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Significance and application of microbial toxicity tests in assessing ecotoxicological risks of contaminants in soil and sediment

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SUMMARY

Micro-organisms are vital for soil fertility and for the degradation of organic matter and pollutants in soils and sediments. Due to their function and ubiquitous presence the microflora can act as a environmentally very relevant indicator of pollution. Microbial tests should be used discriminatory for the establishment of soil and sediment quality guidelines. This review gives an evaluation of microbial toxicity tests and a novel method to derive quality guidelines.

Long term microbial tests are generally less sensitive than short term tests. The toxic effects can be obscured by the activity of a few resistant micro-organisms, when for example soil respiration is used as a sum parameter during a long incubation period. Mineralization tests with high substrate concentrations which enable growth, are less sensitive than similar tests with low concentrations of substrate. The latter tests are more relevant for natural ecosystems.

The often applied microbial toxicity tests can be categorized as single species tests, biomass measurements, carbon and nitrogen transformations, enzymatic tests and tests measuring changes in microbial diversity. Comparisons between tests can only be indicative because the relative sensitivity depends on the toxicants and soils used. The respiration rate per unit of biomass is a more sensitive indicator of toxic effects than the respiration rate or the amount of biomass alone. The autotrophic nitrification and acetylene reduction tests can be sensitive when short incubation times are used. The nitrogen mineralization, denitrification and many enzymatic tests are often not very sensitive. The urease activity is a relatively sensitive enzymatic test in many studies.

The replacement of sensitive micro-organisms by different resistant species can have serious ecological consequences. Some species become extinct while others appear in bulging numbers. Adaptation of a community to a pollutant must be considered as the very process which disturbs a polluted ecosystem. The resistant micro-organisms often fail to perform specific ecological functions. The occurrence of resistant species can be used as an sensitive and ecologically relevant indicator for deterioration from environmental pollution. Persistent toxic effects on the microflora can be caused by zinc, cadmium and copper at concentration levels lower than the current European Community limits.

Tests with anaerobic sediment processes were orders of magnitude more sensitive for some clorinated aliphatic compounds than aquatic toxicity tests. The addition of a few mg zinc per kg soil can inhibit the more sensitive microbial processes (like chloroform or 4-chlorophenol degradation), whereas soil invertebrates and some plants are less sensitive to zinc.

After the evaluation of the tests, a novel method is described to derive soil and sediment quality guidelines using microbial toxicity tests. The results of single species tests with micro-organisms can be incorporated into the contemporary risk assessment method for higher organisms which is based on the extrapolation from single species tests to the protection of 95% of all species in an ecosystem. This method uses the No Observed Effect Concentrations (NOEC) of a number of toxicity test to calculate a Hazardous Concentration 5% (HC5). The HC5 is calculated from more than 5 NOEC values. In analogy the Effect Concentration 10% (EC10) can be used to calculate the Dangerous Concentration 5% (DC5). The DC5 is calculated from more than 5 EC10 values. The DC5 should give protection to 95% of the microbial processes. The DC5 of a number of pollutants are calculated and compared with the HC5 values from the literature. Microbial toxicity tests can be used for risk assessment because micro-organisms are among the most sensitive organisms for the effects of pollutants.

SAMENVATTING

Micro-organismen zijn belangrijk voor de bodemvruchtbaarheid en voor de afbraak van organisch materiaal en vervuilingen in bodems en sedimenten. Door hun functie en alomtegenwoordige aanwezigheid kan de microflora dienen als een zeer milieurelevante indicator voor milieuverontreiniging. Microbiële testen moeten selectief gebruikt worden voor het vaststellen van kwaliteitsrichtlijnen voor bodem en sediment. Dit literatuuroverzicht geeft een evaluatie van microbiële toxiciteitstesten en een nieuwe methode om kwaliteitsrichtlijnen af te leiden.

Lange termijn-testen zijn over het algemeen minder gevoelig dan korte termijn-testen. De toxische effecten kunnen verdoezeld worden door de activiteit van een paar resistente micro-organismen, wanneer bijvoorbeeld de bodemrespiratie als een somparameter gebruikt wordt gedurende een lange incubatieperiode. Mineralisatietesten met hoge substraatconcentraties die groei mogelijk maken, zijn minder gevoelig dan vergelijkbare testen met lage substraatconcentraties. Deze laatste testen zijn meer relevant voor natuurlijke ecosystemen.

De meest toegepaste microbiële toxiciteitstesten kunnen worden gecategoriseerd als testen met één soort, biomassa bepalingen, koolstof- en stikstofomzettingen, enzymatische testen en testen die de veranderingen in de microbiële diversiteit meten. De vergelijking van verschillende testen kan slechts een indicatie van de relatieve gevoeligheid geven, omdat deze afhankelijk is van de gebruikte toxicanten en bodems. De respiratiesnelheid per eenheid biomassa is een gevoeliger indicator voor toxische effecten dan de respiratiesnelheid of de biomassa apart. De autotrofe nitrificatie-en acetyleenreductie-testen kunnen gevoelig zijn wanneer korte incubatietijden worden gebruikt. De stikstofmineralisatie, denitrificatie en veel enzymatische testen zijn vaak niet erg gevoelig. De urase-activiteit is een relatief gevoelige enzymatische test in veel studies.

Het vervangen van gevoelige micro-organismen door andere resistente soorten kan ernstige ecologische gevolgen hebben. Dit gaat gepaard met het uitsterven van gewenste soorten en het woekeren van ongewenste soorten. De aanpassing van een levensgemeenschap aan een vervuiling moet dan ook gezien worden als het proces dat een vervuild ecosysteem verstoort. De resistente micro-organismen kunnen vaak specifieke ecologische functies niet vervullen. Het voorkomen van resistente soorten kan gebruikt worden als een gevoelige en ecologisch relevante indicator voor de achteruitgang door milieuvervuiling. Persistente toxische effecten op de microflora kunnen door zink, cadmium en koper worden veroorzaakt bij gehalten die lager liggen dan de huidige normwaarden van de Europese Gemeenschap.

Testen met anaerobe sedimentprocessen zijn vele orden van grootte gevoeliger voor gechloreerde alifatische verbindingen dan aquatische toxiciteitstoetsen. De toevoeging van enkele mg zink per kg bodem kan de meer gevoelige sedimentprocessen (zoals de afbraak van chloroform en 4-chloorfenol) remmen terwijl bodeminvertebraten en sommige planten minder gevoelig zijn voor zink.

Na de evaluatie van de testen, wordt een nieuwe methode beschreven om bodem en sediment kwaliteitsrichtlijnen af te leiden uit microbiële testen. De resultaten van testen met één soort micro-organisme kunnen ingebracht worden in de huidige risico-evaluatiemethode voor hogere organismen die gebaseerd is op de extrapolatie van testen met een enkele soort naar de bescherming van 95% van alle soorten in een ecosysteem. Deze methode gebruikt de "No Observed Effect Concentration" (NOEC) van een aantal toxiciteitstesten om een "Hazardous Concentration 5%" (HC5) te berekenen. Uit meer dan 5 NOEC's wordt dan één HC5 berekend. In analogie kunnen de "Effect Concentration 10%" (EC10 waarden) worden gebruikt om een "Dangerous Concentration 5%" (DC5) te berekenen. Uit meer dan 5 EC10 waarden wordt dan één DC5 berekend. Deze DC5 zou bescherming van 95% van de microbiële processen moeten geven. De DC5 van een aantal milieuverontreinigende stoffen is berekend en vergeleken met de HC5 waarden uit de literatuur. Microbiële toxiteitstesten kunnen gebruikt worden voor risico-evaluatie omdat micro-organismen tot de gevoeligste organismen voor effecten van verontreingingen behoren.

1. INTRODUCTION

Persistent contaminants in the environment affect human health and ecosystems. Heavy metals, for more than 300 years, and pesticides, for more than 50 years, are quantitatively the most important pollutants. For environmental policy it is important to assess the risks of these pollutants. Many toxicity tests have been developed at a variety of levels of ecological organization. These tests range from single species laboratory tests to field enclosures. In ecotoxicology it was recognized that only a few species can be tested in the laboratory while a lot of species are present in a polluted ecosystem. This has prompted regulators to apply safety factors between the lowest results of different toxicity tests and the maximum tolerable concentrations in the environment [144]. These safety factors were used on both microbial, plant and animal toxicity tests. Instead of safety factors one can also use statistical extrapolation methods to compensate for the fact that only a few species can be tested in laboratory toxicity experiments while very many species have to be protected in nature [97]. In the Netherlands a statistical method was developed to extrapolate a relatively safe pollutant concentration from the results of relatively few ecotoxicological experiments with a small number of species. Using this method a soil pollutant concentration HC5 (Hazardous Concentration 5%) can be calculated from a number of toxicity test data. At a concentration lower than the HC5 it is expected that 95% of the species are protected [179]. This approach is illustrated in Figure 1.

