starvation or stress values. At the end of the incubation, respiration rates were similar in the controls and the treatments with ciprofloxacin. Therefore, ciprofloxacin almost totally inhibited the initial microbial activity. This can be explained by its biostatic nature that targets growing microorganisms. In contrast, the biotoxic effect which should reduce the number of living cells is negligible, similarly to what has been discussed by Thiele-Bruhn (2005).

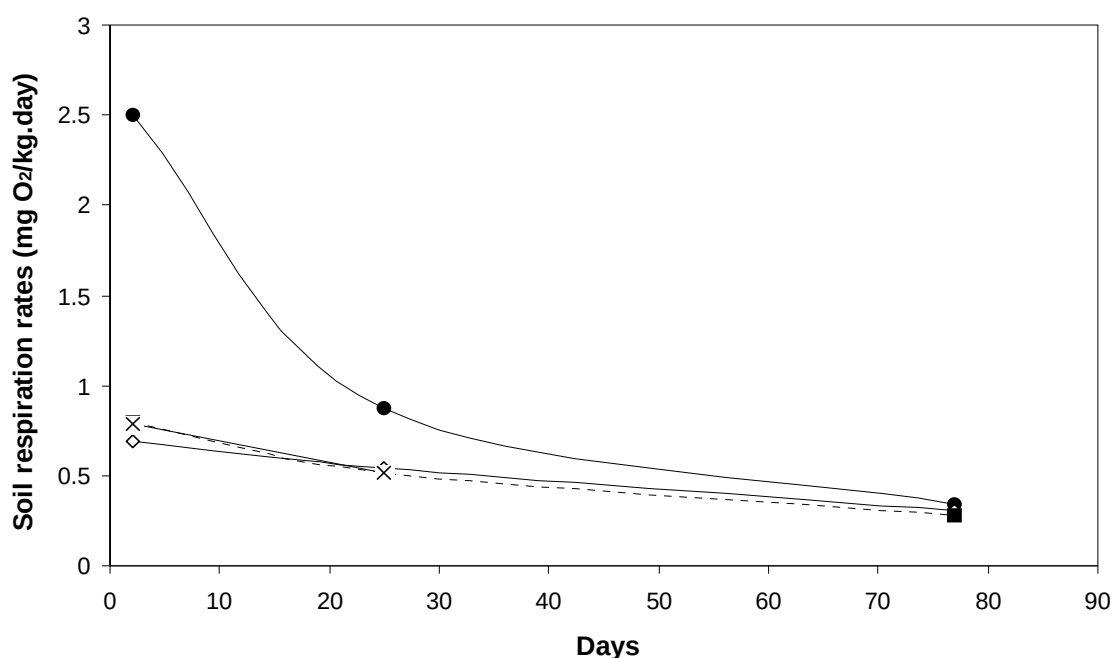


Figure 23. Soil respiration rates in the inhibition test. (●) 0 mg kg⁻¹ CIP, (◊) 0.2 mg kg⁻¹ CIP, (■) 2 mg kg⁻¹ CIP, (x) 20 mg kg⁻¹ CIP.

T-RFLP analyses were performed in order to study the induced effects of ciprofloxacin on the soil bacterial community. The results analysed by multidimensional scaling statistics (MDS; **Figure 24**) revealed a shift in microbial community composition, including both abundance and diversity. The relevant factors driving this change were the presence of antibiotic and the time of incubation. Statistical analysis revealed that these two factors were uncorrelated ($p=0.431$), demonstrating that both factors were acting

Thus, although the effect of the antibiotic on microbial communities was evident, no clear concentration effect was visible, which is consistent with the soil respiration data presented above. Moreover, when Jaccard index was used instead of Bray-Curtis for the analysis of the changes in the community, also significant differences were found between the control and the treatments (data not shown) and this indicates a shift in species composition.

3.3.3.2 EC₅₀ for bacteria (*Pseudomonas putida*)

The inhibition of a relevant bacteria strain for the soil environment by ciprofloxacin was also studied. The EC₅₀ of CIP for *Pseudomonas putida* mt-2 in pure culture was 0.25 mg L⁻¹ (**Figure 25**) and 1 mg L⁻¹ completely inhibited bacterial growth. In contrast to these results, acetate was still mineralised at much higher concentrations in the inhibition test in aqueous media (**Table 14**). One reason for this difference is the higher diversity in activated sludge compared to only one species, which increases the chance to have microorganisms that are not or less affected by the toxic. Additionally, the fraction adsorbed to sludge (10% of initially added ¹⁴C-label; **Table 10**) could also contribute to the reduced toxicity against microorganisms by reducing the dissolved concentration in MM. Although a direct comparison between bacterial growth and mineralisation of a compound often is not really adequate, the comparison in this case may be possible due to the high concentration used in MM, which is 20 times more than the concentration of total growth inhibition. Moreover, *P. putida* did not grow at 1 mg L⁻¹ of ciprofloxacin (**Figure 25**). However, soil microorganisms were still active at the concentration of total growth inhibition of *P. putida* (**Table 14**), presuming a different toxicity extent in soil, aqueous systems, and pure cultures.

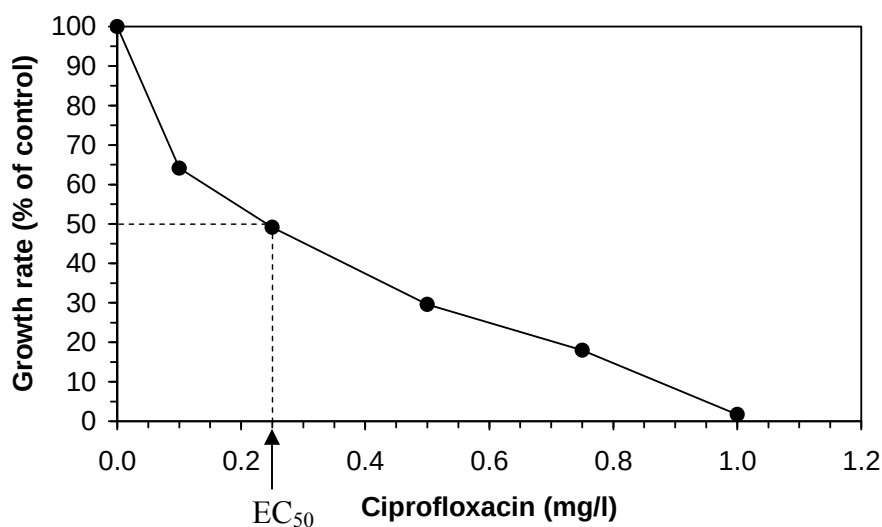


Figure 25. Effect of CIP on the growth of *Pseudomonas putida* mt-2 in pure culture.

3.3.3.3 Induction of antibiotic resistance in soil

In order to evaluate the adaptation of soil microbiota to ciprofloxacin by means of induction of antibiotic resistance, samples from different incubation times were tested for three different ciprofloxacin resistance genes (qnr A, B, S). In none of the samples genes qnr A (580 bp) and qnr B (264 bp) were detected (**Figure 26**). Gene qnr S (428 bp) was neither detected in the control (non amended soil samples) nor in incubations with different ciprofloxacin concentrations after 3 days. However, the qnr S gene was detected in samples treated with 20 mg kg⁻¹ of the antibiotic on day 14 (lane 4), 29 (lane 10) and 65 (lane 19); in samples treated with 0.2 mg kg⁻¹ on day 29 (lane 7); and in incubations with 2 mg kg⁻¹ on day 65 (lane 16) and 113 (lane 22).