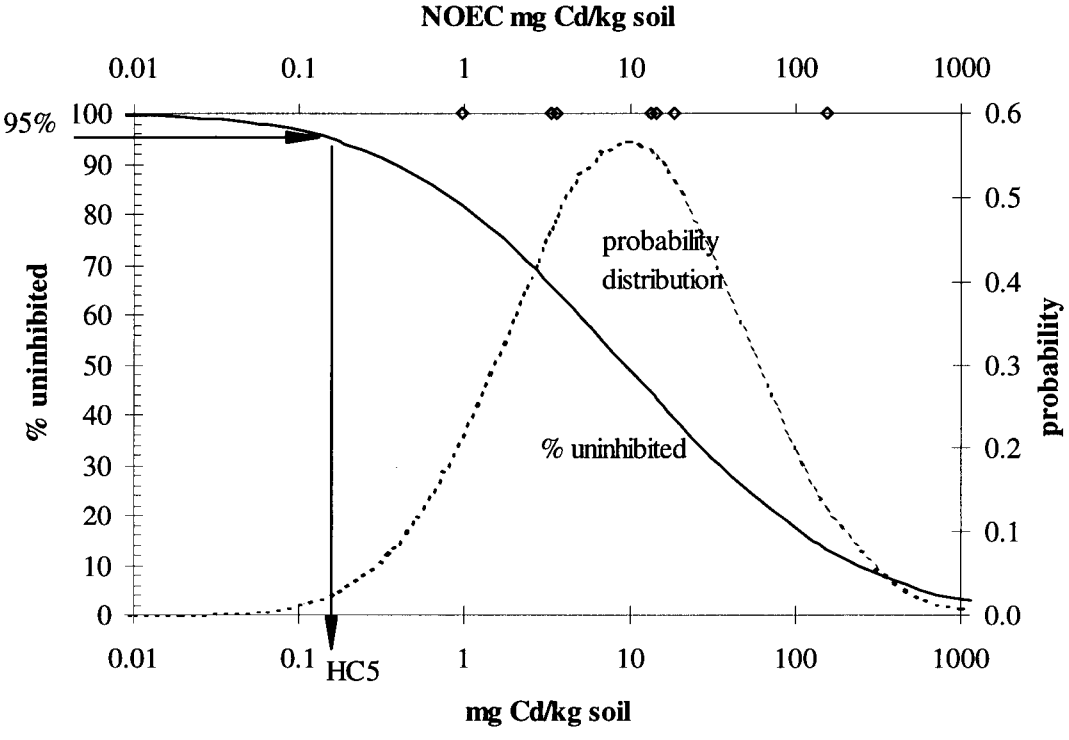

Figure 1 The extrapolation method [179] from single species tests to the Hazardous Concentration 5% (HC5). From the 7 NOEC data points, indicated at the upper axis, a normal probability distribution has been calculated which is the dotted bell shaped curve. This distribution is used to calculate a cumulative probability distribution, the descending curve, which indicates the percentage uninhibited species at each cadmium concentration.

The data points above at the upper axis in Figure 1 for example are the NOEC concentrations for cadmium on 7 different species of soil invertebrates [179] placed on a logarithmically scaled axes. From these data points a normal probability distribution was estimated with the same average and standard deviation as the data points. This is the bell shaped dotted curve in Figure 1. The descending line in Figure 1 is a cumulative probability distribution with the same average as the data. The curve was normalized to start at 100% and descend to 0%. The standard deviation of this distribution was obtained from the standard deviation of the log(NOEC) data multiplied by an extrapolation factor which is dependent on the number of tests [3]. The horizontal arrow indicates the point on the curve where 95% of the species is uninhibited. The vertical arrow indicates the resulting HC5 cadmium concentration (0.16 mg Cd/kg). The remaining 5% are exposed above their NOEC and might show observable effects caused by the pollutant.

The results of ecotoxicological tests with microbial processes can not be used together with single species tests in a single extrapolation method because a process test is fundamentaly different from a single species test. It is obvious that microbial toxicity tests should be used in the risk assessment of environmental pollutants because micro-organisms perform a multitude of specific functions in all ecosystems. Their very large biochemical diversity enables micro-organisms to live in many habitats. Different types of micro-organisms specifically adapted to their environment can be found everywhere on earth. Micro-organisms play an indispensable role in the geochemical cycli of many elements and are vital for the elimination of environmental pollutants. These geochemical and mineralization reactions are often performed by specialized bacteria that can not easily be replaced by other species. The symbiotic and pathogenic relationships between micro-organisms and higher organisms also play an important role in the ecosystem. Many plants for example would not survive in nature without their specific root fungi that form the mycorrhiza [137,142]. Each plant lives in association with a specialized rhizosphere microflora which is vital for the competitive abilities of the plant [39] and the resistance to root pathogens [113,49]. In addition micro-organisms are important food sources for many representatives of the soil fauna [118]. Soil animals like *Collembola* [83] and *Enchtraeidae* graze on very specific microbial species that grow on leaf litter or roots [98]. Replacement of these micro-organisms by other microbial species can lead to starvation of the animals. The activity of the soil microflora is a prerequisite for a healthy soil since micro-organisms are indispensable for the degradation of organic substances in soil and the subsequent formation of soil aggregates [4]. A number of *Streptomycete* species give a soil its specific odor [66] which also attributes to our perception of a healthy soil.

Due to their functions and ubiquitous presence micro-organisms can be used in a very large variety of toxicity tests [57]. Toxicity tests are, often routinely performed, experiments to quantify the effect of a toxicant on a specific organism or process. Earlier reviews describe side-effects of pesticides on soil fertility [58], effect of metals on microbial processes in soil [11] and the influence of environmental factors on the toxicity of polluted soils and sediments for micro-organisms [80]. So a large number of toxicity data are available to be used in the above mentioned risk assessment method.

This review describes the critical and selective application of microbial toxicity data into a novel risk assessment method. Chapter 2 describes some common issues in the interpretation of most microbial toxicity tests. The chapters 3-7 categorize various toxicity tests with micro-organisms. These categories are: Single species tests, biomass tests, carbon and nitrogen mineralization tests, enzymatic tests and the determination of changes in microbial diversity. Chapter 8 and 9 describe the use of these tests for the establishment of sediment and soil quality guidelines.

2. IMPORTANT ASPECTS FOR MICROBIAL TOXICITY TESTS

Before the description of the different categories of microbial toxicity tests, there is the question whether we can correct for soil characteristics between different test. Furthermore the effect of the incubation time, substrate concentration and the consequences of degradation kinetics will be considered. Finally the usefulness of the NOEC or some effect concentration as the input for the extrapolation method will be discussed.

2.1 The use of soil correction factors

The soil or sediment matrix can decrease the toxicity of pollutants by binding due to sorption, precipitation or complexation. For the HC5 extrapolation method each experimental NOEC concentration of a toxicant on a single species is normalized, to a NOEC for a standard soil with 25% clay and 10% organic matter [179]. This NOEC correction factor can be calculated from the clay and organic matter content of the soil with a formula which is specific for each pollutant [179]. For example the toxic effect of chlorophenols in different soils on two single species of earthworms depended on the chlorophenol concentration in pore water [177], so the concentrations in pore water were used to normalize the effect. For most microbial toxicity tests the situation is much more complex because each soil will have its own microflora with its own intrinsic sensitivity. Soil properties do not fully explain the large differences in sensitivity between soils. For example the large differences in toxic effects of pentachlorophenol and zinc on the mineralization of ^{14}C acetate in different soils could not be explained by sorption alone. Even when the effects were expressed as pore water concentrations the differences in sensitivity were many orders of magnitude [170]. Therefore the correction for soil characteristics still faces a number of complicating factors and more research is needed before this correction can be applied for microbial toxicity tests.

2.2 The effect of toxicants on the kinetics of microbial reactions

2.2.1 The inhibition of natural unstimulated microbial communities

Starvation is a common phenomenon in natural ecosystems [78] and microbial growth is relatively rare [6,7]. Therefore a toxicity test using the natural starving microbial communities in soil will be closer to the field conditions compared to a substrate enriched soil. To illustrate the implications of this difference an experiment with a non-enriched natural soil amended with a very small amount of acetate was performed. Figure 2 shows the toxic effect of pentachlorophenol (PCP) on the mineralization of ^{14}C-acetate to $^{14}CO_2$ [166]. The control soil showed a slower acetate consumption than the soil with 25 mg PCP/kg. For the test 10 g (fresh weight) soil was incubated with 10 ml of water and 45 ml air to facilitate the dispersion of 15 ng ^{14}C-acetate. The relatively high amount of biomass in a soil (1-10 mg biomass Carbon/10 g soil) [27] can not increase significantly by the addition of the minute amount of carbon from 15 ng of acetate. This addition will not enable the microflora to grow and therefore resistant species will not have a chance to grow rapidly and obscure toxic effects. In unamended soils many micrograms of acetate are produced and consumed in a gram soil daily [90] so that the addition of a few nanogram extra will not make a difference. So the measurement of the effects of toxicants on the first order degradation rates of low substrate concentrations is relevant for the natural situation in the field. Therefore the results of these tests should be incorporated into risk assessment procedures since they reveal the true sensitivity of the microbial processes.

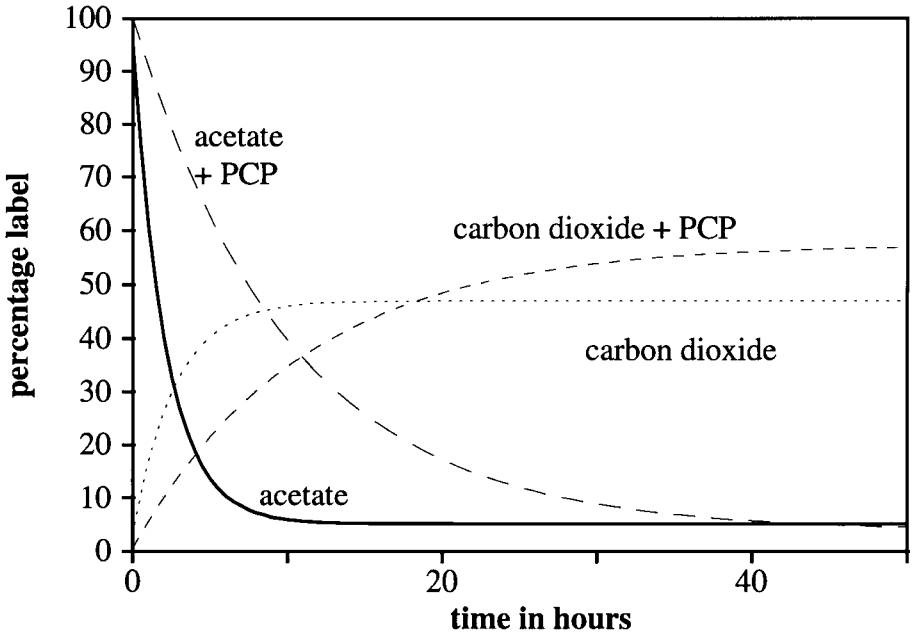

Figure 2 The toxic effect of 25 mg PCP/kg soil on the mineralization of 1 µg/l ^{14}C acetate. The descending curves show the acetate consumption and the ascending curves the corresponding carbon dioxide production.

2.2.2 The inhibition of growing microbial communities

Ecotoxicological responses are easier to detect in a metabolically activated soil. This activation can be performed by the addition of substrates like sugars and amino-acids which can be used by a wide variety of micro-organisms. Glutamic acid is often used as a substrate to activate the soil microflora [69,187,121]. In Figure 3 the effect of chlorpyrifos on the mineralization of a high concentration (1 mg/g) of glutamate in soil (Haanstra, *et al.*, unpublished results) is shown. Glutamate is added to soil and the carbon dioxide evolution is measured as an indicator for the growing amount of biomass. After an initial period the mineralization rate increases exponentially and can be plotted on a logarithmic scale as a straight line. The first line is a control where no chlorpyrifos is added and the soil microflora can grow uninhibited on the added glutamate. At 10 or 1000 mg chlorpyrifos/kg soil the lag time increases but the growth rate remains relatively similar since the three straight lines in Figure 3 are almost parallel. At 10 mg/kg the lag time increased by 2.2 h. The lag time can be interpreted as a decrease of the initial activity of the specific micro-organisms, responsible for the glutamate mineralization. A lag time equal to one doubling time would correspond to a decrease of 50% of the initial activity. The doubling time of the glutamate respiration is 3.7 hour. The ratio between the lag time and the doubling time is 2.2/3.7=0.6. Therefore this lag time corresponds with a decrease of 0.6*50% = 30% of the initial community degrading the glutamate. Some species might be more sensitive than others so one can assume that a number of species is seriously inhibited while others are not. If there are microorganisms with an important ecological function among the inhibited species there can be serious ecological consequences. So the effect of a toxicant on a growing microbial community results in an increase in lag time which can be interpreted as a percentage decrease in the initial activity of the community due to the inhibition of sensitive species.

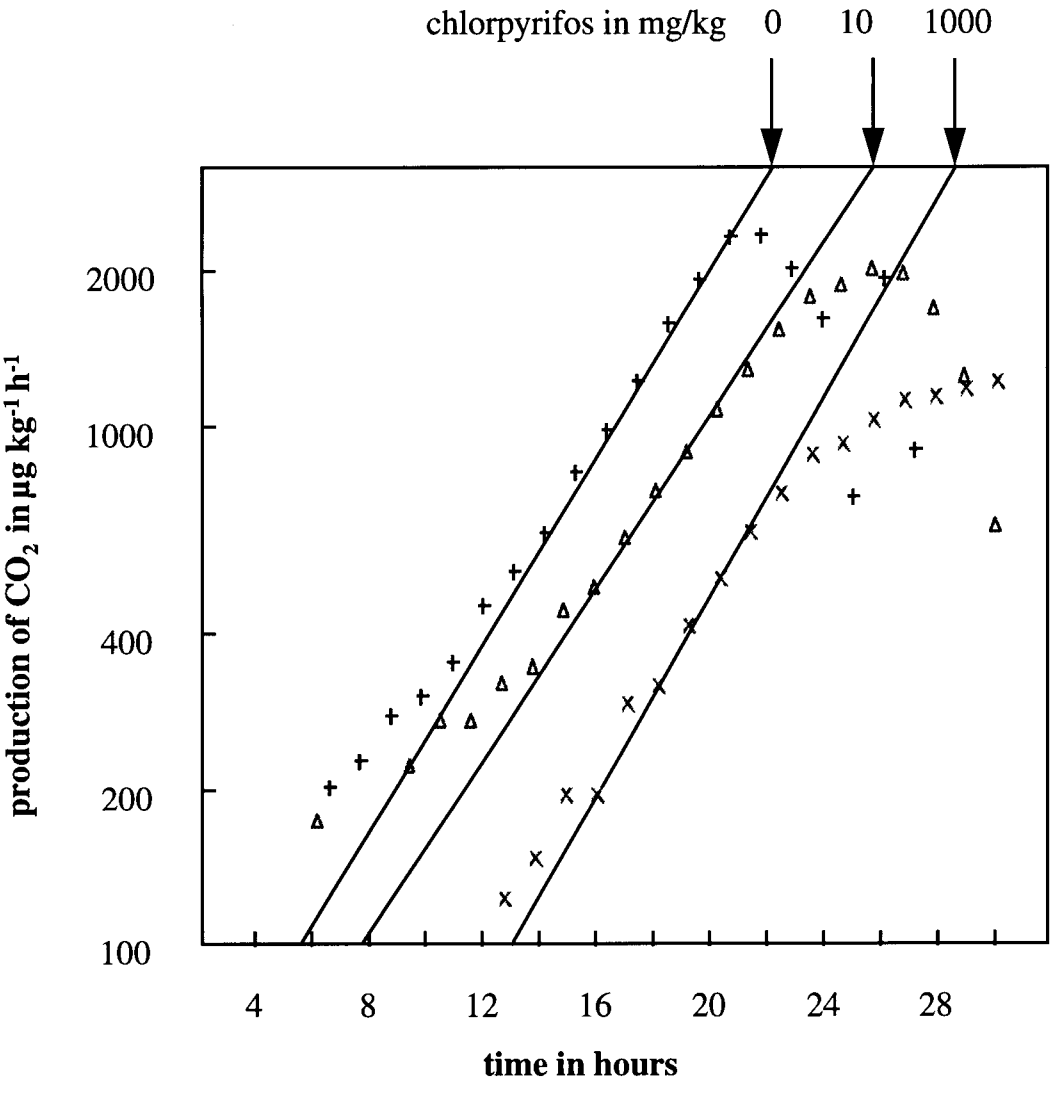

Figure 3 The toxic effect of chlorpyrifos on the respiration rate of glutamate. + is the control, Δ is 100 mg/kg and × is 1000 mg/kg .

2.2.3 Rate measurements versus measurements of toxic effects at a single incubation time

The effects at a single incubation time gives limited information and may lead to an erroneous interpretation because the measured effects can decrease at prolonged incubation time. The rule learned from animal toxicity tests that short-term tests are less sensitive than chronic tests due to the slow uptake of the toxicant by the animals does not hold for micro-organisms. The uptake is much faster in micro-organisms than in animals or plants [79]. Equilibrium is reached relatively rapid due to the larger surface to volume ration of micro-organisms. Long term toxicity tests with micro-organisms often obscure the inherent sensitivity of the different species of micro-organisms because the surviving species continue to degrade the substrate. In Figure 2 for example the carbon dioxide production in the samples with 25 mg PCP/kg will be similar to the control after an incubation time longer than 40 hours. In Figure 3 the CO_2 production after 24 h will be higher in the samples with 1000 mg chlorpyrifos/kg than in the control.

On the other hand, rate measurements can be very laborious because a number of measurements have to be made at each toxicant concentration. With first-order kinetics a

good approximation can be made when the carbon dioxide production is measured before most of the substrate is utilized. The first-order process can be described by $S(t)=S(0)*e^{(-rate*t)}$ with S(t) is the substrate concentration at time t and S(0) is the initial substrate concentration. An exponential function can be calculated by a Taylor approximation: $e^x = 1 + x/1 + x^2/4 + x^3/6 + x^4/24 + ...+ x^n/n! + ...$ for all x. The first two terms of the Taylor approximation of the above exponential function are 1- rate*t since x=-rate*t. Hence S(t)=S(0)*(1-rate*t). The latter terms can only be neglected when (rate*t) is small. This means that the first part of the first-order mineralization curve can be approximated with a linear equation (see Figure 2). The fraction of the converted acetate equals S(t)/S(0)=1-rate*t. This rate constant can then be estimated by a single short term measurement at each toxicant concentration. In this paper "short term" is short in relation to the process rate, *e.g.* (rate*t) must be small. For slow processes with a rate of 0.001 per month a "short" term measurement can take many months whereas 1 hour can be a long incubation time for a process which takes minutes to proceed.

We conclude that measurement of the rates of processes at a large number of toxicant concentrations is a better way to study toxic effects than the measurement of the effect at a single incubation time. For reactions which show first order kinetics, the measurement of the percentage substrate degraded after a short incubation time, is a good alternative for measuring the rates of the process at each toxicant concentration.

2.3 The selection of a single effect concentration as input for the extrapolation method

For the interpretation of the toxic effects of a single pollutant on a number of processes and single species it is convenient to summarize a complete dose effect curve into a single effect concentration. In order to compare the results of different tests a similar toxicity endpoint should be chosen. In the past, some microbial toxicity tests were developed using a risk philosophy which differs from present ideas regarding ecotoxicological effects [181]. The side effects of pesticides were considered to be reversible and therefore acceptable when they were in the same order of magnitude as the natural fluctuations of microbial processes caused by drought, heat, frost or other natural disturbances [59]. This might be true in an agricultural soil where all organisms have been exposed to regular pesticide applications for many years, but in a more natural ecosystem the local organisms will only be adapted to the prevailing natural influences. There is no guarantee that toxic effects in the same order of magnitude as the natural fluctuations are indeed reversible and have no impact on natural ecosystems [181]. The contemporary extrapolation procedures are based on NOEC's [83,180]. and not on the 90% or even 99% Effect Concentrations (EC90 or EC99) [59], which are in the same scale as natural fluctuations. Therefore it seems logical to use the NOEC as endpoint of microbial, plant and animal toxicity tests.

The NOEC can be defined as the higest toxicant concentration which does not show a statistically significant difference with the controls. The use of the NOEC is problematic since it is not only dependent on the sensitivity but also on the accuracy of the test [81]. This is a major drawback for microbial toxicity tests since the accuracy of different types of microbial test varies tremendously. A enzymatic test for example can be very precise giving an error of 1- 8% while the results of plate count experiments show a large error which can be as large as 48% [28]. The EC10 can replace the NOEC as input for statistical extrapolation procedures used to derive environmentally safe pollutant concentrations [97,179]. The EC10 is a commonly used effect parameter in microbial tests [70,191,169,165]. At the EC10 concentration there is a 10% inhibition which might not be very different from the NOEC concentration but the EC10 is not dependent on the accuracy of the test. Therefore the EC10 is to be preferred as endpoint of toxicity tests with microbial processes.

3. TOXICITY TESTS USING A SINGLE MICROBIAL SPECIES

The most simple way of toxicity testing is studying the effect of an added toxicant on growth or metabolism of a single microbial species in a defined medium [101]. The extrapolation of these results to the situation in soil is difficult due to the likely differences in bioavailability of the toxicant in the soil and in the medium. More specifically, growth media may contain EDTA or other compounds which chelate metals [140,21] or reduce toxicants like Hg^{2+} to volatile Hg [1]. High phosphate concentrations may lead to precipitation of toxic metal ions in the medium or to enhancement of the resistance of the cell [2]. Therefore correction factors are needed to translate the toxicant concentrations in medium to the corresponding concentrations in the water-phase and eventually in soil.