The low intensity of the qnr S bands indicates a low copy number of this gene in soil. Unfortunately, due to the low amount of template and its instability, the obtained PCR fragments could not be confirmed by sequencing. Nevertheless, these results prove that ciprofloxacin induced resistance can be present in soils contaminated by this antibiotic, that the induction of resistance is independent of the concentration at the concentration range used in these experiments, but dependent on the contact time with the antibiotic, since the earliest detection was after 14 days.

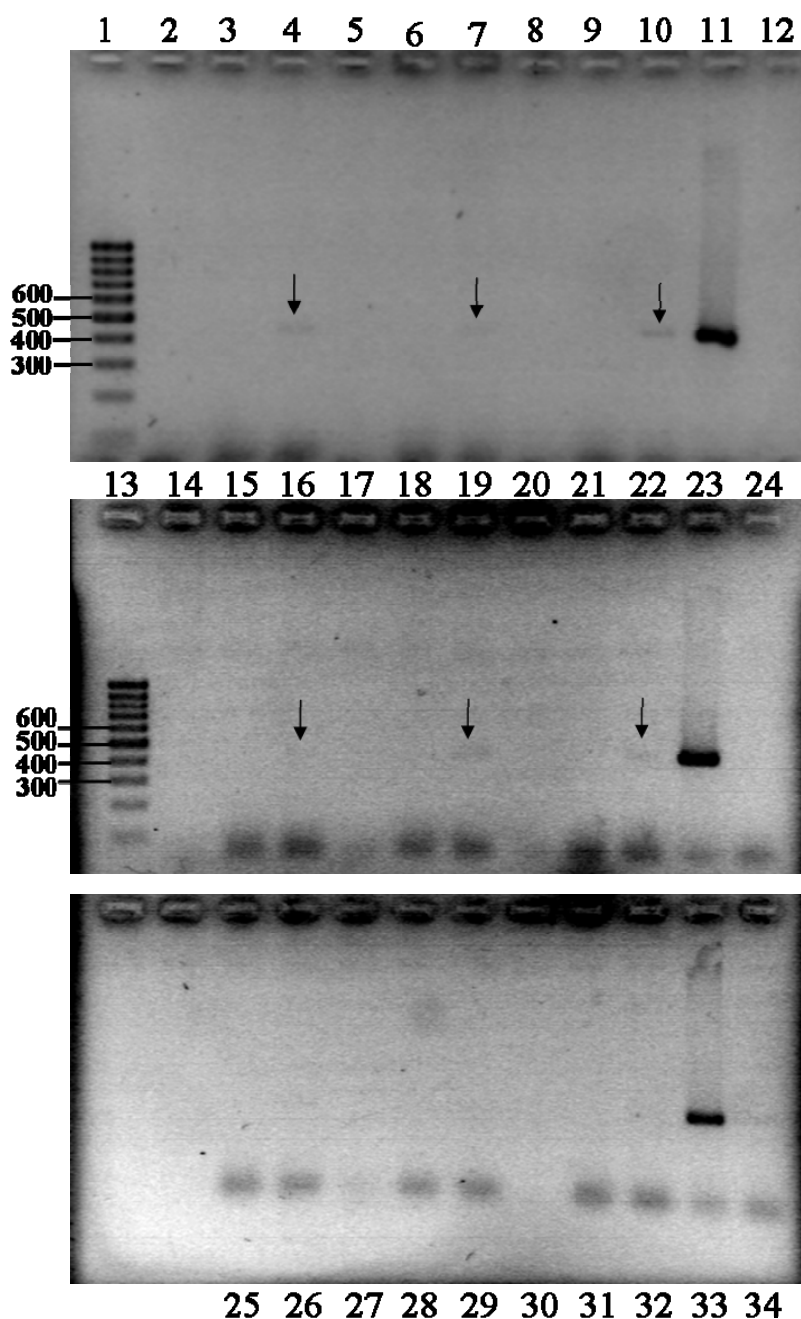


Figure 26. PCR of ciprofloxacin resistance genes qnr A (580 bp), B (264 bp) and S (428 bp) from soil incubations with ciprofloxacin. Agarose gel electrophoresis (2%). Lanes: 1 and 13, Molecular size marker; 2, 3 and 4, qnr genes B, A,S respectively CIP 20 mg/kg day 14; 5, 6 and 7, qnr genes B,A,S respectively from CIP 0.2 mg kg⁻¹ day 29; 8, 9, 10; qnr genes B,A,S respectively from CIP 20 mg/kg day 29; 14, 15 and 16, qnr genes B, A, S respectively CIP 2 mg/kg day 65; 17, 18 and 19, qnr genes B,A,S respectively from CIP 20 mg/kg day 65; 20, 21, 22, qnr genes B,A,S respectively from CIP 2 mg/kg day 113; 25, 26, 27 qnr genes B A,S respectively from CIP 20 mg/kg day 3; 28, 29 qnr genes A,S from non spiked soil day 3; 30, 31, 32 qnr genes B,A,S from non spiked soil day 113; 11, 23 and 33 qnr S in resistant strain (positive control); 12, 24 and 34 no DNA (negative control)..

Discussion

We compared results from OECD ready biodegradability tests (OECD 301) for the herbicide 2,4-D and the pharmaceuticals ibuprofen and ciprofloxacin with those from simulation tests in soil (OECD 307) in order to evaluate the environmental fate of these compounds. According to the aims of the work, not only mineralisation rates were determined but also metabolite profiles and carbon distribution during the biodegradation process. In addition, we evaluated the risk of these compounds for the environment, particularly the effects of ciprofloxacin on microbial communities.

4.1 Biodegradation of 2,4-D in aqueous medium and soil

2,4-D can be classified as readily biodegradable and thus not persistent because it met the criterion of more than 60 % mineralisation (71.2%) within a 10-day window according to the OECD 301B test (OECD, 1992). In soil, no adaptation period was observed and the mineralisation of 2,4-D started immediately after the beginning of the experiment, very likely due to a mixture of specific (use of 2,4-D as carbon and energy source) and unspecific (cometabolism) microbial activities (Robertson and Alexander, 1994). Apparently, specific 2,4-D degrading microorganisms were already present in the used soil since it had been treated with structurally related herbicides (MCPA, Dichlorprop, I. Merbach, UFZ, personal communication 2010) which might have induced an adapted microflora. Nonetheless, 2,4-D degrading organisms have been also found in soils never exposed to the herbicide (Vieuble-Gonod et al., 2003). In agreement with previous studies (Lerch et al., 2009a, Vieuble-Gonod et al., 2003), the period of highest biodegradation (mineralisation rate) in soil coincided with the time where 2,4-D was still available and thus the growth of its degraders was not limited by substrate availability

(section 3.1.2). Biodegradation rates decreased very rapidly after 8 days when no more 2,4-D or metabolites were found in the soil extracts.

Low amounts of NER were detected at the beginning of the experiment in both, biotic and abiotic incubations, indicating that 2,4-D was not physico-chemically stabilised in soil to a significant degree. This is consistent with the previously reported low sorption of the herbicide to soil (Benoit and Barriuso, 1997; Boivin et al., 2005). Consequently, adsorption of 2,4-D to soil does not seem to play a major role in the dissipation of the herbicide and, apparently, does not control its bioavailability. Instead, microbial activity was the main process driving the elimination of the compound. Analyses of NER for biomolecules (fatty acid [FA] and aminoacids [AA]) in these samples were performed by Karolina Nowak and showed that microorganisms derived activity was responsible for the majority of NER formation from this compound (**Figure 27B**; Nowak et al., 2011). The pesticide-derived carbon was converted to microbial biomass (fatty acids and amino acids) with a peak in the living fraction on day 8 (2.5% of the initial ^{13}C ; Nowak et al., 2011). At the end of the experiment, biogenic NER reached around 40% of the initially added $^{13}\text{C}_6$ -2,4-D equivalents and were finally stabilised within non-living soil organic matter. These results challenge a previous study (Boivin et al., 2005) where the increased sorption of 2,4-D over time with an unexpected increase of mineralisation during NER aging was explained by the physical entrapment of the molecule.

In addition, around 19% of NER were found under abiotic conditions indicating abiotic NER formation (Pignatello and xing, 1996; Alexander 2000; Kästner et al., 1999; Gevaio et al., 2000; Katayama et al., 2010). No biogenic residues were found when analysing NER from sterile incubations (Nowak et al., 2011).

Consistent with the results of purified extracts analyses obtained in this study, Lerch et al. (2009a) extracted only 6% of the applied $\text{C}_{2,4\text{-D}}$ after 8 days and 0.1% at the end of their experiment. Moreover, the difference in the amounts of ^{13}C -label in the extracted fractions before and after purification presented here can be explained by the extensive extraction of the label bound to particulate organic matter including biomass residues extracted using high temperature and pressure, which was then efficiently removed by our purification method (SPE).

Overall, biotic mineralisation was higher in water (84%) than in soil (58%) systems where, similar to earlier observations (Boivin et al. 2005), around 40 % of the applied ^{13}C -label was converted to NER. However, the majority of these residues corresponded to biogenic bound residues which do not pose any risk for the environment (Nowak et al. 2011). In addition, only very low amounts of 2,4-D (2% of initially applied) were found after 8 days of incubation. Consequently, the extent of biodegradation in water and soil (sum of mineralisation and transformation of the molecule to innocuous products) are comparable for the readily degradable herbicide 2,4-D.

Under abiotic conditions however, degradation was higher in soil than in aqueous systems, probably due to redox reactions with soil minerals. For instance, abiotic processes are important in the degradation of PAHs in soil (Park et al. 1990) and the herbicide 1,3-D was abiotically degraded in soil, mainly by hydrolysis. Water and organic matter promote the degradation of the compound via direct substitution reactions in sterile soils (Guo et al., 2004). Nevertheless, the sterile systems might not completely reflect the abiotic processes actually occurring in the biotic systems. Given that degradation of 2,4-D is relatively fast, this process efficiently competes with sorption and abiotic NER formation. It was already reported by Kästner et al. (1999) that higher abiotic NER formation was obtained from anthracene when fungal and bacterial metabolism was inhibited in comparison to the active biotic turnover.

4.2 Biodegradation of ibuprofen in aqueous medium and soil

Ibuprofen was ultimately biodegraded to an extent of 68% after 28 days; however, its biodegradation did not fulfil the criterion of 60% mineralisation in a 10 day-window. Therefore, this pharmaceutical should be classified as easily degradable and not readily biodegraded unless this requirement is not considered for compounds occurring as a mixture of isomers, as recommended in the last revision of the OECD guidelines (OECD, 2006). This recommendation is applicable in this case because ibuprofen occurs as a mixture of two isomers in the environment, with the pharmacologically inactive (*R*)-(-)-isomer being more persistent (Buser et al., 1999).

In soil biodegradation experiments, 45.2% of ibuprofen was mineralised after 90 days. The degradation rates were lower than for 2,4-D in soil. However, the period of high microbial activity continued for longer time than in the case of 2,4-D, and also coincides with the periods of highest availability of ibuprofen and of highest abundance of the metabolite 2-hydroxy-ibuprofen (**Table 9**). The limited biodegradation of ibuprofen during the first 14 days is consistent with the high amounts of ibuprofen in the extracts and maybe related to low microbial growth during this period, as indicated by the low incorporation into the living biomass and into biogenic NER reported by Nowak et al., (submitted, **Figure 28B**) within this period. Analyses of NER for biogenic molecules (FA and AA) in these samples were performed as for 2,4-D by Karolina Nowak.

At the end of the biotic incubations, around 30% of the initially applied ^{13}C -label was found in NER. This amount corresponded to the amount of ^{13}C -label found in biomolecules within the non-living SOM fraction, i.e. in biogenic residues (Nowak et al., submitted). Consequently, biogenic residues represent almost the complete residual fraction of C labelled carbon from the degradation of ibuprofen in soil. In addition, NER are much higher in the biotic systems than in the abiotic ones. Similarly to 2,4-D, around 15% of the initially applied ^{13}C -label were found in NER in the abiotic incubations, indicating the abiotic formation of NER due to aging processes of the compound in soil (Pignatello and xing, 1996; Alexander 2000; Gevao et al., 2000; Katayama et al., 2010). Again, in the abiotic incubations, no ^{13}C -label was detected in the microbial biomass (Nowak et al., submitted). Once more, these results illustrate the importance of microbial contribution to non-extractable residue formation in soil systems and that abiotic NER may be overestimated because metabolisation is absent (no competition).

A relatively high sorption tendency was reported for ibuprofen in a soil with similar texture than our soil (Kreuzig et al., 2003). Our results, however, illustrate low sorption or entrapment of this compound to soil which also agrees with results from previous studies (Xu et al. 2009). Moreover, in another study (Richter et al., 2007), NER formation and mineralisation were faster during the first 20 days of incubation than in our study, but the final mineralisation was lower (38%). This may be due to the position of the C label in the parent molecule they used, located in a methyl group whereas we applied ring labelled ibuprofen. In addition, a degradation pathway of ibuprofen by bacteria isolated

from a waste water treatment plant was already reported, where the first reaction resulted in the loss of the C₃-methyl group (Murdoch and Hay, 2005). Another controversial result was the fast elimination of ibuprofen reported by Xu et al. (2009), which could be attributed to the use of unlabelled ibuprofen, which does not allow differentiating between sorption to and degradation processes.

The comparison of the water-based and soil tests revealed that, as for to 2,4-D, both elimination and mineralisation of ibuprofen were faster in MM than in soil. Thus, the whole biodegradation process does occur slightly faster in water than in soil. However, abiotic degradation was higher in soil than in aqueous systems.

4.3 Biodegradation of ciprofloxacin in aqueous medium and soil

Ciprofloxacin was not degraded in water under biotic and abiotic conditions because it is highly toxic and inhibits the microbial action. In addition, it is resistant to abiotic degradation reactions such as hydrolysis (Thiele-Bruhn 2003). Therefore, ciprofloxacin is not readily biodegraded but recalcitrant to degradation in aqueous systems.

In soil however, a low but significant mineralisation was observed. In non-sterile soils, 0.9% of the initially added [2-¹⁴C]-ciprofloxacin was mineralised after 93 days of incubation. The biotic contribution to the ultimate degradation of the compound represents 50% of the total mineralisation, indicating the participation of microorganisms in the process. Thus, to some extent, ciprofloxacin can be biodegraded. However, since the radiochemical purity of ciprofloxacin was 99.4 %, this low mineralisation or part of it could also correspond to mineralisation of accompanying compounds or impurities. Similar low mineralization extents (0.49-0.58%) were reported for 4 mg kg⁻¹ another fluorquinolone (sarafloxacin) after 80 days of incubation in soils of varying texture (Marengo et al., 1997). The low mineralization was attributed to the strong sorption of the antibiotic and the resulting low bioavailability; however no inhibition test of microbial activity was performed. In contrast, our results indicate that the low mineralization was mainly due to the toxicity of ciprofloxacin. We assume that the biotic mineralization in our study was mainly related to the activity of fungi (Wetzstein et al.,

1999), archaea, yeasts (Chen et al. 1997), ciprofloxacin-resistant bacteria, and to the reduction of the toxicity by sorption. The last two aspects are further analysed in the following sections. In addition, the relatively faster degradation at the beginning of the incubation may be related to the higher bioavailability of the compound for its degraders, i.e. fungi (Wetzstein et al., 1999; Parshikov et al., 1999; Parshikov et al., 2001), to fast abiotic reactions with soil components (Stevenson, 1994; Yaron et al., 1996; Marengo et al. 1997) or to fast photoinduced degradation (Lam et al. 2003; Calza et al., 2008) during preparation of the experiments.