A more realistic physico-chemical approach is testing under soil conditions by introducing specific strains in sterilized soil. This has been not applied often because sterilization will change some soil properties that might affect the chemical speciation of the soil pollutants. A few data have been published. For example a pure culture of *Pseudomonas putida* was introduced into sterile soil and the mineralization of ^{14}C glutamate to $^{14}CO_2$ by this strain was monitored. A very high concentration of 100 mg/kg of isopropyl carbanilate, methyl-N-3-hydroxycarbanilate and 3,4-dichloroaniline gave about 50% inhibition of the glutamate mineralization rate [133]. In another experiment, three different *Pseudomonas* species were added to sterilized soil and the inhibition of the denitrification by various metals was monitored. The lowest tested concentration of 10 mg of Cd, Cu and Zn per kg soil inhibited the activity of two of the *Pseudomonas* species whereas 10 mg Pb/kg was not inhibitory [23].

A few data are available on strains of micro-organisms introduced into non-sterile soil. Its survival or activity was measured as a function of the toxicant dose. In these experiments there is the problem to measure the activity of the introduced micro-organism among the other microflora. The monitoring of the survival of a single species in natural soil may become practical in the future due to the developments of the polymerase chain reaction technique [82]. In this method total DNA is extracted from soil and a specific part of that DNA is hybridized with a species specific DNA probe. This hybridized DNA is amplified enzymatically and can then be separated and detected. This makes the detection possible of a single species among all other soil micro-organisms [19].

A fourth approach may be the extraction of the pollutant from soil and testing the extracts on several strains as in Microtox solid phase test. The light emission of the marine bacterium *Vibrio fisheri* (formerly known as *Photobacterium phosphoreum*) is measured in buffer [22]. However the experimental setup is artificial and therefore the test is mainly used as a screening tool for polluted soils and sediments [41].

4. TOXICITY TESTS USING MICROBIAL BIOMASS

In many tests with micro-organisms the sum of the activities of different microbial species in a community is measured. These activities are used as sumparameters in microbial toxicity tests. Measuring activities in relation to biomass is even more relevant. The total microbial biomass is an important parameter in ecology since micro-organisms form a vital part of the soil food web [42]. This chapter describes the application of biomass measurements for the risk assessment of pollutants.

4.1 Inhibitory and stimulatory effects of pollutants on soil microbial biomass

The total microbial biomass is the sum of the biomass of each separate microbial species in a soil being bacteria, actinomycetes or fungi which can be dormant or active. When a chemical is added to a soil, the various micro-organisms may have different types of response. Some micro-organisms become intoxicated and lyse. Other micro-organisms are resistant to a pollutant and can increase their numbers and biomass because of decreased competition. Specific micro-organisms will actually grow on organic pollutants. The observed increased biodegradation of pesticides after repeated applications is due to the selection of pesticide degrading micro-organisms [33]. Decreased predation by intoxicated nematodes or protozoa, might also be a factor that causes changes in the original numbers [85]. So microbial biomass is an ecologically important parameter but it can be relatively insensitive to pollutants because of the combination of stimulatory and inhibitory effects.

4.2 The use of the fumigation method for the evaluation of toxic effects

The determination of the total biomass of all living micro-organisms in a soil is a relatively difficult task because of the diversity of the organisms involved and their different responses to treatment. Some methods are only suited for the biomass determination of a specific group. The fumigation method does not have these drawbacks and is generally accepted although results vary [14,5]. In the chloroform fumigation procedure most (>99%) of the soil micro-organisms are killed. The stimulation of the carbon dioxide production after the removal of chloroform is a yardstick for the available organic carbon, derived from the affected microbial biomass [88]. Chloroform disintegrates the cell and the lysed micro-organisms excrete soluble organic compounds into the soil which can also be measured directly. This has been described as the fumigation extraction method [184,64]. This technique can also be applied for the measurement of microbial nitrogen or phosphorus. The fumigation technique can be used to assess the toxic effects of pollutants on the total microbial biomass in soil. The total microbial biomass as measured by the fumigation extraction method is not a sensitive parameter but the ratio between the respiration and the biomass can be used as a moderately sensitive indicator of the effects of pollutants [25,63,100,17].

5. TOXIC EFFECTS ON CARBON AND NITROGEN TRANSFORMATIONS IN SOIL

The carbon and nitrogen transformations in soil are mostly determined in relation to soil fertility. Therefore toxicity tests were developed to study the effects of pesticides and metals on these transformations. Here we will indicate which tests are useful for ecological risk assessment.

5.1 The toxic effects on unamended soil respiration

In these tests the natural CO_2 evolution of a soil is measured as a function of the toxicant dose applied. The rate of the unamended soil respiration is limited by the amount of readily degradable organic material. Soil respiration can be performed by a multitude of species which are not equally vulnerable and therefore this respiration is not very sensitive, neither to metals [11,52,53,68] nor to organic chemicals [189]. It can even be stimulated by the addition of pesticides while a number of other processes and enzymatic reactions do become inhibited [157,160,161].

5.2 Amended soil respiration

Addition of easily degradable substrates enhances respiration. Higher activity facilitates the measurement of the effects of pollutants. Glucose, acetate, lucerne and many other compounds have been exploited to stimulate soil respiration. Methods with radioactive substrates show first-order kinetics whereas the non radioactive methods use higher substrate concentrations and exhibit growth kinetics. In the latter the CO_2 production is larger than the natural respiration. Prolonged incubation times can obscure toxic effects. For example the Alfalfa meal amended long term respiration test was considered too insensitive to use for the evaluation of side effects of pesticides and was replaced by a short term respiration test [65,61] which can be quite sensitive for metals (see Table 1) [31]. The inhibition of the soil respiration by metals decreased upon prolonged incubation from 2 to 70 weeks [53]. The normal application rate of dinoseb acetate induced a reduction of the short-term (12 h) respiration whereas the long term (14 days) respiration did not show any effect [108]. Table 1 shows a large range in sensitivity for a single toxicant like pentachlorophenol, Zn Cr or Cd. This might be attributed to differences in the properties of the microbial communities and the sorption capacity of the soils.

Table 1 Toxicity tests measuring respiration in soils amended with a substrate.

Method	Chemicals	EC50 in mg/kg	Refer-ences	Method	Chemi-cals	EC50 in mg/kg	Refer-ences
^{14}C-gluta-mate	Chlorpropham	100	[133]	Gluta-mate[a]	Cd	55->1000	[69]
	Monuron	100			Cr	55-1000	
	3,4-dichloroaniline	>100			Cu	<55-400	
^{14}C-acetate	pentachlorophenol	0.1-950	[169]		Ni	>55-400	
	Chlorite	51			Pb	400->1000	
	Cd	97			Zn	55-1000	
	pentachlorophenol	15-1500	[168]		As	100	[121]
	triphenyltinhydroxide	1700		Alfalfa meal[b]	Cd	50	[31]
	3,4-dichloroaniline	480			Cr	50	
	orthoxylene	5500			Pb	400	
	Zn	87->1000	[170]		Zn	150	
	pentachlorophenol	57-1207			Cu	150	
glucose	Pb	>1000	[44]		Mn	150	
	Dinoterb	20	[107]				

[a] For the glutamate mineralization similar analysis had to be performed as described in Figure 3 since the authors did not report an EC50 value.

[b] The alfalfa meal mineralization [31] showed first order kinetics and the EC50's were estimated graphically.

High substrate concentrations can also lead to an underestimation of the sensitivity of the natural microflora. Radiotracer experiments were used to compare the sensitivity of the tests at both high and low substrate concentrations. The effect of added pentachlorophenol on the mineralization of ^{14}C acetate was larger at 3 µg acetate/kg compared to 0.3 or 3 g acetate/kg. The EC50 was 2 or 2.5 times lower at 3 µg acetate/kg [169]. The same trend was also shown in experiments with metal contaminated soils. The total CO_2 production was not influenced whereas the mineralization of ^{14}C glucose to $^{14}CO_2$ was strongly inhibited [16]. So long term tests with high substrate concentrations are often less sensitive than the short term tests and the tests with low substrate concentrations.

5.3 Nitrogen transformation in soil

Figure 4 gives an overview of the soil nitrogen cycle. The conversion of organic nitrogen compounds to NH_4^+ and NO_3^- is called nitrogen mineralization. When only the formation of NH_4^+ is measured it is referred to as ammonification. The oxidation of NH_4^+ to NO_3^- is called nitrification and is normally performed by a few autotrophic species of bacteria. Nitrogen immobilization occurs when nitrate or ammonia are consumed by soil micro-organisms which are growing on an added substrate which does not contain enough nitrogen for their growth. All the nitrate available in the soil is then incorporated into microbial biomass. The reduction of NO_3^- to N_2 under anaerobic conditions is called denitrification and can be performed by a multitude of microbial species. The reduction of atmospheric N_2 to organic nitrogen compounds is called nitrogen fixation and is performed under anaerobic conditions by specialized bacteria with an enzyme which can be measured by the reduction of acetylene.

For the evaluation of toxicity tests using nitrogen transformations it is important to know that nitrification and nitrogen fixation are performed by a few species of specialized bacteria while the nitrogen mineralization, ammonification, nitrogen immobilization and denitrification can be performed by a multitude of species.

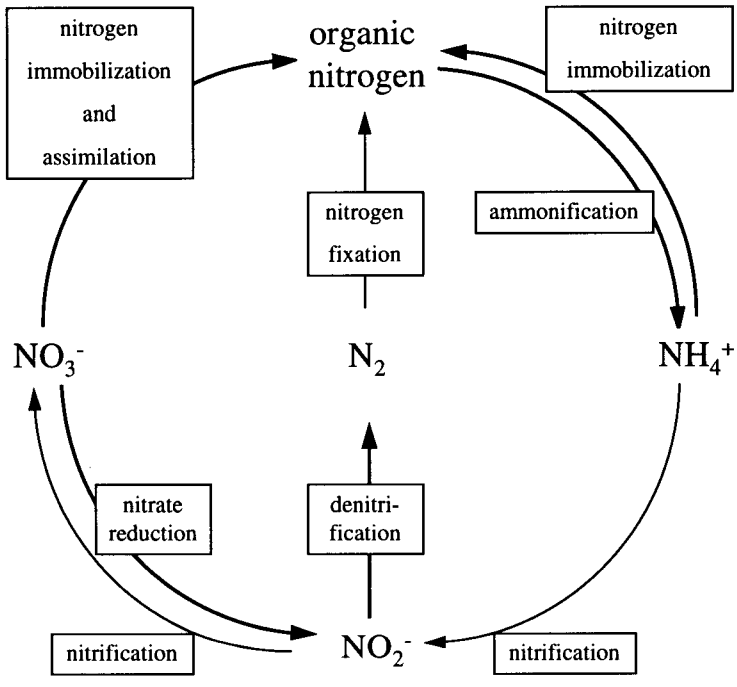

Figure 4 The soil nitrogen cycle. The broad arrows indicate general processes performed by many species.