From our results we conclude that the decline of the extractable fraction of ciprofloxacin-derived radioactivity is mainly governed by sorption and formation of non-extractable residues, and only partially by degradation. Three metabolites were detected (F6, F9 and M311). Two of them (F6 and F9) were reported during the biodegradation of ciprofloxacin by the brown rot fungus *Gloeophyllum striatum* (Wetzstein et al. 1999) also indicating fungal degradation in the present experiments. Degradation pathways mediated by hydroxyl radicals attack (Wetzstein et al., 1999) were apparently predominant in our incubations. The unknown metabolite M311 most probably corresponds to a degradation product of ciprofloxacin and could be formed after the loss of the cyclopropyl group since it was also found in the degradation of the fluoroquinolone norfloxacin by white-rot fungus (Prieto et al., unpublished results). Metabolite F9, which is formed by the loss of the piperazine moiety, was also found in photodegradation experiments of ciprofloxacin (Calza et al. 2008). Fluorquinolones are reported to be rapidly photodegraded with a half-life 13 ± 2 min in surface water (Lam et al. 2003). Furthermore, sarafloxacin was quickly degraded abiotically by surface-catalyzed hydrolysis or oxidation resulting in a polar transformation product. This transformation product was even present in samples immediately after the spiking of soil (Marengo et al. 1997). Similarly, the three metabolites determined in this study were found on day 0 samples. This rapid transformation is presumably a result from fast abiotic reactions with soil components, which is consistent with the relatively high initial mineralisation rate (**Figure 19**). Additionally, alkaline hydrolysis (Martins et al. 2001) or photodegradation of the molecule (Lam et al. 2003; Calza et al., 2008) may occur in the extracts during sample preparation (purification, long time concentration, etc...) before analysis.

During the whole incubation period, the amounts of NER were similar in biotic and abiotic incubations, suggesting that NER formation is mainly due to abiotic processes, i.e. sorption and aging (Pignatello and xing, 1996; Kästner et al., 1999; Alexander 2000; Gevao et al., 2000; Uslu et al, 2008; Vasudevan et al., 2009; Katayama et al., 2010). This is consistent with the low mineralisation rate obtained under biotic conditions (section 3.3.2).

Even though ciprofloxacin can be degraded abiotically, we conclude that it is highly persistent in soil. This high persistence is mainly governed by sorption, while photo and microbial degradation are of minor importance. For instance, humic acids decrease the photodegradation of enrofloxacin (Schmitt-Kopplin et al. 1999). The interaction of ciprofloxacin with humic substances thus might stabilise it and protect it from biodegradation. In addition, ciprofloxacin proved to be rapidly and extensively degraded by fungi present in soil (Wetzstein et al. 1999; Parshikov et al., 1999). However, it seems that this only occurs under artificial conditions and thus presumably not in the real environment. A degradation pattern was proposed for ciprofloxacin in soil (Golet et al., 2003) including an initial phase of biodegradation followed by long term persistence. The authors explained this behaviour by aging of ciprofloxacin residues in soil or by reaching the biodegradation concentration threshold. Again, the toxicity of the compound, and how this toxicity inhibits the relevant action of microorganisms was not considered.

In spite of the fact that ciprofloxacin was not extensively degraded in soil and contrary to the well accepted rule that degradation of antibiotics is hampered by sorption to the soil matrix (Thiele-Bruhn 2003), ciprofloxacin biodegradation including mineralisation was higher in soil than in water. Sorption to soil particles may have reduced the bioavailability of the compound and thus its toxicity (Alexander, 1994; Welp and Brümmer, 1999). It has been described that the association of the bioactive functionalities of the molecule to soil exchange sites is particularly efficient in this respect (Thiele 2000). Another explanation can be the participation in the biodegradation of extracellular enzymes avoiding the transport of the toxic within the cell.

4.4 Toxicity of ciprofloxacin and bioavailability

Due to the high toxicity of ciprofloxacin in aqueous medium, further investigation about ciprofloxacin effects were performed in the soil system. Ciprofloxacin derived toxicity was higher in aqueous systems than in soil, since sorption of toxicants is one of the main mechanisms in controlling their bioavailability and toxicity (Welp and Brümmer, 1999). The lower toxicity in soil in terms of bioavailability can be explained by the lower mass transfer of the compound to the bacteria in soil due to sorption or entrapment in comparison to aqueous systems, where mass transfer is not limited (Sikkema et al., 1995; Ehlers and Luthy, 2003). In soil therefore, limited mass transfer of the toxic compound allows detoxification mechanisms to compete with mass transfer and eliminate to a certain extent the antibiotic from the cell.

Toxicity in soil declines with time most probably because of sorption, aging and transformation to less toxic molecules. It has been reported that sorption and desorption of compounds in soil systems plays a key role for their environmental fate (Welp and Brümmer, 1999) and that formation of NER leads to a decrease in their toxicity (Barriuso et al., 2008). However, sorption and NER formation from ciprofloxacin and its related residues does not completely reduce the effects of this compound on the soil microbial community. One possible explanation is that since 80-90% of the microorganisms inhabiting soil are attached to solid surfaces (Hattori, 1973) and thus can still be in contact with them even if it is sorbed to soil particles.

Golet et al. 2003 reported that ciprofloxacin is not degraded and thus very stable even after stabilization of activated sludge under methanogenic conditions. However, it can lose its antibiotic potential under such conditions (Cordova-Creylos et al., 2007). As presented before, we detected some transformation products of ciprofloxacin in soil and according to Wetzstein et al. (2009), toxicity of ciprofloxacin is not relevant in soils and metabolite F9 has almost no antibacterial activity. This, however, does not agree with our results because in general, the decline in the antimicrobial potential was slow and incomplete as previously reported in activated sludge (Halling-Sorensen 2003). Possible reasons are the incomplete transformation of the molecule and the stability of the fluorine substituent at the aromatic C-6 position which is crucial for the antibiotic potency (Pico

and Andreu, 2007). Carbon-fluorine bonds are among the strongest bonds in nature and therefore hardly cleaved during metabolism (Murphy et al., 2009). These results disagree with the loss of antibacterial activity of ciprofloxacin after its defluorination, decarboxylation, hydroxylation or oxidation of the amine moiety by the brown rot fungus suggested by Wetzstein et al. (1999). As mentioned above, these processes are unlikely to occur in our soil and in the real soil environment. Moreover, although ciprofloxacin is transformed in soil, this process is limited and cannot be seen as a process radically reducing the compound's toxicity.

Extensive degradation was reported in experiments with enrofloxacin labelled in the piperazine moiety, or the carboxyl group, which are suggested as good indicators for the antibiotic activity and degradability of the compound (Wetzstein et al., 1999; Wetzstein et al., 2009). Our conservative approach using the label in one of the most stable carbon positions of the molecule contradict this extensive degradation, and provided consistent results of both low degradation and inactivation of the antibiotic.

Limited bioavailability influences the effects of antibiotics on the soil microbial community by reducing their toxicity. The presence of multivalent cations in soil was reported to inhibit the antimicrobial potential of fluoroquinolones (Marshall and Piddock, 1994). This may explain the lower toxicity in soil when comparing the concentration and effect of ciprofloxacin in the soil solution and in mineral medium. Furthermore, in addition to the presence of cations and induced resistant strains, some soil microorganisms are naturally tolerant towards antibiotics (Esiobu et al. 2002) such as pseudomonades (Krieg and Holt, 1984). In addition, the high microbial diversity in soil may be responsible for the weaker effects of antibiotics in soil than in water (Schauss et al. 2009). Moreover, microorganisms like *Achaea* and fungi for example are not targeted by the antibiotic. Also, the activity of soil bacteria is close to dormancy (Stenström et al., 2001; Nannipieri et al., 2003) and thus they cannot be affected by antibiotics exerting a biostatic effect (Thiele-Bruhn and Beck, 2005). And finally, antibiotic resistance may develop and spread by gene transfer (Beaber et al., 2004). Altogether, these reasons may also contribute to the difference in toxicity for “active” activated sludge bacteria and soil bacteria.

Reported EC₅₀ values of ciprofloxacin in the literature vary over a wide range of concentrations. Two studies by Halling-Sorensen et al. (2000, 2003) reported an EC₅₀ of 0.006 mg L⁻¹ and 0.61 mg L⁻¹ for sewage sludge bacteria. The EC₅₀ for the cyanobacterium *Microcystis aeruginosa* was 0.005 mg L⁻¹ but 2.97 mg L⁻¹ for the algae *Selenastrum capricornutum* (Halling-Sorensen et al. 2000). Moreover, ciprofloxacin has phytotoxic effects on the aquatic plant *Lemna gibba* with an EC₂₅ of 271 µg L⁻¹ (Brain et al. 2004) and thus at relevant environmental concentrations (Larsson et al., 2007; Golet et al., 2002). The EC₅₀ of 0.25 mg L⁻¹ we determined for *Pseudomonas putida* mt-2 is in the same range as these reported values. This is another proof of the strong antibiotic power of ciprofloxacin and the hazardous consequences it can generate on the environment.

Ciprofloxacin inhibited particularly the initial microbial activity as determined by O₂ consumption in soil (**Figure 23**). This activity arises from the exposure of microorganisms to new available C and energy sources after the setup of the experiments, this means growing microorganisms or active bacteria. This is consistent with its mechanism of action inhibiting DNA gyrases, thus DNA replication, recombination and transcription (Moore et al 1995). These results and the fact that 80-90% of the soil processes are mediated by microbes (Nannipieri and Badalucco, 2003; Coleman et al., 2004) stress the important impacts of ciprofloxacin for microbial ecosystem services, such as nutrient recycling and the carbon cycle. In general, the fact that we did not observe a clear dose-response demonstrates that, the antibiotic was already fully effective at the lowest concentration employed, and thus indicates how effective the compound actually is. Similar results were obtained by Kotzerke et al. (2008) for sulfadiazine in soil. In addition, small concentrations of bioavailable antibiotics might cause considerable effects on soil microorganisms (Thiele-bruhn and Beck, 2005). Therefore, it would be interesting to know more about the lowest observed effect concentration (LOEC) in soil and compare those with the normal concentrations reported in the environment, e.g. in WWTP. These concentrations, however, might be below the detection limit of chemical analyses. Nevertheless, the concentrations used in this study are comparable to environmental ones reported elsewhere (Larsson et al., 2007; Golet et al., 2002 and Martinez-Carballo et al., 2007).

In conclusion, we provided strong evidence for the negative effects that ciprofloxacin can exert on soil and water ecosystems, which contradicts the recent assessment of low persistence and low ecological risk, if any, of ciprofloxacin in the soil environment (Wetzstein et al., 1999, 2009). Conversely, our results are consistent with a life cycle assessment of priority and emerging pollutants in waste water which demonstrated that ciprofloxacin is one of the main pharmaceutical and personal care products of environmental concern, being the main contributor to ecotoxicity in terrestrial systems, and one of the main in freshwater systems (Muñoz et al., 2008). Finally, although extractable amounts of antibiotics in soils are usually small (this study; Wetzstein, 2009; Hamscher et al., 2002), initial concentrations can be high enough to affect soil microorganisms immediately after addition (Thiele-Bruhn and Beck, 2005).

In our experiment, the *qnr S* resistance gene appeared after 14 days of exposure, independent of the ciprofloxacin concentration studied. Resistance development is promoted by continuous exposure of bacteria to concentrations below therapeutic levels (Picó and Andreu, 2007). This is exactly what can occur in soil, where due to a decrease of compound's bioavailability, bacteria are exposed to lower effective concentrations of ciprofloxacin. Therefore soils can be an important source of resistant bacteria that can transfer the corresponding genes to other bacteria living in ground or drinking water. Eventually these genes can be transferred by plasmids to pathogenic microorganisms (Esiobu et al., 2002; Martinez, 2009; Zhang et al., 2009).

4.5 Implications of fluorquinolone contamination for human health and human related activities

There is evidence that fluoroquinolones can affect the yield and the quality of agricultural crops. For example, enrofloxacin was found in carrots grown in soils contaminated with this antibiotic (Boxall et al., 2006). Moreover, a decline in growth was observed when plants were exposed to enrofloxacin (Migliore et al. 2003). This might be related to interference of fluoroquinolones with photosynthesis (Aristilde et al., 2010). An additional explanation can be the inhibitory effects of these antibiotics on key microbial populations for plant growth. Hence, fluoroquinolones can have also negative

implications for human health and human related activities, like agriculture. Therefore, our knowledge on the fate and effects of pharmaceuticals, particularly for antibiotics, and their degradation products in the environment must be improved for proper environmental risk assessment and human risk assessment, e.g. for mixtures of pharmaceuticals which have stronger effects than single compounds (Cleuvers et al., 2003). As well, better strategies to remediate sludge and manure contaminated with antibiotics or restrict their application to agricultural fields are necessary to avoid input of these compounds into the soil ecosystem.

4.6 General rules for prediction of biodegradation in soil

In a recent study, a new database concerning the fate of pharmaceuticals in wastewater treatment plants was published (Miège et al., 2009). Removal percentages during waste water treatment are reported for 50 compounds, including 70% removal for ibuprofen and ciprofloxacin, although with high variation of 30% between the studies. Our results, clearly illustrate that degradation of these compounds is radically different. Meanwhile ibuprofen is easily or readily degradable, ciprofloxacin is recalcitrant to biodegradation. Contrary to the well accepted hypothesis that mineralisation is higher in water than in soil mainly due to sorption processes (Ladd et al., 1996), mineralisation of ciprofloxacin was higher in soil than in aqueous media, which we assign to the reduced toxicity in soil due to sorption. Therefore, for toxic compounds in soil, a reduced bioavailability can result in increased degradation potential. These results illustrate the strong variability between different studies and also demonstrate how the lack of data coming from soil studies impedes understanding the real fate of contaminants in the environment.