5.4 Autotrophic nitrification

The oxidation of NH_4^+ to NO_3^- is one of the best studied [152] and standardized toxicity tests [9]. Moreover, it is relatively sensitive compared to many other microbial toxicity tests [59,58,11]. This sensitivity is generally attributed to the small number of species like *Nitrosomonas* or *Nitrospira* which are able to obtain their metabolic energy from the oxidation of NH_4^+ to NO_2^- [138,75]. The oxidation of NO_2^- to NO_3^- by *Nitrobacter* and other bacteria is less sensitive to chlorinated anilines [152] and metals [11]. Table 2 shows the sensitivity of the nitrification for toxicants. Most pesticides are tested at a single application dose and therefore only the percentage of inhibition (ECx) is given and not the EC10 or EC50.

Table 2 The sensitivity of the nitrification for toxicants.

Chemicals	effect	mg/kg	References	Chemicals	effect	mg/kg	References
aniline	NOEC	25	[152]	cinmethylin	EC12	50	[109]
4-chloroaniline	NOEC	10		cyanazine	EC95	50	
3-chloroaniline	NOEC	10		dimethazone	EC80	50	
paraquat	EC50	2500	[147]	ethalfluralin	EC90	50	
	EC50	720		metoachlor	EC90	50	
acetylene	EC32	0.1 Pa*	[114]	oryzalin	EC79	50	
propyne	EC40	1 Pa		propachlor	EC97	50	
1-butyne	EC36	1 Pa		acifluorfen	EC10	5	
2-ethylpyridine	EC85	1		diclofop methyl	EC20	5	
phenylacetylene	EC78	1		fenoxyprop methyl	EC20	5	
3-butyn-2-one	EC40	5		fluazifop butyl	EC80	50	
1-phenyl-2-propyn-1-ol	EC40	10		haloxyfop methyl	EC80	50	
1-pentyne	EC30	10		mefluidide	EC63	50	
3-phenyl-2-propyn-1-ol	EC25	10		tridiphane	EC30	5	
4-phenyl-1-butyne	EC21	10		Cu	EC45	320	[103]
diphenyl-acetylene	EC28	10		Cd	EC77	560	
methane	EC50	10 kPa	[115]	Pb	EC14	1035	
ethane	EC50	5 kPa		Zn	EC40	325	
ethylene	EC50	250 Pa		Ni	EC64	295	
acetylene	EC50	0.1 Pa		Cr	EC81	260	
2,4-D	EC10	4	[125]	nitrapyrin	EC44	15	[37]
ethylenethiourea	NOEC	0.1	[185]	2-ethynylpyridine	EC82	15	
nitrapyrin	EC25	30	[160]	4-amino-1,2,4-triazole	EC36	15	
nitrapyrin	EC27	30	[164]				

* For gaseous chemicals the concentrations are expressed in Pascals

5.5 Non-symbiotic nitrogen fixation

The nitrogen fixation is difficult to measure directly therefore acetylene is used to monitor the activity of the nitrogen fixing enzyme nitrogenase. In this test gaseous acetylene is added which is reduced by the nitrogenase enzyme to ethylene. This method is relatively easy to perform but the relationship between acetylene reduction and nitrogen fixation is still under debate [96]. Since acetylene reducing enzymes are not available in all soils, this test is unsuitable as a general test [105]. In Table 3 the sensitivity of this process for a number of chemicals is shown. The acetylene reduction is more sensitive for a number of pesticides and metals than respiration, plate counts, urease, dehydrogenase, phosphatase or sometimes even nitrification [156,11,129]. However, the effects of some insecticides were much stronger on phosphatase and invertase compared to acetylene reduction [158].

Table 3 The sensitivity of the non-symbiotic nitrogen fixation (measured as acetylene reduction) for toxicants.

Chemicals	effect	mg/kg	References	Chemicals	effect	mg/kg	References
ethoprop	NOEC	5	[155]	Zn	EC50	50	[26]
leptophos	NOEC	5		Cu	EC50	20	
chlordane	NOEC	5		Ni	EC50	2.5	
parathion	NOEC	5	[160]	Cd	EC50	3	
telone II	NOEC	5		methylpyrimifos	NOEC	10	[111]
maneb	NOEC	5		alachloron	NOEC	2	[130]

5.6 The relative sensitivity of the different nitrogen transformations

Respiration, carbon mineralization, nitrogen mineralization and ammonification are highly intertwined processes because the oxidation of a specific organic compound by a single organism can consume O_2 and yield CO_2, NO_3^- and NH_4^+ at the same time. The nitrate and ammonia formed are consumed immediately when there is a high carbon to nitrogen ratio in the substrate. Nitrogen mineralization and immobilization can lead to production and consumption of inorganic nitrogen in the same experiment [32]. Denitrification is also not very sensitive [24,162,163,164]. Table 4 shows that the effect concentrations relatively high. Therefore nitrogen mineralization, ammonification, nitrogen immobilization and denitrification are not very suitable for toxicity testing.

Table 4 The sensitivity of some nitrogen transformations for toxicants

Process	Chemi-cals		mg/kg	Refer-ences	Process	Chemicals		mg/kg	Refer-ences
N-miner-alization	Ag	EC56	540	[102]*	N-miner-alization	Cd	NOEC	336	[77]
	Hg	EC45	1000			Cd	EC50	1120	
	Cu	EC27	320		Ammoni-fication	tridemorph	EC50	45	[15]
	Cd	EC25	560		Denitri-fication	potassium azide	EC39	50	[24]
	Pb	EC19	1035			2,4-diamino 6-trichloro-methyl-s-triazine	NOEC	10	
	Mn	EC18	275						
	Fe	EC15	280						
	Zn	EC14	325						
	Ni	EC14	295						
	Sn	EC12	590						
	Cr	EC18	260						
	Fe	EC17	280						
	Al	EC16	135						
	B	EC10	55						
	V	EC12	255						
	Mo	EC27	480						

*The effect of metals (5 mmol/kg) on the nitrogen mineralization in three unamended soils was measured by determining the amount of nitrate, nitrite and ammonia. Since only 1 dose was given the effect is calculated from the percentage of inhibition of the most sensitive soil.

6. TOXIC EFFECTS OF POLLUTANTS ON SOIL ENZYMES

Free or particle bound extracellular enzymes can catalyze a number of reactions which are important for the ecological functioning of the soil. Here we will describe the ecological function of soil enzymes, their measurement, the use of enzymatic tests for the risk assessment of pollutants and which tests might be more sensitive for toxicants.

6.1 Ecological function of soil enzymes

The majority of enzymes are active in living cells but both micro-organisms and plants commonly excrete a number of enzymes in soil. Large polymers as lignins, cellulose, and large proteins can not be transported through the cell-membranes of micro-organisms. Therefore specific oxygen dependent lignin peroxidases [73], cellulases [104] and proteases are excreted as exo-enzymes to perform extracellular digestion. All extracellular enzymes in soil are subject to degradation but binding to clay can stabilize enzymes [35]. The enzymes dehydrogenase, glucosidase, urease, sulphatase, phosphatase can be used as a parameter for the cycling of macro-elements.

6.2 The measurement of soil enzymatic activities

Most enzymatic tests are artificial and refer to the potential activity of soil enzymes. A defined amount of soil is incubated in a generally aqueous environment with a specific substrate and sometimes in a buffer [22]. The enzyme converts the specific substrate to another compound that can be extracted and quantified by light spectroscopy for example. When a sufficient amount of a toxicant is added the color production decreases compared to the control. This dose dependent inhibition can be measured and an EC10 or EC50 can be calculated. Many enzymatic activities are easier to measure than the corresponding soil processes. For example, the effect of metals on the phosphatase or arylsulphatase activity can be measured readily by monitoring the conversion of p-nitrophenylphosphate or p-nitrophenylsulphate respectively [55,70]. Detailed analysis of the effect of pollutants on the sulfate or phosphate cycle is far more extensive. The main advantage of enzymatic tests is the easy way of measuring but a disadvantage might be their indirect approach.

An overview of the effect of metals on a number of different soil enzymes in various soils was reported earlier [11]. It is difficult to select a enzymatic reaction which is more sensitive than other microbial toxicity tests since most studies are performed with different soils at different incubation times and different compounds. Therefore only a limited number of comparisons can be made between different tests in the same soils. The dehydrogenase activity seems to be similarly sensitive [51,192,136] as the respiration in the same soils. Effects of metals on urease [54], phosphatase [55] and arylsulphatase [70] have been measured in the same soils and arylsulfatase was the most sensitive.

In the literature a large number of tests are applied on a single dose of pesticides [18,38] [74,91,107,126,156,157,158,160,164]. This allows only for a limited, qualitative comparison of different tests within one study. In many studies urease was relatively sensitive for pesticides. Phosphatase, arylsulphatase and amylase were less sensitive in most studies, whereas dehydrogenase and amylase were insensitive.

7. EFFECTS OF POLLUTANTS ON MICROBIAL DIVERSITY AND RESISTANCE

In the preceding chapters the toxic effects of pollutants on sumparameters like microbial biomass, different processes involved in the carbon and nitrogen cycle and on enzymes have been quantified. Sumparameters reflect the actions of many species in the microbial community of a soil. Theoretically species diversity should be a more sensitive indicator of the effects of environmental pollution than a sumparmeter like the process rate, since a replacement of a sensitive species by an equally active resistant species does not change the process rate. In practice, however, measurement of a specific part of the species diversity is often too laborious since it has to be determined in taxonomic detail [50,11]. When the micro-organisms are only determined at the genus level and not at the species level there is a high probability that the replacement of a sensitive species by a resistant one remains undetected [11]. The development of resistance against pollutants in a community is also a change in species diversity but it can be measured more easily. This chapter describes the different forms and ecological consequences of the occurrence of resistance. Moreover, it will be shown that the development of resistance against pollutants in a community is an indicator of ecological deterioration.