In addition, Aronson et al. (2006) suggested that estimated degradation half-lives should not be used for risk assessment, only for banning and prioritising chemicals. Also, given that small differences in the chemical structure of pollutants and in experimental conditions may result in pronounced differences of degradation rates, statistically established correlations may help to roughly classify chemicals, but detailed insight into their environmental fate definitely needs experimental studies. Therefore, the understanding of biodegradation, including processes and pathways in the environment

must be improved significantly (Tunkel et al. 2000). A fundamental assumption implemented for ERA within the REACH and EMEA initiatives for example, is that ready or easy biodegradability in water can always be transferred to soil. However, in screening tests in aqueous systems, e.g. OECD 301 test (OECD, 1992), the concentration of the studied compound is high and microorganisms utilise the chemical as the only or primary source of carbon and energy since no other C sources are added. However, in soil, which is a complex system (Nannipieri and Badalucco, 2003), the high organic carbon content may result in lower degradation rates of the compound. Moreover, natural carbon sources are degraded simultaneously and therefore the pollutant may be degraded as a secondary substrate (Ahtiainen et al 2003). Consequently, first-order models derived from the Monod equation, may thus not be adequate to describe the mineralisation kinetics for low concentrations of chemicals in soil (Scow et al. 1986), or of toxic compounds, where degradation strongly depends on the initial concentration of the compound. In the same way, Scow and Johnson (1997) stated that models and equations describing metabolism in aqueous media do not include all the biological interactions that may occur in soil. Besides, screening tests can provide fairly reproducible qualitative results for chemicals that are either easily biodegradable or recalcitrant; however, for chemicals of intermediate biodegradability the results are extremely variable and difficult to interpret, as illustrated by the inconsistent data for ibuprofen degradation.

Simulation tests in complex environmental systems such as soil in comparison to screening tests, will exhibit a broader range of degradation and abiotic interactions (e.g. compound-soil minerals), leading to contradictory data. Different bioavailability, inoculum, levels of microbial activity, concentrations of the chemical due to the soil heterogeneity, type of soil etc. will lead to data variation, and thus direct comparison with ready biodegradability data is hampered due to the many variables involved.

Another crucial aspect is that in soil, compound dissipation has to be clearly differentiated from biodegradation. As an example, the half life for Tylosin in soil was much shorter than in water when determined by dissipation, but 80% of the compound was sorbed to soil (Hu and Coats, 2007). However, the stability and effects of the adsorbed fraction are unknown. Therefore, only isotopically labelled compounds with the labelled atom in the most stable position(s) of the molecule should be used in simulation

studies to obtain valuable biodegradation and fate data. In order to distinguish the two processes in soils and sediments, half lives of chemicals should be assigned either as dissipation half lives (DisT50) or as degradation half lives (DegT50), being the last one the most adequate for risk assessment purposes. Moreover, the proportion of NER formed depends on the position of the labelling in the compound chemical structure, i.e. if the label is positioned in a labile molecular fragment, the NER formation will tend to be low. In contrast, if label is in a stable moiety the NER amount will be high.

In general, there is a common misconception on the classification of a chemical as persistent or non-persistent, i.e. the environmental fate of a substance is mostly considered an inherent property of the compound which can be as easily determined as solubility, melting point or K_{ow} . This approach is not adequate, since the fate and degradation of a chemical is determined by a combination of compound-specific properties and environmental conditions (Boethling et al. 2009) and thus a systems property. Misconceptions are also found in regulatory guidelines, e.g. in the EMEA guideline on the risk assessment of medicinal products for human use (EMA, 2006). In this guideline, a two-phase approach for risk assessment is suggested: in the first phase, only consumption and $\log K_{ow}$ data are taken into account. This means for example that substances having a $\log K_{ow}$ higher than 4.5 should be screened in a step-wise procedure for persistence, bioaccumulation and toxicity. However, as demonstrated in this study the use of K_{ow} is not a good predictor for the environmental fate or toxicity. Ciprofloxacin was demonstrated to be a hazardous pollutant due to its low degradation in the environment and high toxicity against relevant environmental microbial communities, even if its K_{ow} is very low ($\log K_{ow}$ -1.1). In addition, Kottler and Alexander (2001) didn't obtain a good correlation when correlating sorption and bioavailability of 21 PAHs in soil. Misconceptions about NER related persistence is discussed in the section below.

As concluded by Boethling et al. (2009), existing guidance places disproportionate emphasis on the screening phase. Although it is recognised that simulation tests have greater environmental relevance, they are triggered only by a negative result in the screening test. A new approach in the environmental risk assessments of chemicals is needed, with a screening and subsequent confirmatory phase (fate-specific simulation tests). Unfortunately, the use of models is seen as a key factor to improve our

understanding of the fate of pollutants in soils, however there is a lack of focused empirical data to develop such models (Braidia et al., 2001). In the Conclusions section of this thesis some general rules derived from empirical data for extrapolating results from screening tests to the biodegradation in soil are presented.

4.7 New concept for assessment of non-extractable residues

NER formation is normally considered as a process contributing to pollutant dissipation and also a process decreasing the pollutant bioavailability. Therefore the decreased availability implies an increase in the persistence of the compound (Barriuso et al., 2008). The actual conception of NER and their related risk for the environment is still controversial (Alexander 2000; Barraclough et al., 2005; Craven and Hoy, 2005; Barriuso et al., 2008) in terms of stability and risk, whether they are bioavailable/non-bioavailable and hazardous/innocuous and how these characteristics may change over time. Currently, in Europe pesticides with > 70% NER formation will not be approved, unless it is shown that they are innocuous for the environment and the current approach is to treat soil NER in the same way as persistent parent compounds (Craven and Hoy, 2005). The guidance to pharmaceutical companies assumes that all residues that cannot be extracted with exhaustive extraction methods should be considered unavailable (Boethling et al. 2009) and not dangerous for the environment. According to this, NER from 2,4-D, ibuprofen and ciprofloxacin should be considered as persistent and not bioavailable. Conventional mass balance and mass balance considering biogenic NER of the biodegradation of ibuprofen and 2,4-D are presented in **Figure 27** and **Figure 28** respectively. NER from ibuprofen are mainly comprised of biogenic residues (**Figure 28B**) and were slowly mineralised (Nowak et al. submitted), thus bioavailable. It has also been reported that bacteria and earthworms are able to access the supposedly unavailable contaminant (Stokes et al., 2006). Similarly, due to the long term toxicity of ciprofloxacin in soil, NER were maybe still bioavailable for bacteria as discussed previously, or just bioaccessible, but then released by microorganisms that can degrade natural organic matter (Ekschmitt et al., 2005). In the case of 2,4-D, NER were also mostly biogenic (**Figure 27B**) and

definitely did not consist of 2,4-dichlorophenol or other degradation products as suggested by Benoit et al. (1997). Ignoring the fact NER can still be bioavailable and that biogenic residues are not hazardous for the environment leads to an underestimation or overestimation of the risk of NER for the environment. The overestimation of the risk related to NER (conventional view versus new view on biogenic residues or NER) is illustrated in **Figure 27** and **Figure 28**.

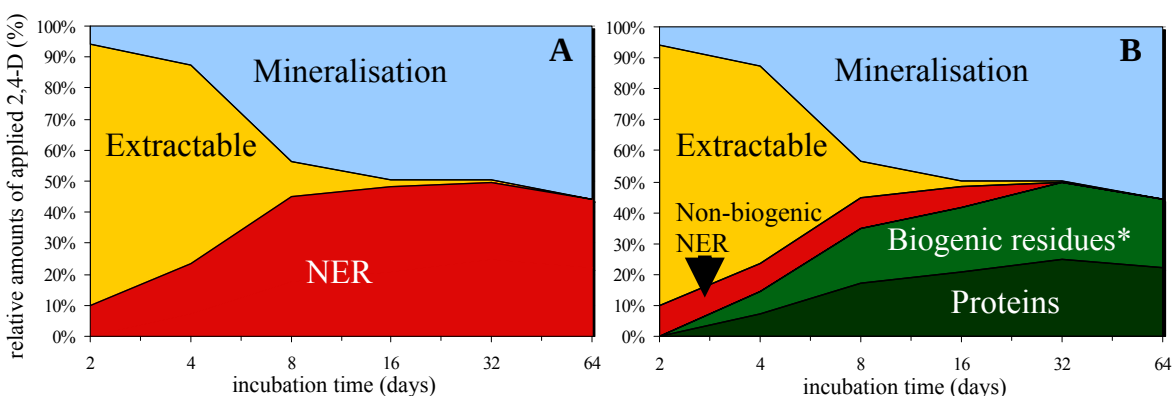


Figure 27. Conceptual scheme of the ^{13}C conversion over microbial degradation of $^{13}\text{C}_6$ -2,4-D in soil. A: conventional mass balance and B: new view; mass balance considering biogenic residues formation. *Biogenic residues were estimated from AA using a conversion factor of 2 (for details see Nowak et al., 2011). This figure was kindly provided by Karolina Nowak.