7.1 Occurrence of resistance

Prolonged exposure to pollutants and pesticides can induce resistance into many micro-organisms [154], plants and animals [95,128]. The main part of the soil biomass does not grow at any measurable rate but has just enough food to support its maintenance requirements under normal conditions [7]. The average number of divisions per microbial cell in an arable soil was calculated to be 2 times per year [27]. It can be assumed that a large number of microbial species does not grow fast under any conditions and is therefore more difficult to study [99,127].

Some taxonomic groups of microorganisms have a more limited capability to become resistant than other groups. For example, the conjugation of resistance plasmids seems to be limited to Gram negative bacteria [149,76]. This might explain the decrease of Gram positive bacteria in metal polluted soils. Metal resistant microbial communities contained more prokaryotes and Gram negative rods while the number of Gram positive bacteria was decreased [50]. Fungi were reported to be more metal resistant than bacteria [13,11].

In general the resistance of communities of organisms to pollutants can be obtained via four different mechanisms. First, organisms can obtain some resistance when they are living under specific conditions which enable them to withstand the toxic stress by limiting uptake, maximizing excretion or by detoxification of the pollutant. Second, a phenotypic physiological resistance can be induced in individuals when they become acclimated to a toxicant for longer periods. Third, genetical resistance can occur in a population when the individuals that are most adapted to the pollutant obtain a competitive advantage and become dominant after many generations of selection [95]. Forth, communities can also become "resistant" because the sensitive species are replaced by resistant ones which might or might not perform similar functions. For example, the fatty acid composition of Zn resistant bacteria was different from the sensitive bacteria [124]. This indicates that the sensitive species were not replaced by resistant relatives but by unrelated species with different properties.

7.2 The ecological consequences of community resistance

The emergence of community "resistance" against a pollutant is in fact the very process which describes the deterioration of ecosystems. A theoretical experiment can illustrate this process. An undisturbed community might be characterized by the log-normal distribution of NOEC values for Cd [179] which is shown in Figure 5. When this community will be exposed to a cadmium pollution of 100 mg Cd/kg soil the species with a NOEC < 100 will become more or less inhibited. This inhibition can lead to the extinction of the sensitive species due to a decreased competitive ability. The remaining resistant community will therefore have an average NOEC value over 100 mg Cd/kg. About 84% of the original species were inhibited by 100 mg Cd/kg. Therefore one might expect that resistant communities will have less species and will show a decrease in ecological functioning.

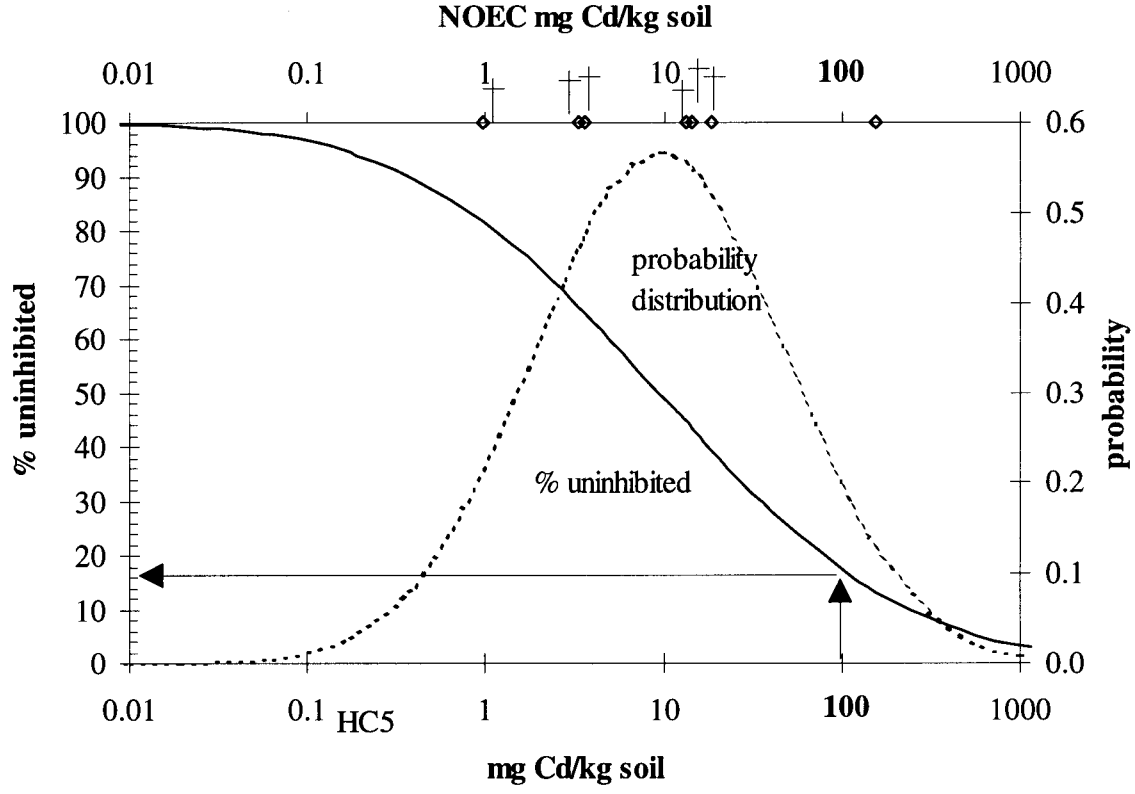

Figure 5 The theoretical effect of the application of 100 mg Cd/kg soil on the distribution of NOEC values of a community [179]. The species indicated with a cross on the upper axis have a NOEC < 100 and are intoxicated. The arrows indicate that at 100 mg/kg only 16% of the species is uninhibited.

Resistant communities show decreased competitive abilities [128], a decreased mineralization rate [170], decreased biodegradation capacities [122,134,56] and a decreased cold resistance [48]. Table 5 indicates that Cd or Zn resistant bacteria from a sandy soil show less metabolic diversity since they were less able to degrade specific substrates [56]. Resistant mycorrhizal fungi can also have a negative influence on plant growth [142,71]. Resistant bacteria can accumulate high concentrations of metals [2,132] which can lead to intoxication of predators like nematodes [53].

The following studies show that replacement of sensitive populations by resistant ones of the same species can also have serious ecological consequences. The effect of metals on the symbiotic nitrogen fixation was demonstrated in long term field experiments. Sludge amendments caused metal pollution which at first did not inhibit *Rhizobia* to fix nitrogen in clover because the higher organic carbon content obscured toxic effects [123]. When the sludge amendments were ceased the organic material was mineralized and toxic effects did occur which were persistent for more than 25 years [116]. At a metal polluted site with 469 mg Zn/kg, 163 mg Cu/kg, 35 mg Ni/kg and 15.4 mg Cd/kg [116], only metal resistant *Rhizobia* unable to fix nitrogen in clover were present [67]. The resistant *Rhizobia* could grow in the polluted field but could not fix nitrogen [34]. Serious and persistent toxic effects on the microflora can be caused by zinc, cadmium and copper concentrations which are lower than the current European Community limits [117]. Glyphosate, tetramethylthiuram-disulfide or zinc treatment can also result in ineffective *Rhizobia* [110,143], whereas paraquat treatment could not [135].

Table 5 Percentage of strains of bacteria sensitive or resistant to Cd or Zn able to grow on various organic substrates from [56].

Substrate	Strains able to grow on the substrate (%)			
	Cd-sensitive (n = 93)	Cd-resistant (n = 64)	Zn-sensitive (n = 84)	Zn-resistant (n = 94)
benzoic acid	18	8	21	10
2-hydroxybenzoic acid	16	16	17	5
3-hydroxybenzoic acid	20	13	19	7
2,5-dihydroxybenzoic acid	8	6	8	0
3,4-dihydroxybenzoic acid	8	5	7	2
3,4,5-trihydroxybenzoic acid	0	0	0	0
2-aminobenzoic acid	11	3	11	2
3-aminobenzoic acid	10	0	8	1
4-aminobenzoic acid	5	3	6	3
4-methoxybenzoic acid	10	0	10	1
3,4-dimethoxybenzoic acid	13	0	13	2
L-phenylalanine	13	11	17	2
phenylacetic acid	16	14	19	2
4-hydroxyphenylacetic acid	4	0	6	3
terephtalic acid	4	0	5	0
benzoglycine	15	13	17	0
salicine	12	6	12	13
quercitine	2	2	2	0
flavon	2	0	2	0
1,2-dihydrobenzol	0	0	0	0
catechine	0	0	0	0
4-hydroxycinamic acid	13	0	17	2
nicotinic acid	0	0	0	3
Mean	8.7%	4.3%	9.4%	2.6%

7.3 Resistance as an indicator of environmental pollution

The occurrence of resistant micro-organisms in the field was proposed as an indicator of environmental pollution since heavy metals can induce a large number of resistant actinomycetes, fungi, and bacteria [89,50,56]. There are a few methods to measure the differences in community resistance from clean and polluted sites. In the first method microbial strains are isolated from soil on agar plates. The percentage pollutant resistant strains from clean soils can then be compared with the percentage from polluted soils. For the determination of the percentage of resistant micro-organisms a proper threshold concentration must be quantified for the toxicant [89,84]. A significant increase of the percentage resistant micro-organisms from polluted soils was only detected when a high threshold concentration was used [60]. This can be due to the high standard deviation in plate count methods since the average metal sensitivity of isolates from a metal polluted soil was lower than from a reference soil [8]. Besides plate counts also a direct microscopic method was developed to distinguish arsenic resistant bacteria [194]. The thymidine incorporation technique can also be used to detect metal resistant bacteria in soil [12,47]. The occurrence of resistance against a pollutant is an excellent method to detect the effects soil pollution on microbial diversity in the field.