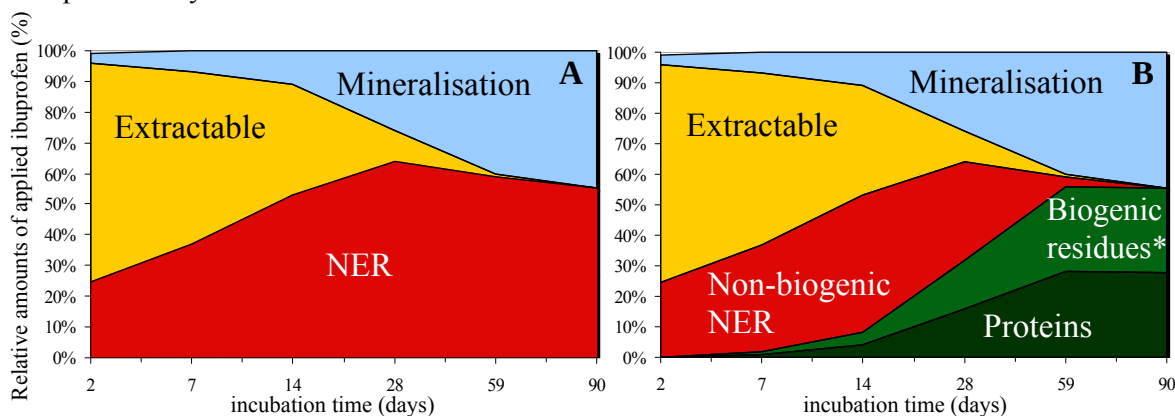


Figure 28. Conceptual scheme of the ^{13}C conversion over microbial degradation of $^{13}\text{C}_6$ -ibuprofen in soil. A: conventional mass balance and B: new view; mass balance considering biogenic residues formation. *Biogenic residues were estimated from AA using a conversion factor of 2 (for details see Nowak et al., submitted). This figure was kindly provided by Karolina Nowak.

Concerning NER formation, ageing enhances this development via chemical and/or physical processes (Sharer et al. 2003, Walker et al. 2005). However, the present results and those obtained by Nowak et al., (2011; submitted) demonstrate that the microbial contribution to NER formation must also be considered. This is particularly true for easily degradable compounds: microbial derived organic compounds such as proteins, amino acids, lipids etc...are stabilised in soil (Fan et al., 2004; Kindler et al., 2009; Miltner et al. 2009) and represent an important fraction of the total NER (**Figure 27** and **Figure 28**).

Additionally, abiotic and biotic NER formations are understood as independent processes, however biodegradation and abiotic sorption are competing and not independent processes as discussed before. Consequently, abiotic and biotic NER can not be considered as simply independent fractions, where the first one is subtracted from the second one to obtain the biotic contribution to NER formation. The implication of this dependency is that sterile controls do not reflect the actual abiotic contribution in biotic systems.

In order to conclude, the risk of NER from easily degradable compounds, determined by means of non-extractable isotope labelling, may be overestimated due to the contribution of biogenic residues. Consequently, a revision of the actual mass balance strategies and biodegradation guidelines, i.e. OECD 307, particularly for the setup of the abiotic controls must be carried out for this type of compounds.

Conclusions

In the present study, a detailed comparison of the degradation in aqueous and soil systems of three labelled compounds, the herbicide 2,4-D, the nonsteroidal antiinflammatory, analgesic and antipyretic ibuprofen, and the antibiotic ciprofloxacin was achieved. The use of ^{13}C and ^{14}C label allowed obtaining a mechanistic overview of the biodegradation of these environmentally relevant compounds in a standard mineral medium and in an agricultural soil. New insights into the microbial degradation processes in soil based on the presented results and literature data were derived, and we identified the chances and limitations for the prediction of biodegradation in soil on the basis of data obtained from tests in aqueous systems. Furthermore, the related risk associated with the presence of these compounds in the environment was assessed.

Understanding the processes potentially affecting the environmental fate of chemicals, and how their fate depends on environmental conditions, is fundamental to assessing and predicting biodegradation. Consequently, a proper understanding of ongoing processes in soil, e.g. bioavailability, biotic and abiotic degradability, toxicity dependence, and NER formation including biogenic residues is indispensable, in particular for soil systems.

In order to overcome the inconsistencies of the existing data, whenever possible, we suggest that biodegradation tests in soil should be performed in order to generate consistent databases and provide a validated assessment of the environmental risk of a chemical. A central remark regarding this point is the necessity of using $^{13/14}\text{C}$ -label in the most stable(s) position(s) of the molecules to obtain conservative but more realistic data. For instance, C-label positions in labile parts such as carboxylic moieties or other highly oxidised positions of the parent compound should be avoided, since label in these positions is lost as CO_2 even without any further transformation of the compound or rapidly formed NER. In these cases mineralisation is overestimated and the proportion of

NER underestimated. In terms of risk, the use of ^{13}C permits the critical differentiation between non hazardous biogenic NER and potentially hazardous non-biogenic NER. Moreover, the difference of compound derived label between crude extracts (parent compound + metabolites + biomass) and purified extracts (parent compound + metabolites) may give an idea about the microbial activity and contribution during biodegradation.

Additionally, the use of C-labelled molecules allows the distinction between dissipation and degradation of a contaminant in soil, which is necessary for risk assessment because only degradation ultimately removes the compound from the system. Another important issue is the need of a revision of the sterile (abiotic) control in soil guidelines, since abiotic and biotic NER formation are competitive processes and such controls do not reflect the actual abiotic contribution in biotic systems. The present conception of abiotic controls in soils leads to an overestimation of the abiotic contribution to NER formation.

However, as the development of an empirical database is a long term issue and simulation tests cannot always be done, we propose some general rules for extrapolating results from water-based ready biodegradability tests to the biodegradation in soil systems:

- For easily or readily biodegradable compounds of low toxicity towards microbes, mineralisation and metabolisation are mostly higher in water systems than in soil, which is due to NER formation in soils, including sorption and sequestration processes. However, the final extent of biodegradation in water and soil, i.e. the sum of mineralisation and biogenic NER, are often comparable in the case of such chemicals.
- For compounds which are highly toxic towards microorganisms, mineralisation and metabolisation is higher in soil systems because (i) sorption of the compound to the soil particles (NER formation) will reduce its toxicity towards degrading microorganisms, (ii) microbial diversity is higher in soils, (iii) tolerance against the toxin is induced and (iv) soils are spatially heterogeneous and compounds interact abiotically with soil components (clay minerals, metal-oxides etc...) catalysing their degradation.