8. TOXIC EFFECTS OF POLLUTANTS ON SEDIMENT PROCESSES

This chapter will show that toxicity tests in soils and sediments are similar in many aspects. Finally an example of a risk assessment procedure based on tests with microbial processes is given. This procedure is similar to the procedure which is described in the introduction to derive the HC5 (Hazardous Concentration 5%) but is based on tests using microbial processes instead of single species tests.

8.1 The similarities and differences between soils and sediments

Sediments are similar to soils with respect to a high biomass of bacteria [167] and a high sorption capacity which makes it advisable to express toxic effects in mg/kg sediment dry weight and not in mg/l sediment. It is difficult to compare the effect of 3 µg Cd/l on methane production [188] with the effect of 100 mg Cd/l on ^{14}C-acetate incorporation or β-glucosidase activity [18] when the sorption capacity of the sediments involved is not known.

Due to the complete water saturation of sediments the available oxygen can become depleted. A low redox potential alters the thermodynamically most favorable biodegradation pathways in a drastic way. When oxygen becomes depleted, nitrate, sulfate and finally carbon dioxide are used as electron acceptors. Denitrification does also occur at low oxygen tensions in soils. Hydrogen sulfide and methane can be formed under strongly reducing conditions in sediments. These processes are performed by specialized, strictly anaerobic bacteria which live in consortia with facultative anaerobes. The speciation of metals is changed because metal sulfides can precipitate [46]. Anaerobic sediments are uninhabitable for animals which can only live close to the surface because they need oxygen. Most ecological functions in anaerobic sediments are performed by bacteria.

8.2 Microbiological toxicity tests in anaerobic sediments

The sensitivity of different microbial toxicity tests in sediment can be compared when a single sediment is used for a number of tests. Methanogenesis seems to be a relatively sensitive test. It was more sensitive for metals than carbon dioxide production or ATP content but less sensitive than short-term sulfate reduction [29]. The EC50 for methanogenesis was 8 mg Cu^{2+} /kg while an addition of 400 mg Cu/kg sediment only partly inhibited phosphatase and not acetylene reduction or dehydrogenase activity [86]. The herbicide simazine partly inhibited methanogenesis at 50 mg/kg and phosphatase at 100 mg/kg [86]. Acetylene, formazan or nitrophenol used in the enzymatic assays might also be reduced by chemical compounds in the sediment [72].

Aside from methanogenesis and enzymes, a number of other tests are also used in sediments. The ^{3}H-thymidine incorporation was more sensitive for naphtalene and pentachlorophenol than the ^{14}C-glucose metabolism [20]. Direct microscopic counts of acridine orange stained bacteria correlated negatively with 2-5 mg Cd /kg sediment [62]. In rice field soil the EC50 of the cellulose degradation was 40 mg trichlamide/kg under flooded conditions and much smaller under upland conditions. Chlorothalonil inhibited at 15 mg/kg under both upland and flooded conditions [93]. The conversion of an oxygenated sediment to a low redox potential can also be sensitive to toxic effects, because of the inhibition of the facultative aerobic micro-organisms which consume oxygen and lower the redox potential. A small amount of 4 mg trichalimide /kg delayed significantly the lowering of the redox potential to -200 mV [92]. Pollution with metals, antibiotics or phenolic compounds could evoke resistance in sediment bacteria [153,43]. This indicates that pollution induced community tolerance can also be used in sediments.

8.3 The use of microbial tests to establish sediment (or soil) quality guidelines

The effect of 5 toxicants on the mineralization of 1-5 µg/l concentrations of ^{14}C-acetate [183], ^{14}C-chloroform [173],^{14}C-4-chlorophenol [172], ^{14}C-benzoate [172], and the formation of methane [182] was measured in sediment. The methanogenesis is linked with the other processes since organic compounds are mineralized to CO_2 and CH_4 under anaerobic conditions in freshwater sediment. Table 6 summarizes the toxic effects of benzene, pentachlorophenol, 1,2-dichloroethane (DCE), chloroform [171]. Each toxicant shows a different sensitivity pattern for the 5 mineralization reactions. On average chloroform is the most toxic compound for these processes while benzene is the least toxic. DCE is very toxic for the ^{14}C-chloroform mineralization, whereas it is only slightly toxic for methanogenesis.

The experimental sediment was sampled in the Rhine which can not be regarded as a pristine river. An elevated background concentration is a common problem in the risk assessment of naturally occurring compounds like metals since it becomes difficult to find a control sample where toxic effects are absent. The background concentration of zinc in the Rhine sediment was relatively high (800 mg/kg) and therefore only the more zinc resistant micro-organisms might be present. A small addition of more than 8 or 11 mg Zn/kg to the high background concentration was able to inhibit the 4-chlorophenol or chloroform mineralization respectively. The added zinc might be more soluble than the background concentration of zinc.

Table 6 The EC10 concentrations of 5 toxicants on the mineralization of 4 substrates and also on the formation of methane

	mineralized substrates					DC5
toxicants	acetate	benzoate	4-chloro-phenol	chloroform	methano-genesis	
benzene	480	150	150	140	>10000	1
PCP	19	6	3.1	15	140	0.4
DCE	0.7	71	23	0.07	860	0.0004
chloroform	0.04	0.04	0.05		5.5	0.0001
zinc	> 3500	42+800	8+800	11+800	1780+800	300

All concentrations are expressed in mg toxicant/kg sediment (d.w.). The background concentration of zinc is 800 mg Zn/kg. The EC10 is that concentration of toxicant that gives a 10% decrease in the mineralization rate constant. The DC5 is the Dangerous Concentration 5% which is derived from five EC10 values for a single toxicant. It is similar to the HC5 concentration which is derived from 5 NOEC values.

The EC10 values in Table 6 can be used as input for an extrapolation method to derive sediment quality guidelines which is analogous to the HC5 method. The outcome is different from an HC5 because an EC10 of a microbial process is used instead of a NOEC of a single species test. Therefore, the quality guideline will be designated as a Dangerous Concentration 5% (DC5). Figure 6 shows for each toxicant a cumulative normal distribution with the same average as the log transformed EC10 concentrations from Table 6 [171]. Figure 6 is analogous to the cumulative probability distribution shown in Figure 1. The log(EC10) in mg/kg sediment is placed on the X axis and the percentage protected reactions is placed on the Y axis. The DC5 values (at 95% on the Y axis) are indicated by arrows in Figure 6 and listed in Table 6. The DC5 values were calculated using the ETX program which is designed to calculate the HC5 from NOEC values [3]. The standard deviation of the log EC10 concentrations for each toxicant was multiplied by an extrapolation factor 1.62 for the calculation of the normal frequency distribution for the reactions shown in Figure 6 [3].

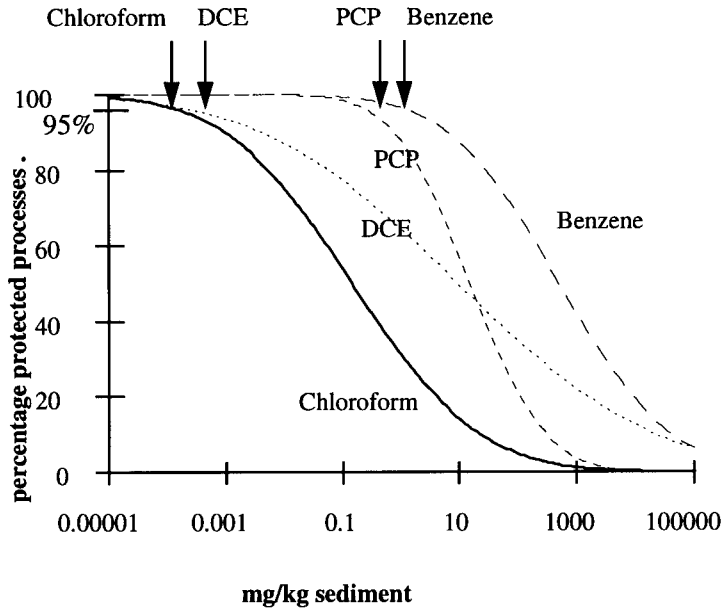

Figure 6 The inhibition of mineralization reactions in a Rhine sediment by chloroform, 1,2-dichloroethane (DCE), pentachlorophenol (PCP) or benzene. The X axis is a logarithmic plot of the sensitivity of the sediment processes expressed as EC10 toxicant concentrations and the Y axis is the percentage of all theoretical mineralization reactions which is protected. The arrows indicate the 95% protection level.

9. THE USE OF MICROBIAL TOXICITY DATA IN RISK ASSESSMENT

Standardization is often a problem with microbial processes because each site contains its own indigenous microflora. Even in the guideline for the determination of nitrogen mineralization and nitrification in soils from the international organization for standardization there is no strict standardization of the microflora because there is no prescribed soil [9]. Differences in microbial activity and soil properties ask for a site specific approach. At heavily polluted sites a resistant microflora might be present which should not be used for the assessment of the sensitivity of the microflora in general. With the HC5 method the average sensitivity of a species can be used as an input for the extrapolation method when more tests are performed with the same species. The differences between the NOEC values for a particular toxicant in a number of tests with the same species are caused by random error and therefore the use of an average NOEC is justified. The differences between the EC10 values for a particular toxicant in a number of tests with the same microbial process are not caused by random error. Each soil or sediment will have its own microflora and a single process will be performed by different species in different soils and sediments. Therefore the EC10 values of a specific process should not be averaged before using the DC5 method.

9.1 The use of different categories of toxicity tests with micro-organisms for the establishment of quality guidelines

The results of toxicity tests with a single microbial species can not be incorporated in the extrapolation procedure for microbial processes (the DC5) but can be included in the

extrapolation method for single species (the HC5 method). It is not the size of the test organism but it is the type of test which determines in what extrapolation procedure the test result should be used. Differences in species diversity in the field can be used directly to derive soil quality standards when a relation with the pollutant can be established (see chapter 7). The occurrence of pollutant resistant strains in the field can also be regarded as direct evidence that the pollutant concentration is causing ecological effects. This can also be used directly to establish or confirm soil quality standards.

9.2 A comparison between 95% protection levels for microbial processes in soils and sediments with the corresponding levels derived from single species tests

In order to derive 95% protection levels for pollutants in soils the DC5 method is applied for 4 pollutants in soil. Table 7 shows an overview of the inhibition of different microbial processes by pentachlorophenol, chlordane, zinc and mercury. Most of these literature data do not show an EC10 but have different endpoints for toxicity testing like a NOEC, LOAC (Lowest Observed Adverse effect Concentration) or ECx. From these variety of endpoints the EC10 has to be estimated before it can be used as input in the DC5 extrapolation method. A stringent selection of the tests which do report an EC10 has the disadvantage that a lot of data are ignored. Therefore an arbitrary classification has been used to derive the EC10 from other effect parameters. The EC5 till EC19 effect concentrations were estimated to be equal to the EC10. Also the NOEC and LOAC were assumed to be equal to the EC10. The EC20 to EC50 were divided by 3 to obtain the EC10. The EC50 to EC95 were divided by 10 to obtain the EC10. The EC95 to EC100 values were not used to estimate the EC10. The conversion factors should be regarded as crude estimates which give an order of magnitude value for the EC10. The DC5 concentrations were calculated from the EC10 concentrations using the ETX program [3].

Table 7 Toxic effects of pentachlorophenol, chlordane, mercury or zinc on microbial processes

Process	Effect	mg/kg	EC10 mg/kg	Refer-ences	Process	Effect	mg/kg	EC10 mg/kg	Refer-ences
pentachlorophenol									
ATP content	NOEC	2	2	[195]	acetate min.[a]	EC10	12	12	[170]
ATP content	LOAC	6-30	17	[141]		EC10	176	176	
nitrification	NOEC	11.4	11.4	[186]	acetate min.	EC10	6	6	[168]
respiration	NOEC	125	125	[186]		EC10	15	15	
iron reduction	LOEC	10	10	[196]		EC10	14	14	
dehydrogenase	EC50	177	59	[45]		EC10	50	50	
glutamate min.	LOAC	10	10	[187]		EC10	0.1	0.1	
glycine degradation	NOEC	120	120	[139]		EC10	1.4	1.4	
acetate min.	EC10	11	11	[169]		EC10	0.3	0.3	
Chlordane									
acetylene reduction	EC42	10	3	[155]	nitrification	EC90	10	1	[159]
acetylene reduction	LOAC	5	5	[156]	sulphur oxidation	EC40	10	3	[159]

Mercury

ATP content	NOEC	1.5	1.5	[195]	glucose min.	NOEC	0.4	0.4	[148]
respiration	NOEC	70	70	[157]	glucose min.	EC20	1.3	0.4	[146]
respiration	NOEC	10	10	[146]		EC25	50	17	
	NOEC	100	100			NOEC	40	40	
	NOEC	100	100		glucose min.	NOEC	0.2	0.2	[94]
	EC11	10	10		glucose min.	NOEC	40	40	[146]
	EC87	10	1			NOEC	200	200	
	NOEC	10	10			NOEC	40	40	
	EC20	74	25			NOEC	200	200	
	EC20	74	25		nitrification	NOEC	7.4	7.4	[146]
phosphatase	NOEC	2006	2006	[146]		NOEC	7.4	7.4	
	EC15	2006	2006		ammonifi-cation	NOEC	7.4	7.4	[146]
	NOEC	500	500			NOEC	7.4	7.4	

Zinc[b]

phosphatase	EC37	108	36	[112]	respiration	EC7	100	100	[30]
phosphatase	EC28	71	24	[11]	respiration	EC25	108	36	[112]
	EC15	590	590		respiration	NOEC	177	177	[119]
phosphatase	EC10	41	41	[55]		NOEC	178	178	
	EC10	1333	1333		respiration	EC21	10	3	[87]
	EC10	2607	2607		respiration	EC45	100	33	[87]
	EC10	159	159		respiration	NOEC	100	100	[120]
pyrophos-phatase	EC8	1635	1635	[11]	respiration	NOEC	80	80	[122]
urease	EC10	70	70	[54]	respiration	NOEC	51	51	[11]
	EC10	30	30			EC16	1000	1000	
	EC10	30	30			EC10	327	327	
	EC10	460	460			EC25	327	109	
urease	EC7	32.7	32.7	[151]	respiration	EC10	11.7	11.7	[31]
	EC6	32.7	32.7		respiration	EC21	10	3	[11]
	EC61	327	33		respiration	NOEC	150	150	[53]
	EC33	327	109			EC11	400	400	
	EC23	327	109		respiration	EC13	100	100	[124]
	EC34	327	109		N min.	EC15	250	250	[11]
						EC32	1000	333	
	EC51	327	33			EC30	700	233	
dehydro-genase	EC30	1000	333	[106]	ethylene production	EC50	10-100	10	[10]
	EC30	200	67		nitrification	NOEC	100	100	[131]
dehydro-genase	NOEC	30	30	[11]	N fixation	EC50	334	111	[116]
phytase	NOEC	590	590	[11]	N fixation	EC50	50	17	[26]
glutamate min.	NOEC	>100 0	1000	[69]	nitrification	EC24	327	109	[103]
	NOEC	55	55		nitrification	NOEC	500	500	[11]
	LOAC	55	55		nitrification	NOEC	10	10	[193]
	LOAC	55	55			NOEC	100	100	
arylsulphat-ase	EC11	164	164	[11]		NOEC	100	100	
arylsulphat-ase	EC10	105	105	[70]	iron reduction	NOEC	80	80	[190]
	EC10	728	728		acetate min.	EC10	0.7	0.7	[170]
	EC10	151	151			EC10	>1000	1000	
	EC10	2353	2353		acetate min.	EC10	>1010	1010	[168]
					glucose min.	NOEC	125	125	[148]

[a] min. = mineralization

[b] The added zinc concentrations are used.

Table 8 compares the DC5 concentrations calculated from the sediment data from Table 6 and the soil data from Table 7. Since zinc is present in high concentrations in the sediment there is a large difference of 800 mg/kg between the total concentration and the added concentration. Both can be used for the DC5 and DC50 calculations. The DC50 is the concentration which inhibits 50% of the microbial processes and is equal to the geometric mean of the EC10 concentrations of a single pollutant. The extremely low DC5 of 0.75 mg added Zn/kg sediment might be an artifact caused by the precipitation of ZnS during the tests which creates large differences in sensitivity depending on the amount of acid volatile sulfide present in the sediment to bind the added zinc. Zinc is more toxic for microbial processes than for plants or animals [36]. The HC5 for soil invertebrates and plants was calculated to be 128 mg added Zn/kg soil while an addition of 30 -70 mg Zn/kg soil was expected to have adverse effects [36]. The DC5 concentration of zinc added to soil was calculated from 67 EC10 concentrations. Due to the large number of tests this DC5 is relatively insensitive to specific errors in a single test. If for example the lowest EC10 of 0.7 mg Zn/kg is replaced by a 10 times higher value the DC5 increases from 7 to 9 mg added Zn/kg soil.

The DC5 concentrations of chloroform and 1,2-dichloroethane are 10 000 times lower than the corresponding HC5 concentrations in the sediment. These chlorinated alkanes are not very toxic for fish with LC50 concentrations ranging from 18-191 mg chloroform/l [176] to 480 - 550 mg 1,2-dichloroethane/l [40]. The high HC5 concentrations of these compounds are attributed to a narcotic mode of action [178] while the low DC5 concentrations might be caused by the formation of toxic intermediates during the reductive dechlorination in the anaerobic sediment. The mineralization of chloroform proceeds via the toxic intermediate phosgene [150] which is not formed under aerobic conditions when chloroform is not degraded. Table 8 shows that the DC5 concentrations of benzene, pentachlorophenol and 1,2-dichloroethane in sediment are in the same order of magnitude as the corresponding HC5 concentrations derived from aquatic toxicity tests. Also the DC5 for PCP and mercury in soil are not very different from the HC5 levels derived from tests with soil animals. The HC5 of the insecticide chlordane is much lower than the DC5 which might be expected since chemicals used as insecticides were selected to be toxic for insects.

Table 8 **A comparison between the 95% protection levels for microbial processes in soil or sediment (DC5) with the corresponding levels derived from single species toxicity tests with animals and plants (HC5)**

	Soil			Sediment		
	DC50 mg/kg	DC5	HC5 mg/kg	DC50 mg/kg	DC5	HC5 mg/kg
benzene				432	1	3.3[a]
PCP	10	0.4	0.2[c]	15	0.4	0.18[b]
DCE				9	0.0004	4[a]
chloroform				0.14	0.0001	5[a]
chlordane	3	0.7	0.0043[d]			
total zinc				1379	300	120[b]
added zinc	104	7.5	128[e]	111	0.75	
mercury	20	0.4	1[f]			

The hazardous concentration 5% (HC5) was derived from soil and aquatic toxicity tests [a][178], [b][174], [c][145],[d][175], [e] the value for soil invertebrates and plants from [36],[f] value based on biomagnification of mercury [146].

10. CONCLUSIONS

Micro-organisms play an indispensable rol in the ecological functioning of soils and sediments. Microbial processes are performed by a community of different microbial species. Some species are sensitive while others are insensitive for a specific pollutant. There are a number of microbial toxicity tests which do not reveal the true sensitivity of the micro-organisms since in these tests the activity of the resistant species obscures the inhibition of the sensitive species. The replacement of sensitive species by insensitive ones in an ecosystem can have serious ecological consequences. This review gives criteria to select relatively sensitive microbial toxicity tests and describes a novel method to derive soil and sediment quality standards from these tests. The method is very similar to a method used to derive a Hazardous Concentration 5% (HC5) which calculates a pollutant concentration which does not inhibit 95% of the animal or plant species. The HC5 method requires the No Observed Effect Concentration from more than 4 toxicity tests with different animals or plants. In analogy our novel method requires the Effect Concentration 10% (EC10) from more than 4 toxicity tests with microbial processes. Using the same statistical procedure as the HC5 method a Dangerous Concentration 5% (DC5) is derived. At pollutant concentrations lower than the DC5 more than 95% of the microbial processes are assumed to be protected. As an example the DC5 is calculated for a number of pollutants in soils and sediments. For chloroform and 1,2-dichloroethane in sediment the DC5 is much lower than the HC5 derived from aquatic toxicity tests. This indicates that microbial processes are much more vulnerable for these compounds than aquatic animals or plants. The DC5 for zinc added to soil is much lower than the HC5 for zinc which indicates that microbial processes are also relatively vulnerable for this metal.

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