- Hydrophobic compounds (high K_{ow}) show lower mineralisation and metabolisation rates in soil than in aqueous systems, and tend to form high amounts of aged NER in soil due to their tendency to sorb to soil particles.
- Compound elimination with low or negligible mineralisation indicates principally abiotic formation of potentially hazardous NER containing toxic parent compounds and/or their primary metabolites mostly due to sorption and aging processes.
- High mineralisation of a compound (over 50%) is generally a consequence of microbial degradation with the concomitant formation of metabolites and microbial biomass, which may later be stabilized in soil organic matter, resulting in biogenic residues. This type of NER may account for a large portion or even all of the NER detected by isotope mass balances, thus leading to an overestimation of their hazardous potential.

The present investigation also contributes to improve the environmental risk assessment of emerging pollutants such as pharmaceuticals, for which not enough or contradictory data on their environmental fate and effects are available. Whereas ibuprofen is readily biodegradable and does not seem to be hazardous for the environment, we clearly demonstrated that ciprofloxacin is persistent, and affects the microbial communities and activities in soil. Thus, it represents a hazardous pollutant for the environment causing bioavailable and toxic NER. Consequently much more attention has to be given to contamination of soils with antibiotics, which often has been neglected.

Moreover, even if soil has a buffering capacity against toxic compounds, it does not inhibit their antimicrobial activity completely. Fluoroquinolones thus can considerably affect environmental processes, particularly soil processes such as nutrient or carbon cycling and generate antibiotic resistance strains.

For a comprehensive assessment of contaminated soil, future investigations of pollutant fate in soil should always combine all the available resources and knowledge from biodegradation and ecotoxicology disciplines. They should particularly focus on multi-component mixtures of compounds, the complexity of interactions that can be generated in terms of bioavailability, biodegradation and toxicity and because they better reflect the

reality. Furthermore, the adaptation of the microbial community to continuous application of toxic compounds with manure or sewage sludge still needs to be studied.

Abbreviations

^{13}C	Labelled (stable isotope)
^{14}C	Labelled (radioactive isotope)
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4-DCP	2,4-Dichlorophenol
AA	Amino Acids
ANOSIM	Analysis of Similarities
ASE	Accelerated Solvent Extraction
BaCl_2	Barium chloride
BOD	Biological oxygen demand
bp	Base pairs
BSTFA	bis-trimethylsilyltrifluoroacetamide
CaCl_2	Calcium chloride
CAS	Chemical Abstracts Service
CEC	Cation exchange capacity
CoSO_4	Cobalt(II) sulfate
CuSO_4	Copper(II) sulfate
DNA	DesossiriboNucleic Acid
DT_{50}	Half -life
EA-C-IRMS	Elemental Analyser-Combustion-isotope ratio Mass Spectrometry
EDTA	Ethylenediaminetetraacetic acid
EC_{50}	Half maximal effective concentration
EMA	European Medicines Agency
EPA	US Environmental Protection Agency
FeCl_3	Iron(III) chloride
FeSO_4	Iron(II) sulfate

GC-C-IRMS	Gas Chromatography Combustion-isotope ratio Mass Spectrometry
GC-MS	Gas Chromatography Mass Spectrometry
H ₃ BO ₃	Boric acid
ha	Hectare
HCl	Hydrochloric acid
HCOOH	Formic acid
HPLC	High performance liquid chromatography
ESI-HR-MS	Electrospray ionization high resolution mass spectrometry
IFEN	Institut Français de l'Environnement
kBq	Kilo becquerel
K _d	Distribution coefficient
KH ₂ PO ₄	Potassium dihydrogen phosphate
KHPO ₄	Potassium hydrogen phosphate
K _{oc}	Soil organic carbon-water partitioning coefficient
KOH	Potassium hydroxide
K _{ow}	Octanol/water partition coefficient
LC-MS	Liquid chromatography-mass spectrometry
LSC	Liquid scintillation counting
M	Molar concentration
MCPA	2-methyl-4-chlorophenoxyacetic acid
MDS	Non-Metric Multidimensional Scaling Analysis
MgSO ₄	Magnesium sulfate
MnSO ₄	Manganese sulfate
MM	Mineral medium
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
Na ₂ HPO ₄	Sodium hydrogen phosphate
n.a.	not analysed
n.d.	not detectable

NaOH	Sodium hydroxide
NER	Non-Extractable Residues
NH ₄ Cl	Ammonium chloride
nm	Nanometre
min	minutes
OD	Optical Density
OECD	Organization for Economic Co-operation and Development
OM	Organic Matter
PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
PEC	Predicted environmental concentration
PNEC	Predicted no-effect concentration
rDNA	Ribosomal DNA
RP	Reversed phase
rpm	revolution per minute
SOM	Soil Organic Matter
SPE	Solid Phase Extraction
SS	Suspended solids
Taq	Termus aquaticus DNA-polymerase
TLC	Thin Layer Chromatography
TOC	Total Organic Carbon
T-RFLP	terminal restriction fragment length polymorphism
UFZ	Centre for Environmental Research
USA	United States of America
v	Volume related
WHC	Water Holding Capacity
WWTP	Wastewater treatment plant
ZnCl ₂	Zinc chloride

Figures

Figure 1.	Scheme of contaminant bioavailability at the soil microscale. Prevalently, only a fraction of contaminants is bioavailable to degrading organisms in heterogeneous soils. A substantial part is only bioaccessible, denoting that the compound is physically or temporally constrained but could become bioavailable, e.g. by aggregate destruction and humic matter degradation. Contaminants can also be occluded and, hence, are non-bioaccessible (cf. legend).	12
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Declaration of Authorship (in German)

Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre eidesstattlich, dass ich die Dissertation selbstständig verfasst und alle in Anspruch genommenen Hilfen in der Dissertation angegeben habe.

Ich erkläre, dass durch die Veröffentlichung als Dissertation der RWTH Aachen bestehende Schutzrechte – insbesondere Urheberrechte – nicht verletzt werden.

Diese Arbeit wurde am UFZ verfasst. Ich erkläre, dass Veröffentlichung der Dissertation bestehende Betriebsgeheimnisse nicht verletzt.

Leipzig, den 22.07.2011

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International Publications

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- Girardi, C., Greve, J., Lamshöft M., Fetzner, I., Miltner, A., Schäffer, A., Kästner, M. Biodegradation of ciprofloxacin in water and soil and its effects on the microbial communities. Submitted.
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Conferences and Proceedings

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- 2010: Girardi, C., Miltner, A., Greve, J., Lamshöft M., Kästner, M., “Biodegradation of Pharmaceuticals and their Effect on Microbial Communities in Soil and Water Systems”. Presentation. International Symposium: Microbial contaminant degradation at biogeochemical interfaces. Leipzig, Germany.
- 2009: Girardi, C., Miltner, A., Kästner, M. “ Biodegradation of Biocides and Pharmaceuticals Compounds in Soil and Water Systems”. Platform presentation. SETAC Europe 19th Annual Meeting. Göteborg, Sweden.
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- 2006: Engel, E., Arredondo, V., Girardi, C., Gonzalez, A., Escobar, P., Fiore, N & Valenzuela P.D.T. “Grapevine Viruses in Chile: Genomics and Detection Based on Immunocapture and Microarray Technologies” pp 761-771 in: Proc 15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine. Stellenbosch, South- Africa.
- 2005: Arredondo, V., Gonzales, A., Girardi, C., Engel, E., Escobar, P. & Valenzuela, P.D.T. “Genomic Analysis of Chilean Isolates of Grapevine Viruses and Development of Immuno-detection Systems”. Biotechnology Habana 2005. La Habana, Cuba.
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